

**STUDIES OF ARBUSCULAR
MYCORRHIZAL (AM) FUNGI IN SOME
COMMONLY OCCURRING MEDICINAL
PLANTS OF GOA**

**THESIS TO BE SUBMITTED TO GOA UNIVERSITY
FOR THE AWARD OF DEGREE OF**

**DOCTOR OF PHILOSOPHY
IN
BOTANY**

By
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Research Guide

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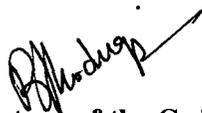
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DECLARATION

I hereby declare that the thesis entitled “**STUDIES OF ARBUSCULAR MYCORRHIZAL (AM) FUNGI IN SOME COMMONLY OCCURRING MEDICINAL PLANTS OF GOA**” submitted to Goa University, for the award of the degree of **DOCTOR OF PHILOSOPHY IN BOTANY** is a record of original and independent work carried out by me during June 2004 - June 2008, in the **DEPARTMENT OF BOTANY, GOA UNIVERSITY** under the supervision of **Dr. B. F. RODRIGUES**, Professor, Department of Botany, Goa University and that it has not previously formed the basis for the award of any Degree, Diploma, Associate-ship or Fellowship or any other similar title to any candidate of this or any other University.



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CERTIFICATE

I hereby certify that the thesis entitled “**STUDIES OF ARBUSCULAR MYCORRHIZAL (AM) FUNGI IN SOME COMMONLY OCCURRING MEDICINAL PLANTS OF GOA**” submitted to Goa University, for the award of the degree of **DOCTOR OF PHILOSOPHY IN BOTANY** is a record of original and independent work carried out by me during June 2004 - June 2008, in the **DEPARTMENT OF BOTANY, GOA UNIVERSITY** under the supervision of Dr. **B. F. RODRIGUES**, Professor, Department of Botany, Goa University and that it has not previously formed the basis for the award of any Degree, Diploma, Associate-ship or Fellowship or any other similar title to any candidate of this or any other University.

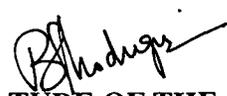
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Dedicated
to my
parents.....



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INTRODUCTION

MEDICINAL PLANTS AND MYCORRHIZAL FUNGI:

A medicinal plant is any plant which, in one or more of its organ, contains substance that can be used for therapeutic purpose or which is a precursor for synthesis of useful drugs (Sofowora, 1982). Plants owe their virtues as medical agents to secondary metabolites that they synthesize and have contributed more than 7000 different compounds that are in use today as heart drugs, laxatives, anti-cancer agents, hormones, antibiotics, analgesics, anesthetics, ulcer compounds and antiparasitic compounds (Biradar and Reddy, 2007).

Kumar and Mahadevan (1984) reported the absence of AM fungal association in 28 medicinal plants belonging to 20 families and attributed it to the toxic substances present in these plants. However many medicinal plants with secondary metabolites have been reported to harbour AM fungi in their root system (Taber and Trappe, 1982; Abbott and Robson, 1982; Rao *et al.*, 1989; Selvaraj and Subramanian, 1990 & 1992; Sullia and Sampath, 1992; Basu and Srivastava, 1998; Prasad and Reddy, 1998; Rao *et al.*, 2000; Hemalatha and Selvaraj, 2003; Manjunath and Reddy, 2003).

Taber and Trappe (1982) reported the presence of AM fungi in rhizomatous tissue and in the scale-like leaves of rhizomes and roots of *Zingiber officinale*. Mukherji and Ardey (1985) recorded AM colonization in *Kalanchoe spicata* and *Eclipta alba*. The presence of AM fungi has also been observed in medicinal

rhizomes (Iqbal and Nasim, 1986) and medicinal bulbs (Iqbal and Firdaus-e-Bareen, 1986). Burrow *et al.* (1990) observed increased fern height, corn weight and bud numbers in *Asparagus officinalis* and correlated positively to the percent AM root colonization. Burani *et al.* (1994) observed maximum AM colonization (100%) in the roots of *Mentha piperita* and *Psoralea corylifolia*. The importance of AM association in *Ancistrocladus korupensis* for the successful cultivation of the plant for production of Michellanine-B (anti HIV alkaloid) has been reported (Thomas and Carpenter, 1995). Some medicinally important wild plants examined from the Magara hills, Islamabad and the salt range of Pothohar revealed that wild plants have enormous ability to establish AM association under stressed conditions particularly under saline environments (Gill *et al.*, 2000).

Rao *et al.* (2000) reported AM fungal association in economically important spice and aromatic crops. Application of AM fungi improved the growth and productivity of *Ocimum* species (Gupta *et al.*, 2000). Gorski (2002) reported the presence of AM fungal association in 76 medicinal plants. Panwar and Tarafdar (2006) reported the presence of AM fungal association in three selected endangered medicinal plant species viz., *Leptadenia reticulata*, *Mitragyna parviflora* and *Withania coagulans* and suggested that mycorrhizal status may prove crucial for re-establishment of such endangered plants.

Growth and yield parameters:

Inoculation of *Mentha piperita* L. with *Glomus fasciculatum* in unsterilized soils produced better root colonization with significantly higher total root length, shoot dry weight, P uptake, organic and inorganic P fractions in leaf tissues and oil yields as compared to the un-inoculated control, both at deficient and high levels of soil P (Sirohi and Singh, 1983). Rao *et al.* (1988) recorded AM fungal association in root samples of 25 medicinal plants growing in red sandy loam soils of Bangalore and no correlation was observed between percent root colonization and the chlamydospore number in the rhizosphere soil. Bass (1990) reported physiological effects of AM colonization in relation to internal P concentration in *Plantago major* and *Pletosperma* species.

Gupta *et al.* (1990) reported the occurrence of AM fungal association in seven cultivated aromatic *Cymbopogon* species where *C. uratus* showed maximum AM fungal root colonization. Gupta and Janardhanan (1991) recorded a two fold increase in growth and three fold increase in biomass production as compared to non-mycorrhizal plants in *Cymbopogon martini* on inoculation with *Glomus aggregatum*. *G. clarum*, *G. intraradices*, *G. monosporum* and *G. vesiculiferum* tested on the growth of *Asparagus* significantly increased plant dry weight both in green house and in the field (Pederson *et al.*, 1991). Sreenivasa and Gaddagimani (1993) recorded better plant height and increased dry matter yield in chilli on inoculation with *Glomus macrocarpum* and 50kg /Ha $\underline{P_2O_5}$.

Oliveria *et al.* (1995) reported an increase in biomass production in eucalyptus seedlings on inoculation with AM fungi. Mohan *et al.* (1995) observed better establishment and survival of neem plants in AM inoculated seedlings even under arid zone conditions. Khaliq and Janardhanam (1997) studied the influence of three AM fungi viz., *Glomus aggregatum*, *G. fasciculatum* and *G. mosseae* on the productivity of six cultivated mint species that showed significant variation and effectiveness on inoculation with different AM fungi, suggesting their potential role in improving the productivity of commercially cultivated mints. Black pepper inoculated with *Glomus fasciculatum* resulted in increased N, P and leaf nitrate reductase activity (Shivashankar and Iyer, 1989).

Kothari *et al.* (1999) reported increased root and shoot biomass, root length, nutrient (P, Zn and Cu) uptake per unit length and nutrient concentration in bergamont mint plants inoculated with *Glomus intraradices*. Inoculation with exotic strains of AM fungi increased early vigour and resistance to infection by *Fusarium* species and also increased rooting and root mass in cardamom seedlings (Joseph *et al.*, 2000). Earanna *et al.* (2001) reported *Glomus caledonium* as the most efficient AM fungi to cultivate *Rauwolfia serpentina* among six different species of AM fungi screened for symbiotic response.

Aparna (2000) recorded improved growth, P nutrition, alkaloid concentration and yield in *Andrographis paniculata* on inoculation with *Glomus mosseae*. Two selected medicinal plants viz., *Phyllanthus amarus* and *Withania somnifera* on inoculation with AM fungi and plant growth promoting rhizomicro-organisms (PGPRS) revealed that the *P. amarus* yield improved through inoculation with *G. fasciculatum* and *Bacillus coagulans* or *Trichoderma harzianum* whereas, *G. mosseae* and *B. coagulans* proved to be the best in improving growth and yield of *W. somnifera* (Earanna, 2001). The application of AM fungi on other beneficial soil microflora is known to improve productivity of a wide range of crops and their potential can be best explored in medicinal and aromatic crops, which could be grown well under poor and marginal soils (Rao, 2001). There are reports on the association of AM fungi in several medicinal plants such as *Cassia siamea* (Habte, 1995), *Calotropis procera* (Rao et al., 1996), *Plectranthus amboinicus*, *Wedelia chinensis*, *Eclipta prostrata* (Selvaraj, 1989) and *Cichorium intybus* (Selvaraj et al., 2001).

Kumar and Murugesh (2002) tested the efficacy of three AM fungal species viz., *Glomus mosseae*, *G. fasciculatum* and *G. monosporum* on 10 medicinal plants and reported that AM inoculation is beneficial in improving the growth. Basu and Srivastava (1998) tested 13 AM fungal species on five medicinal plants and observed that AM fungal association not only enhance the growth of medicinal plants but also improved the productivity of medicinal compounds.

Arbuscular mycorrhizal fungi are known to play a pivotal role in nutrition and growth of plants mainly in oriented agricultural systems. However, little is known about their potential effects on secondary metabolites in medicinal and aromatic plants (Copetta *et al.*, 2006; Kapoor *et al.*, 2002a, b & 2004; Khaosaad *et al.*, 2006). Until recently very little attention has been paid on the accumulation of secondary compounds in the aerial parts of AM plants. There have been reports on the activity of antioxidants in AM plants, but mainly in response to abiotic stresses (Schutzendubel and Polle, 2002; Porcel and Ruiz Lozana, 2004). It has also been known for several years that different species of AM fungi can contribute to higher production and yield of essential oils in plants with medicinal virtues such as mint (Sirohi and Singh, 1983). A recent study showed that increase of essential oils in sweet basil colonized by *Gigaspora rosea* is correlated to a large number of peltate glandular trichomes per surface area (Copetta *et al.*, 2006). Another study reported increase in essential oil concentrations in Oregano plants colonized by *Glomus mosseae*, which was not related to improved P nutrition (Khaosaad *et al.*, 2006). Recently Toussaint *et al.* (2007) reported that *Glomus caledonium* and *G. mosseae* induced the production of antioxidants in the aerial parts of sweet basil.

Although there is enormous data on the yield and growth increase in several ornamental and vegetable crops as well as trees, limited research has been conducted to investigate the contribution of arbuscular mycorrhizal fungi on the quantitative and qualitative profile of the secondary metabolites typical of the plant (Strack *et al.*,

2003; Xin *et al.*, 2006). Hence, there is a need for research to improve the quality and quantity of drugs produced from native medicinal plants in relatively shorter period and at lower expense by using selective AM fungi. Therefore the present study was undertaken with following aims and objectives:

1. To study root colonization of AM fungi associated with the medicinal plants selected for the study.
2. To isolate, identify and study the diversity of AM fungal spores from the rhizosphere soil of medicinal plants.
3. To identify dominant indigenous AM fungi and their multiplication in the roots of compatible host in pot cultures.
4. To study the histochemical localization of polyphosphate granules and lipid bodies in intraradical mycelium of selected AM fungal species.
5. To investigate mycorrhizal status of medicinal plants selected for the study as influenced by phenology.
6. To study the response of selected AM fungal species on growth of selected medicinal plant species.

REVIEW OF LITERATURE

SECTION I - HISTORY OF ARBUSCULAR MYCORRHIZAL FUNGI:

Arbuscular Mycorrhizal (AM) fungi have been described as early as 1842 (Nageli, 1842) but most of Nageli's drawings only remotely resembled the AM fungi. Trappe and Berch (1985) and Rayner (1926, 1927) cited other earlier observations of the symbiosis during the period 1875-1895. Extensive surveys of host plants and anatomical descriptions on AM are given by Schlicht (1889), Dangeard (1896), Janse (1897), Petri (1903), Gallaud (1905), Peyronel (1924) and Lohman (1927). As early as 1889, Schlicht had already observed the basic anatomical relationships between the host and fungal tissues. Janse (1897), called the intramatrical spores "vesicles" and determined that other structures, named "arbuscules" by Gallaud (1905), were located in the inner cortex. Gallaud (1905) made a very accurate observation of the arbuscule and concluded that it is surrounded by a host membrane, which was later confirmed by Cox and Sanders (1974) using transmission electron microscopy. Gallaud (1905) also noted that partial digestion of the arbuscule resulted in a structure called the "sporangiole" by Janse (1897). This observation was confirmed by electron microscopy (Cox and Sanders, 1974). Gallaud (1905) further distinguished between Arum and Paris types of arbuscules (Smith and Smith, 1997). Jones (1924) described the term "appressorium". Link (1809) established the genus *Endogone*. Tulasne and Tulasne (1844) were the first to describe the genus *Glomus*, known only from spore clusters found in soil. Dangeard (1896) was the first to describe an arbuscular mycorrhiza, which happened to have formed from poplar roots.

Frank (1885) gave the name “mycorrhiza” to the peculiar association between tree roots and ectomycorrhizal fungi. A thorough discussion of the derivation of the word “Mycorrhiza”, including the incorporation of the second ‘r’ is given Kelly (1931, 1950). The name for the AM symbiosis has changed throughout the years. The symbiosis was once frequently called “Phycomycetous endomycorrhiza” to distinguish it from the endomycorrhizal symbiosis formed between members of the Ericaceae or Orchidaceae and higher fungi. The recognition that not all fungi formed vesicles led to the proposal that this symbiosis should be renamed Arbuscular mycorrhiza (Koide and Mosse, 2004).

The evolution of mycorrhizal symbiosis:

Paleobiology is a field dealing with the biological and ecological functions that can be deduced from fossils. Both paleobiological and molecular evidence indicate that AM is an ancient symbiosis that originated 460 million years ago. Arbuscular mycorrhizal symbiosis is ubiquitous among land plants, which suggest that mycorrhizae were probably present in the early ancestors of land plants. This positive association with plants may have facilitated the development of land plants (Simon *et al.*, 1993).

The Rhynie chert (siliceous rock of chalcedonic or opaline silica occurring in limestone) of the lower Devonian has yielded fossils of the earliest land plants in which AM fungi have been observed. The fossilized plants containing mycorrhizal

fungi were preserved in silica. They are prepared for observation by cementing pieces of the rock to microscope slides and then grinding the rock with carbide powder to a thickness of 50-150 μ m (Remy *et al.*, 1994).

The Early Devonian saw the development of terrestrial flora. Plants of the Rhynie chert from the lower Devonian (400 mya) were found to contain structures resembling vesicles and spores of present *Glomus* species. Colonized fossil roots have been observed in *Aglaophyton major* and *Rhynia*, which are ancient plants possessing characteristics of vascular plants and bryophytes with primitive protostelic rhizomes (Remy *et al.*, 1994). Intraradical mycelium was observed in root intracellular spaces and arbuscules were observed in the layer of thin cell walls similar to palisade parenchyma. The fossil arbuscules appear very similar to those existing AM fungi (Remy *et al.*, 1994). The cells containing arbuscules have thickened walls, which were also observed in colonized cells. Kar *et al.* (2005) have recently uncovered mycorrhizae from the Miocene, which exhibit a vesicular morphology closely resembling that of the present Glomerales. The need for further evolution may have been lost due to the readily available food source provided by the plant host (Kar *et al.*, 2005). The nature of the relationship between plants and the ancestors of AM fungi are characterized as follows:

- Mycorrhizal symbiosis may have evolved from a parasitic interaction which developed into a mutually beneficial relationship.

- An alternate hypothesis is that mycorrhizal fungi developed from saprophytic fungi that became endosymbiotic (Remy *et al.*, 1994).

There is some fossil evidence that the parasitic fungi did not kill the host cells immediately upon invasion although a response to the invasion was observed in the host cell. This response may have evolved into the chemical signaling processes required for symbiosis (Remy *et al.*, 1994). In both cases, the plant fungal interaction is thought to have evolved from a relationship in which fungi was taking nutrients from the plant into a symbiotic relationship where the plants and fungi exchanged nutrients.

The symbionts:

Arbuscular mycorrhizae are thought to be ecologically important to most vascular plants. Mycorrhizal fungi are found in most of the herbaceous plants and also in tree species (Harley and Smith, 1983). Plants classified as bryophytes, pteridophytes, gymnosperms and angiosperms have been found to form mycorrhizas. Most plants are capable of forming mycorrhizae with numerous species of AM fungi. The persistence of the relationship indicates that mycorrhizae confer some evolutionary advantage to plants in the form of nutrient uptake. Plants with roots that present little branching or lack of fine root hairs, relatively inefficient at seeking out P may receive maximum benefit from mycorrhizal symbiosis (Harley and Smith, 1983).

SECTION II- CLASSIFICATION:

Arbuscular mycorrhizal fungi comprise a monophyletic group of soil fungi, recently reclassified from the polyphyletic phylum Zygomycota to a newly proposed phylum Glomeromycota (Schußler *et al.*, 2001). This phylum was proposed after analysis of a large data set of 18S rRNA gene sequences of all known groups of fungi. To date, more than 190 AM fungal species have been described. They are classified in four orders encompassing 10 families and 14 genera (**Table 1**). *Glomus* is the largest genus containing 54.8% of all described species. In phylogenetic tree based on rDNA, Glomeromycota are sister group to Asco- and Basidio-mycota. The original taxonomy of the AM fungi was based on the morphology of the large soil borne spores, which were found near colonized plant host roots. Distinguishing mycorrhizal spore characteristics used in classification include wall morphologies, size, shape, colour, hyphal attachment and reaction to staining compounds (Wright, 2005).

The ancient phylogenetic origin of Glomales is confirmed by fossil findings, with symbiotic structures within fossil roots from Devonian (Remy *et al.*, 1994; Taylor *et al.*, 1995) and fossilized glomalean spores from the Ordovician, about 460 million years ago (Redecker *et al.*, 2000). Since molecular phylogenetic methods have been used to elucidate the phylogenetic relationships among these fungi, their classification has been in a rapid transition. Molecular studies have also revealed a large number of putative new species suggesting that the 150 morphologically-

Table 1: The classification of AM fungi by Schüßler *et al.* (2001) with emendations of Oehl and Sieverding (2004), Sieverding and Oehl (2006), Spain *et al.* (2006), and Walker and Schüßler (2004), Walker *et al.* (2007a, b) and Palenzuela *et al.* (2008).

PHYLUM

CLASS

ORDER

FAMILY

GENUS

GLOMEROMYCOTA Walker & Schüßler

GLOMEROMYCETES Cavalier-Smith

1. **ARCHAEOSPORALES** Walker & Schüßler

AMBISPORACEAE Walker, Vestberg & Schüßler

AMBISPORA Spain, Oehl & Sieverd.

ARCHAEOSPORACEAE Morton & Redecker emend. Oehl & Sieverd

ARCHAEOSPORA Morton & Redecker

INTRASPORA Oehl & Sieverd.

GEOSIPHONACEAE Engler. & Gilg emend. Schüßler

GEOSIPHON (Kütz.) Wettst

2. **DIVERSISPORALES** Walker & Schüßler

ACAULOSPORACEAE Morton & Benny

ACAULOSPORA Gerd. & Trappe emend. Berch

KUKLOSPORA Oehl & Sieverd.

DIVERSISPORACEAE Walker & Schüßler

DIVERSISPORA Walker & Schüßler

OTOSPORA Oehl, Palenzuela & Ferrol

ENTROPHOSPORACEAE Oehl & Sieverd.

ENTROPHOSPORA Ames & Schneid. emend. Oehl & Sieverd

GIGASPORACEAE Morton & Benny

GIGASPORA Gerd. & Trappe emend. Walker & Sanders

SCUTELLOSPORA Walker & Sanders

PACISPORACEAE Walker, Blaszk., Schüßler & Schwarzott

PACISPORA Oehl & Sieverd.

3. **GLOMERALES** Morton & Benny

GLOMERACEAE Piroz. & Dalpe

GLOMUS Tul. & Tul

4. **PARAGLOMERALES** Walker & Schüßler

PARAGLOMACEAE Morton & Redecker

PARAGLOMUS Morton & Redecker

defined species may vastly underestimate species diversity (Vandenkoornhuise *et al.*, 2002). Traditionally, glomeromycotan taxonomy is mainly based on the morphology of spores. The way the spore is formed on the hypha (mode of spore formation) has been important to circumscribe genera, families, and spore wall structure to distinguish species (Walker, 1983; Morton, 1988).

Prior to 1974, most AM fungi were in the genus *Endogone*. However, Gerdemann and Trappe (1974) removed AM fungi from *Endogone* and placed them in four separate genera viz., *Glomus*, *Sclerocystis*, *Acaulospora* and *Gigaspora*. Unlike the putatively asexual members of the Glomeromycota, *Endogone* species reproduce sexually via zygospores, indicating their phylogenetic link with the phylum Zygomycota. Phylogenetic analysis of the nuclear small subunit ribosomal RNA strongly suggest that *Endogone* (Endogonales) and the Glomeromycota do not form a clade (Gehrig *et al.*, 1996).

The order Glomales was erected by Morton and Benny (1990) for AM fungal classification with two more genera, *Scutellospora* and *Entrophospora* and three families (Glomaceae, Gigasporaceae and Acaulosporaceae). These families were characterized by the mode of spore formation and were initially supported by molecular data (Simon *et al.*, 1993).

Spores of *Glomus* and *Acaulospora* types were reported to be produced by several distinct, deeply divergent lineages (Redecker *et al.*, 2000a) subsequently, described as two new genera *Archaeospora* and *Paraglomus* (Morton and Redecker, 2001) and placed in separate families. Because some species in *Archaeospora* were dimorphic, members of this genus were classified originally in separate families (Morton *et al.*, 1997).

Molecular phylogenetic analysis has also shown that the species which form complex sporocarps formerly placed in the genus *Sclerocystis* are actually phylogenetically nested within well characterized *Glomus* species with simple spores (Redecker *et al.*, 2000b). The genus *Pacispora* comprising of some former *Glomus* species was erected by Oehl and Sieverding (2004). The spores of *Pacispora* have characteristics intermediate between *Glomus* and Gigasporaceae. Another emerging genus split off from *Glomus* is *Diversispora* (Morton and Benny, 1990). Only one *Glomus* species has been formally renamed so far, mainly based on ribosomal small subunit signatures (Walker and Schubler, 2004). The new classification includes the Geosiphonaceae; order Archaeosporales, which presently contain one fungal species that forms endosymbiotic association with the cyanobacterium *Nostoc punctiforme* and produce spores typical to AM fungi (Schubler, 2002).

The Glomeromycota are very old group with an estimated origin of at least 600 to 620 million years ago. Spores and hyphae of Glomalean fungi were discovered

in 460 million year old rocks from the Ordovician thus making them the oldest recognized fungal fossils to date (Redecker *et al.*, 2000b).

Phylogenetic Relationships:

In the recent classification, the phylogeny erected is based entirely on analyses of the small subunit RNA gene. rDNA phylogenies have shown that the genus *Glomus* is several times polyphyletic (Redecker *et al.*, 2000b; Schwarzott *et al.*, 2001). Species forming *Glomus*-like spores can be found in six different lineages within the Glomeromycota. *Paraglomus* appears to be the earliest-diverging glomeromycotan lineage in rDNA phylogenies, although sometimes receiving relatively weak bootstrap support. The separation of *Pacispora* and the *Diversispora* clade from other "*Glomus*" lineages is well supported by rDNA data. *Glomus* groups A and B are exemplified by the well-known species of *Glomus mosseae* and *G. claroideum* respectively. The two groups are genetically relatively distant but still form a monophyletic group in rDNA phylogenetic trees (Schwarzott *et al.*, 2001).

The formation of a "sporiferous saccule" was once thought to be characteristic of Acaulosporaceae (*Acaulospora* and *Entrophospora*), but now is known to occur in at least one additional lineage, namely *Archaeospora*. The Gigasporaceae (*Scutellospora* and *Gigaspora*) are distinguished by the formation of their spores on a "bulbous suspensor" and are well supported by molecular data. Gigasporaceae and Acaulosporaceae form a clade in most rDNA phylogenies, which is in conflict with

previous morphology-based analyses that placed *Glomus* and Acaulosporaceae together (Morton and Benny, 1990). The fungi of the Glomeromycota have coenocytic to sparsely septate mycelia. They reproduce asexually through blastic development of the hyphal tips and form symbiotic relationships with photoautotrophs.

Unusual polymorphism of ribosomal RNA in individual spores has led to the concept of internuclear variation in single spores, defining AM fungi as heterokaryotic organisms (Trouvelot *et al.*, 1999; Kuhn *et al.*, 2001). Heterokaryosis has been assumed to be of importance to ecology and application of AM fungi. This concept however has recently been challenged by experiments suggesting that single spores contain uniform population of nuclei characterized by intranuclear polymorphism (Pawłowska and Taylor, 2004).

Taxonomy:

Peyronel (1923) discovered that the regular occurrence of associations of spores and sporocarps of the Endogonaceae with AM fungi of plants and suggested fungi to be the originators of the mycorrhizae. Valuable data on the biology of fungi of the family Endogonaceae has been obtained from studies using pot cultures. The mode of germination of spores of these fungi, their life cycles, subcellular spore structures and the manner of colonization of roots has been recognized (Mosse, 1959,

1970). Mosse and Bowen (1968) prepared the first key for the recognition of the types of isolated endogonaceous spores.

Gerdemann and Trappe (1974) revised the family Endogonaceae in the order Mucorales, where 44 species belonging to seven genera were characterized. Among them, many taxa were redefined, and two genera (*Acaulospora*, *Gigaspora*) and 12 species were described as new. The genus *Endogone* contained 11 species with zygospores arranged in sporocarps. Tulasne and Tulasne (1845) erected the genus *Glomus* with 19 species with two varieties of *Gl. macrocarpus* Tul. & Tul., i.e., *Gl. macrocarpus* var. *macrocarpus* and *Gl. macrocarpus* var. *geosporus*, and also the genus *Sclerocystis* with four taxa containing species forming chlamydo spores blastically at hyphal tips. In contrast to sporocarpic *Sclerocystis* spp., the chlamydo spores of members of the genus *Glomus* have been considered to occur mainly in loose aggregates or singly in the soil, although the genus also included species forming compact sporocarps with or without a peridium. The distinctive property of genus *Sclerocystis* was the production of chlamydo spores arranged in a single layer around a central plexus.

The genera *Acaulospora* and *Gigaspora* have been defined by Gerdemann and Trappe (1974) as forming azygospores singly in the soil, although no parthenogenetical process of spore development was observed. Species of *Acaulospora* produced spores laterally on the neck of a sporiferous saccule and

species of the genus *Gigaspora* formed spores terminally at the tip of a bulbous sporogenous cell.

Ames and Schneider (1979) erected a new genus in Endogonaceae, *Entrophospora* with *E. infrequens*, a species earlier existing in genus *Glomus*, as *Gl. infrequens*. Spores of *E. infrequens* were formed inside the neck of a sporiferous saccule.

Walker and Sanders (1986) separated the genus *Gigaspora*, containing species with spores lacking an inner wall having no physical contact with their main structural wall, from genus *Scutellospora* with fungi forming spores having at least one inner wall.

Morton and Benny (1990) located soil-borne fungi forming arbuscules in roots of terrestrial plants in new order, Glomales consisting of two suborders, Glomineae and Gigasporineae. The former suborder consisted of the type family Glomaceae with genera *Glomus* and *Sclerocystis* and Acaulosporaceae comprising of the genera *Acaulospora* and *Entrophospora*. The latter suborder was proposed to include the Gigasporaceae with the genera *Gigaspora* and *Scutellospora*. Apart from differences in the mode of formation of spores and their sub cellular structure, the taxa of the suborder Glomineae distinguished the ability to form vesicles that did not occur in mycorrhizae of members of the suborder Gigasporineae.

Almeida and Schenck (1990) concluded that except for *Sclerocystis coremioides* a continuum of morphological properties exists between sporocarpic *Glomus* species and the other members of the genus *Sclerocystis*. As a result, the five-species genus *Sclerocystis* was reduced to single-species.

Redecker *et al.* (2000) utilizing both morphological and molecular data transferred *S. coremioides* to genus *Glomus* and thereby eliminated the genus *Sclerocystis* from Kingdom Fungi. Morton and Redecker (2001) based on data from molecular, morphological and biochemical investigations, erected two new families in the order Glomales, i.e., Archaeosporaceae and Paraglomaceae. Each of these families was phylogenetically distant from other glomalean families, despite similarities in mycorrhizal morphology. The family Archaeosporaceae contained one genus, *Archaeospora* with three species forming typical *Acaulospora*-like spores from the neck of a sporiferous saccule. Two of these species, *Ar. gerdemannii* and *Ar. leptoticha* were considered to dimorphic, forming *Glomus*-like spores. The genus *Paraglomus* in the family Paraglomaceae consisted of two species producing spores indistinguishable from those of *Glomus* species.

The fungi of the order Archaeosporales form endocytosymbioses with photoautotrophic prokaryotes [*Geosiphon pyriformis* (Kütz.) Wettstein emend. Schüßler] produce mycorrhizae with arbuscules with or without vesicles. Their spores are colourless and do not react in Melzer's reagent. Glomoid spores (identical to those

of fungi of the genus *Glomus*) form singly or in clusters on or under the soil surface. Acaulosporoid spores (similar to those of members of the genus *Acaulospora*) develop singly in the soil. They differ from other AM fungi by the possession of the rRNA SSU gene signature YCTATCYKYCTGGTGAKRCG, corresponding to homologous position 691 of *Saccharomyces cerevisiae* SSU rRNA sequence J01353, with the nucleotides being specific for the taxon. The order Archaeosporales contains two families, Archaeosporaceae with the genera *Appendicispora*, *Archaeospora* and *Intraspora* and Geosiphonaceae with the genus *Geosiphon*.

Members of the order Diversisporales form mycorrhizae with arbuscules, frequently lacking vesicles, with or without auxiliary cells. Spores develop either inside (entrophosporoid spores of the genera *Entrophospora* and *Kuklospora*) or laterally on the neck of a sporiferous saccule (acaulosporoid spores of the genus *Acaulospora*), from a bulbous base on the sporiferous hypha (gigasporoid spores of the genera *Gigaspora* and *Scutellospora*), or blastically at the tip of a sporogenous hypha (glomoid spores of the genera *Diversispora* and *Pacispora*). They differ from other AM fungi by the possession of the rRNA SSU gene sequence signature YVRRYW/1-5/NGYYYGB, corresponding to homologous position 658 of *S. cerevisiae* SSU rRNA sequence J01353 SSU rRNA, GTYARDYHMHYY/2-4/GRADRKKYGWCRAC, corresponding to homologous position of *S. cerevisiae* SSU rRNA sequence position 1346 of *S. cerevisiae* SSU rRNA sequence J01353, TTATCGGTTRAATC, corresponding to homologous position 650 of *S. cerevisiae*

rRNA SSU sequence J01353, and ACTGAGTTMATYT, corresponding to homologous position 1481 of *S. cerevisiae* rRNA SSU sequence J01353 with the nucleotides being specific for the taxon. The order Diversisporales is represented by five families, Diversisporaceae with the genus *Diversispora*, Acaulosporaceae with the genera *Acaulospora* and *Kuklospora*, Entrophosporaceae with the genus *Entrophospora*, Gigasporaceae with the genera *Gigaspora* and *Scutellospora* and Pacisporaceae with the genus *Pacispora*.

Fungi of the order Glomerales usually are hypogeous, rarely epigeous. They produce mycorrhizae with arbuscules, vesicles and spores. Spores form either blastically at the tip of a sporogenous hypha or intercalary inside them. Spores occur singly, in clusters or sporocarps having a peridium. They differ from other AM fungi by the possession of the rRNA SSU gene sequence signature **YTRRY/2-5/RYYARGTYGNCARCTTCTTAGAGGGACTATCGGTGTYTAACCGRTGG**, corresponding to homologous position 1353 of *S. cerevisiae* SSU rRNA sequence J01353, with the nucleotides being specific for the taxon. The order Glomerales includes one genus, *Glomus*.

Species of the order Paraglomerales form arbuscular mycorrhizae, rarely with vesicles. Spores are glomoid and colourless. The fungi differ from other AM fungi by the possession of rRNA SSU gene sequence signature **GCGAAGCGTCATGGCCTTAACCGGCCGT**, corresponding to homologous

position 703 of *S. cerevisiae* SSU rRNA sequence J01353, with the nucleotides being specific for the taxon. The order Paraglomerales is represented by one family Paraglomeraceae containing one genus, *Paraglomus*.

Genera of Arbuscular Mycorrhizal fungi:

Arbuscular mycorrhizal fungi are placed in four orders, viz., Archaeosporales, Diversisporales, Glomerales and Paraglomerales belonging to the class Glomeromycetes of the phylum Glomeromycota (Schußler *et al.*, 2001) under 14 genera viz., *Acaulospora*, *Archaeospora*, *Ambispora*, *Diversispora*, *Entrophospora*, *Gigaspora*, *Glomus*, *Intraspora*, *Kuklospora*, *Pacispora*, *Paraglomus*, *Scutellospora*, *Otospora* and *Geosiphon*.

The different genera of AM fungi are described below:

***Acaulospora* Gerdemann & Trappe emend. Berch.** Berch SM.1985. *Mycotaxon* 23: 409-418.

Etyomolgy: Greek, *a-*(without), *caulos* (stem), and *spora* (spore) - referring to the sessile spores.

Spores of fungi of the genus *Acaulospora* develop laterally from the neck of a sporiferous saccule (Morton and Benny, 1990; Morton 2000). The spores are sessile, i. e. no pedicel (a short branch of the sporiferous saccule neck) is formed. The wall of the most juvenile spores consists of only one layer continuous with the wall of a sporiferous saccule hypha. Spores produced singly in soil, generally globose to

subglobose with oily contents. Spore composed of two distinct, separable wall groups; outer wall is continuous laminated; variously ornamented, inner wall composed of one or more walls that are membranous, hyaline, laminated and ornamented. Spore walls are continuous except for a small-occluded pore. Spores of the genus *Acaulospora* germinate by germ tubes emerging from a plate-like germination orb formed by centrifugally rolled hyphae (Blaszkowski, 1994). The germ tubes penetrate through the spore wall. The mycorrhizae of *Acaulospora* species consist of (1) arbuscules with cylindrical or slightly flared trunks (2) irregular and knobby vesicles, and (3) straight and coiled intraradical hyphae with coils mostly concentrated at entry points (Morton, 2000).

***Gigaspora* Gerdemann & Trappe emend. Walker & Sanders.** Walker C, Sanders FE. 1986. *Mycotaxon*. 27: 169-182.

Etymology: Greek, *giga* (giant) and *spora* (spore). Referring to the exceptionally large spores typically produced by the members of the genus.

Azgosporos produced singly in soil, generally globose to subglobose, with oily contents, usually with a narrow hypha extending from the suspensor cell to the pore. Spores of *Gigaspora* develop blastically from a bulbous sporogenous cell formed at the end of a fertile hypha connected with mycorrhizal roots (Bentivenga and Morton, 1995; Walker and Sanders, 1986). The wall of the most juvenile, expanding spores consists of two layers of equal thickness. The inner layer thickens due to the synthesis of new sub layers (laminae). At the end of ontogeny, a warty or

knobby one-layered germination wall is formed, from which germ tubes arise. This wall tightly adheres to the inner surface of the laminate spore wall layer. The outermost spore wall layer of all the *Gigaspora* species is smooth. Apart from spores, *Gigaspora* species also form clusters of auxiliary cells. They are echinulate with spines. The mycorrhizae of *Gigaspora* species consist of only arbuscules and hyphae staining darkly in trypan blue; no vesicles are produced (Bentivenga and Morton, 1995). Arbuscules generally form fine branches directly from a swollen basal hypha. Intraradical hyphae are straight to coiled and vary in diameter because of the presence knob-like projections and inflated areas.

***Scutellospora* Walkers & Sanders.** Walker C, Sanders FE. 1986. *Mycotaxon*. 27: 169-182.

Etymology: Latin *scutellum*- small-shield and *spora*, spore referring to the production of germination shield in spores of members of the genus.

Spores produced singly in soil variable in shape, usually globose or subglobose often ovoid, obovoid, pyriform or irregular borne on a bulbous sporogenous cell formed at the end of a fertile hypha connected with mycorrhizal roots (Walker and Sanders, 1986) usually with a narrow hypha extending from one or more peg-like projections towards the spore. Spore wall structure consists of two wall groups of equal thickness. The inner layer thickens due to addition of new sub layers (laminae). The formation of spore wall ends the differentiation of a third thin, flexible layer, which is tightly adherent to the laminate layer. Germination by means of one or

more germ tubes produced from the spore base through the germination shield formed upon or within a flexible inner wall. The mycorrhizae of *Scutellospora* species consist of only arbuscules and hyphae staining darkly in trypan blue; no vesicles are produced (Morton, 2000). Arbuscules develop from swollen basal hyphae. Intraradical hyphae are straight or coiled and vary in diameter because of the presence of knob-like projections and inflated areas. Thin-walled, knobby or broadly papillate auxiliary cells borne in soil on straight or coiled hyphae, formed singly or in clusters.

Glomus Tulasne & Tulasne. Gerdemann JW, Trappe JM. 1974. *Mycologia Memoir* No.5: 76.

Etymology: Latin, *glomus* (a ball of yarn), possibly in reference to sometimes rounded and cottony appearance of the species.

Spores of *Glomus* species develop blastically at the end of sporogenous hyphae, although intercalary spore formation has also been reported (Declerck *et al.*, 2000). In most species, the sporogenous hyphae develop from extraradical hyphae of mycorrhizal roots. The surface of spores of *Glomus* species may be smooth (in most species) or ornamented. Some species produce spores enveloped in a hyphal mantle 'Gleba' consisting of interwoven 'Peridium'. The wall layers of a subtending hypha are continuous with spore wall layers. At the end of spore development, the lumen of the subtending hypha usually becomes closed by either (1) a curved septum continuous with the innermost lamina of the laminate spore wall layer (2) an invaginated flexible innermost layer (3) an amorphous plug and (4) thickening

subtending hyphal wall. Spores of the genus *Glomus* germinate by emergence of the germ tube through the lumen of the subtending hypha (most species) or the spore wall. Most species of the genus *Glomus* produce spores singly in the soil. Other taxa form more or less compact spore aggregates consisting of spores and a peridium. The mycorrhizae of *Glomus* species consist of arbuscules, vesicles (not always formed), and intra- and extra-radical hyphae. Arbuscules have cylindrical or slightly flared trunks with branches progressively tapering in width toward tips. Vesicles usually are thin-walled and ellipsoid. Intraradical hyphae usually spread along roots and frequently form Y-shaped branches, H-shaped connections and coils mainly occur at entry points.

***Intraspora* Oehl & Sieverd.** Sieverding E, Oehl F. 2006. *Journal of Applied Botany and Food Quality*. 80: 69-81.

Spores occur singly in the soil or in roots. The spores develop inside the neck of a sporiferous saccule at some distance from the saccule. The sporiferous saccule originates terminally or intercalary in extra- and intra-radical hyphae. The spores are globose to subglobose and frequently pyriform. Their sub cellular structure consists of two walls, a spore wall and an inner germination wall. The spore wall is composed of two layers, of which the outer layer sloughs with age and is continuous with the wall of the neck of the sporiferous saccule. The inner layer of this wall is persistent, semi flexible and closes two opposite pores of spores. The inner germination wall is semi-flexible and laminate. The mycorrhizae comprise of arbuscules, vesicles as well

as intra- and extra-radical hyphae. Vesicles form rarely and all the mycorrhizal structures stain faintly in trypan blue.

***Kuklospora* Oehl & Sieverd.** Sieverding E, Oehl F. 2006. *Journal of Applied Botany and Food Quality*. 80: 69-81.

Spores develop inside the neck of a sporiferous saccule at some distance from this saccule and originate from the neck and saccule contents. The sporiferous saccule originate terminally or intercalary inside mycorrhizal extraradical hyphae by their swelling and are globose to subglobose. The sub cellular structure consists of a 3-layered, coloured spore wall and two inner colourless germination walls. The outermost spore wall layer is colourless, and is continuous with the wall of the sporiferous saccule neck. The second structural layer of this wall consists of coloured, tightly adherent, thin sub layers (laminae). This layer occasionally develops towards the saccule, forming a stalk supporting the wall of the sporiferous saccule neck. The first inner germination wall consists of two adherent flexible to semi-flexible layers. The second germination wall is composed of three layers, of which the outermost one is ornamented with small granules. The spores forms typical mycorrhizae intensively stained in trypan blue.

***Pacispora* Oehl & Sieverd.** Sieverding E, Oehl F. 2006. *Journal of Applied Botany and Food Quality*. 80: 69-81.

Spores of fungi of the genus *Pacispora* develop blastically at the end of cylindrical sporogenous hyphae (subtending hyphae) continuous with extraradical hyphae of AM fungi. The spores of members of this genus consist of three wall layers. The ontogenetic development of spores of *Pacispora* species is by the formation of a uniform, plate-like germination shield on the surface layer of the inner germination wall. A germ tube grows from this shield and penetrates through the spore wall. The mycorrhizae consist of arbuscules, vesicles, intra- and extra-radical hyphae, as well as of auxiliary cells. The arbuscules, vesicles and hyphae morphologically resembled those of *Glomus* species and stained intensively in trypan blue. The auxiliary cells occur both outside and inside roots and are knobby.

***Paraglomus* Morton & Redecker.** Morton JB, Redecker D. 2001 *Mycologia* 93: 181-195.

Etymology: Resembling "*Glomus*" with identical spore morphotypes.

Spores of species of the genus *Paraglomus* develop blastically at the tip of extraradical hyphae. The spores of the known species of this genus occur singly in the soil. They are globose to irregular and colourless to pale coloured. The sub cellular structure of spores of *Paraglomus* consists of a spore wall comprising two to three layers continuous with those of their subtending hyphae. Spores of *Paraglomus* species germinate by germ tubes emerging from both the lumen of the subtending

hypha and the spore wall (Morton and Redecker, 2001). Arbuscules of *Paraglomus* species are cylindrical or slightly flared trunks with branches progressively tapering in width towards the tips (Morton, 2002; Morton and Redecker, 2001). The mycorrhizae of *Paraglomus* species do not contain vesicles and their intraradical hyphae are frequently coiled within and between cortical cells. The main visible evidence of mycorrhizae of *Paraglomus* species is their light staining or the lack of any staining reaction in trypan blue or other stains.

***Archaeospora* Morton & Redecker.** Morton JB, Redecker D. 2001 *Mycologia*. 282-285.

Etyomology: Greek, "archaios" = ancient, referring to the ancient position of this genus in Glomales.

Archaeospora is dimorphic, forming both acaulosporioid and glomoid spores (Morton and Redecker, 2001; Sieverding and Oehl, 2006; Spain *et al.*, 2006). Acaulosporioid spores develop laterally, directly on the neck of a sporiferous saccule and are sessile. Two-layered glomoid spores origin blastically at the tip of or intercalary in fertile hyphae, as spores of *Glomus* species. Germination of *Archaeospora* spores is by a germ tube emerging from an irregular germination structure (Spain, 2003). Mycorrhizae of *Archaeospora* (1) do not contain intraradical vesicles or they form rarely, (2) have intraradical hyphae with many coils located within and between cortical cells, (3) stain lightly or not at all in trypan blue and other stains, and (4) are patchily distributed along roots (Morton, 2002).

***Diversispora* Walker & Schußler.** Walker C, Schußler A. 2004. *Mycological Research*. 108: 979-982.

Spores of *Diversispora* develop blastically at the tip of cylindrical to slightly flared sporogenous hyphae continuous with extraradical hyphae of AM fungi. The mycorrhizae of most *Glomus* species consist of arbuscules, vesicles and hyphae staining intensively in trypan blue whereas those of *D. spurca* lack vesicles and stain variably, from almost no staining to intensive staining (Morton, 2002).

***Entrophospora* Ames & Schneid. emend. Oehl & Sieverd.** Ames RN, Schneider RW. 1979. *Mycotaxon* 8: 347-352.

Spores occur singly in the soil or inside roots (Blaszkowski *et al.*, 1998; Sieverding and Oehl, 2006). The spores develop inside the neck of a sporiferous saccule directly at or at a short distance from the saccule originating from the neck. The sporiferous saccule originate terminally or intercalary inside extra- and intraradical hyphae by their swelling. The spores are globose to subglobose and coloured; their sub cellular structure consists of a multilayered, coloured spore wall and one inner 3-layered, colourless germination wall. In spores lacking the sporiferous saccules, two opposite cicatrices resembling small rings with a slightly raised border are visible. The cicatrices are frequently accompanied by stalks developed from the permanent spore wall layers. The mycorrhizae of *Entrophospora* showed intense staining in trypan blue (Sieverding and Oehl, 2006).

***Ambispora* Walker Vestberg & Schüßler.** Walker C, Vestberg M, Schüßler A. *Mycological Research*. 111: 253-255.

Species of the genus *Ambispora* are dimorphic producing both acaulosporoid and glomoid spores i.e. spores originating similarly to those of *Acaulospora* and *Glomus* species (Morton and Redecker, 2001; Spain *et al.*, 2006). The acaulosporoid spores occur singly in the soil and the glomoid ones are formed singly or in loose clusters in the soil and develop terminally from the thin walled hyphae grown from either the wall of a pedicel or branched germ tubes (Spain *et al.*, 2006). In contrast to the sessile acaulosporoid spores of the genus *Acaulospora* and *Archaeospora*, those of *Ambispora* species develop blastically at the tip of a short branch formed at the distal end of the neck of a sporiferous saccule. This branch is called appendix or pedicel. The sporiferous saccule of *Ambispora* species originate terminally from mycorrhizal extraradical hyphae by their swelling. The spores of the known species of *Ambispora* are globose to subglobose and coloured. The sub cellular structure consists of three layered, coloured spore wall and two inner colourless germination walls. The outer spore wall completes development subsequent to the formation of the outer layer of the first inner germination wall. The spore wall and the outer layer of the first inner germination of the spores of *Ambispora* species are continuous with the pedicel wall layer. The mycorrhiza of the species *Ambispora* consists of arbuscules, vesicles as well as intra- and extra-radical hyphae. All these structures stain faintly in trypan blue (Spain *et al.*, 2006).

***Archaeospora* Morton & Redecker.** Morton JB, Redecker D. 2001 *Mycologia*. 93: 181-195.

Archaeospora trappei the only member of the genus *Archaeospora* is a dimorphic fungus, producing both acaulosporoid and glomoid spores (Morton and Redecker, 2001; Sieverding and Oehl, 2006; Spain, 2003; Spain *et al.*, 2006). Acaulosporoid spores develop laterally on the neck of a sporiferous saccule, are sessile similarly as most spores of the genus *Acaulospora*. Two layered glomoid spores originate blastically at the tip of intercalary in fertile hyphae as spores of *Glomus* species. The sub cellular structure of acaulosporoid spores *Archaeospora trappei* comprises of a spore wall and one inner germination wall, each consisting of two to three layers. Germination of *Archaeospora trappei* spores is by germ tube emerging from an irregular germination structure (Spain, 2003).

Mycorrhizae of *Archaeospora trappei* 1) do not contain intraradical vesicles or they form rarely, 2) have intraradical hyphae with many coils located within and between cortical cells 3) Stain lightly or not at all in trypan blue and other stains and 4) are patchily distributed along roots (Morton, 2002).

SECTION III- LIFE CYCLE OF ARBUSCULAR MYCORRHIZAL

FUNGI:

The life cycle of an AM fungus can be divided in three main stages:

A. Establishment of symbiosis: This involves propagule activation, host search, appressorium formation, root penetration and formation of arbuscules.

B. Vegetative growth phase: This involves intra- and extra-radical mycelial growth, increase of fungal biomass, formation of mycelial structures and expansion of mycorrhizal colonization within and between plants.

C. Reproductive phase: This involves the formation of reproductive structures.

Resting spores are the major type of propagule.

The AM fungal mycelium is dimorphic and non-septate or coenocytic (Mosse, 1981). The non-septate hyphae allow a fast cytoplasmic flow in a bi-directional way, not only carrying resources from source to sink regions of the fungal colony and/or symbiotic root, but also transporting fungal organelles, such as mitochondria and nuclei (Bago *et al.*, 1998b; Bago *et al.*, 1999).

The major mycelial structures are characterized as follows:

Intraradical hyphae: Intraradical hyphae consists of inter- and intra-cellular hyphae in roots that contain storage materials and take part in transportation of substances absorbed by extraradical hyphae from the soil to arbuscules or directly to root cells of the host plant (Bieleski, 1973). Intraradical hyphae may be straight or with H- or Y-shaped branches. They may also form coils, whose frequency of occurrence depends on their location in root and the generic affiliation of the AM

fungal species (Morton, 2000). Generally, coils more abundantly occur at entry points. Intraradical hyphae of *Glomus* species are infrequently coiled in the other regions of a mycorrhizal root. In contrast, coils produced by species of the other genera of AM fungi are abundant and evenly distributed along mycorrhizal roots.

Arbuscules: Arbuscule is a specialized morphological structure hypothetically shared by all AM fungal species (Morton and Benny, 1980). Arbuscules are haustoria-like structures that are formed by profuse dichotomous hyphae branching after penetration into inner plant cortical cell walls, forming an interface between fungal tissue and the plant plasma membrane. This interface is thought to be the major site for nutrient and carbon exchanges between both partners, and it is considered the key structure for establishment of a functional symbiosis (Smith and Read 1997; Harrison, 1999). The arbuscules are usually short-lived (1 to 3 weeks), and are preferentially found in young thin roots during early stages of root colonization (Mosse, 1981; Smith and Read 1997; Harrison, 1999). However, long-lived arbuscules have also been reported in woodland plants (Brundrett and Kendrick, 1990). The arbuscule formation is genetically controlled by the host plant, and the numbers of arbuscules formed is dependent on plant species, availability of nutrients, as well as on the fungal partner (Harrison, 1999). Arbuscules are the main sites of nutrient exchange between a plant host and a fungus (Gianinazzi *et al.*, 1979). They are formed within the cells of the inner root cortex (Mosse, 1973) and are indicators of active mycorrhizae. Arbuscules differ in morphology, depending on the generic

affiliation of the AM fungal species (Morton, 2000). Fungi of the genera *Acaulospora*, *Ambispora*, *Appendicispora*, *Diversispora*, *Entrophospora*, *Glomus*, *Intraspora*, *Kuklospora* and *Paraglomus* produce arbuscules with cylindrical or slightly flared, narrow trunks, whose branches progressively taper in width towards tips. Arbuscules of members of the genera *Gigaspora* and *Scutellospora* generally have swollen trunks with branches tapering abruptly at tips (Morton, 2000).

Types of arbuscules:

Gallaud (1904, 1905) surveyed microscopically endomycorrhizas of many plant species and divided them into the following four classes based on types of internal fungal structures and named after plant species or plant taxa in which the type structures were found.

1. *Arum maculatum* series (Arum type) in which initial fungal penetration into epidermis and hypodermis is followed by development of hyphae along the cortical intercellular airspaces and then penetration of cortical cells to form simple and terminal intracellular arbuscules, the walls of which are modified by becoming very thin and which have an amorphous chitin deposition (Bonfante- Fasolo *et al.*, 1990).

2. *Paris quadrifolia* (Paris type) in which the fungus is entirely intracellular with irregular coiled hyphae on some of which are formed 'composite' (compound arbuscules) that are not terminal and are localized in definite layers. In paris type, both hyphal coils and arbusculate coils are involved in

nutrient exchange, and the surface area of hyphal coils is equal to that of arbuscules in arum type mycorrhizas (Dickson and Kolesik, 1999) as well as by the presence of a membrane and interfacial matrix around hyphal coils and arbusculate coils (Armstrong and Peterson, 2002). Arbuscule and arbusculate coils are separated from the cortical cell cytoplasm by a periarbuscular membrane and interfacial matrix material, both derived from the plant symbiont (Armstrong and Peterson, 2002). The Arum type has been reported to be abundant in agricultural crops whereas the Paris type has been found to be more frequent in plants in natural ecosystems (Smith and Smith, 1997; Yamato and Iwasaki, 2002; Ahlu *et al.*, 2005; Tsuyuzaki *et al.*, 2005).

3. Hepatic (liverwort) series, resembling Paris type but with arbusculate structures not organized in layers. Gallaud observed this type in gametophytes of *Pellia epiphylla* and *Conocephalum (Fegalleta) conicum*.

4. Orchid series, in which the fungus is intracellular and tightly coiled, forming 'pelotons'.

Vesicles: Globose or ovoid, thin-walled vesicles are storage organs filled with lipids and glycolipids (Mosse, 1981). They are formed by an intercalary or terminal swelling of hyphae of AM fungi. Vesicles may be inter- or intra-cellular and may be found in both the inner and the outer layers of the cortical parenchyma. In *Glomus* species, vesicles generally are ellipsoid, whereas those of *Acaulospora*, *Entrophospora* and *Kuklospora* highly vary in shape and frequently have knobs and

concavities on their surface (Morton, 2000). Vesicles are never produced by members of the genera *Gigaspora* and *Scutellospora*. No vesicles are found in any species of *Archaeospora*, *Ambispora* and *Intraspora*.

Auxiliary cells: Auxiliary cells are formed by short ramifications occurring simultaneously at both sides of the extraradical hyphae. Each ramification generates several branches that swell and form clusters, which are composed of two to more than 20 balloon-like structures, of about 12-39µm in diameter. Auxiliary cells are metabolically active structures, rich in nuclei, organelles and lipids (Jabaji-Hare *et al.*, 1986; Bonfante and Bianciotto, 1995). However, little is known about their biological function. It has been suggested that auxiliary cells are reminiscent of relict reproductive spores (Morton and Benny, 1990) and has been found only in *Gigasporaceae*. They have a spine or smooth surface in *Gigaspora* and *Scutellospora*.

Extraradical hyphae: Extraradical hyphae significantly increase the absorptive area of roots (Bielecki, 1973) and form hyphal bridges transferring nutrients between co-occurring plants (Newman, 1988). They are also important fungal propagules colonizing plant roots (Jasper *et al.*, 1989, 1991). The extraradical mycelium is of key importance for the fungus and to the function of the symbiosis. The majority of spores are formed in the external mycelium. The extraradical hyphae are also responsible for spreading the root colonization within and between plants, generating

an underground link between plants in a community (Smith and Read, 1997). They found evidence for distinct intra- and extra-radical mycelium development strategies which could be related to taxonomic differences between the families, and the results were independent of the host plant used.

Spores: Spores are multinucleate single cells mainly produced blastically at the tip of extraradical hyphae. Sometimes spores also occur inside roots (Koske, 1985), on the soil surface (Berch and Fortin, 1983), and on plants or their decaying fragments (Blaszkowski *et al.*, 1998). Arbuscular mycorrhizal fungi form spores ranging from 22 to 1050 μ m in diameter (Schenck and Perez, 1990). The number of spores produced depends on the fungal species (Blaszkowski, 1993), the plant species and its variety (Blaszkowski, 1993; Hetrick and Bloom, 1986), soil fertility and fertilizer application (Hayman, 1970), host phenology (Giovannetti, 1985), light intensity (Daft and El Giahmi, 1978) and competitive abilities of co-occurring AM fungal species (Gemma *et al.*, 1989). The reproduction of AM fungi is stated to be clonal (Morton, 2000) and the role of spores is to sequester the genetic information of a given fungal species, disperse the information to new habitats, and initiate new individuals spatially separated from the parent organisms (Morton, 1993). Because many components of the subcellular structure of spores are stable in different environmental conditions, they are the most important structures considered in classification of AM fungi.

Spore formation: The genera of AM fungi are separated on the basis of asexual (anamorphic) spore formation and spore characteristics. The term azygospore or chlamydospore describe the anamorphic stage of AM fungi. Spores of AM fungi are multinucleated and are heterokaryotic. The spores can germinate at different times and some taxa reproduce sexually by forming zygo spores if proper mating types are present (Tommerup, 1987).

Arbuscular Mycorrhizal fungi can produce ectocarpic spores free in the soil or in sporocarps, also in dead animals (Rothwell and Victor, 1984), in dead seeds (Taber, 1982), in plant roots (Morton and Walker, 1984; Koske, 1985), in dead spores of other AM fungi (Koske, 1984) or in soil surface (Berch and Fortin, 1983a; McGee, 1986). Spores in the soil may be produced terminally or laterally on subtending hyphae or on a single suspensor cell. The sporocarpic species produce spores in loose arrangement or in a highly ordered arrangement around a hyphal plexus (Gerdemann and Trappe, 1974; Berch and Fortin, 1983a; McGee, 1986). Spores in *Glomus* are formed terminally on one or more hyphae. *Glomus* species form single spores or spores in sporocarps where the spores are arranged randomly in the matrix hyphae around the central plexus of sterile hyphae (Gerdemann and Trappe, 1974).

Acaulospora and *Entrophospora* tend to form spores associated with a small hyphal chamber. Spores in *Acaulospora* are formed laterally on the stalk of a large terminal and thin walled hyphal chamber (Berch, 1985). However in *Entrophospora*,

spores are produced completely within the neck of the hyphal chamber (Ames and Schneider, 1979).

Gigaspora and *Scutellospora* are separated from other AM fungi by mode of spore germination. In *Scutellospora* germination is via the germination shield found within the spore. In *Gigaspora*, no germination shield is formed, and germination is by the direct growth of one or more germ tubes through the spore wall (Walker and Sanders, 1986; Walker, 1987).

Spore germination: Spores of AM fungi are able to germinate and grow from a quiescent state in response to different edaphic and environmental conditions, irrespective of the presence of host plants. Germ tubes are not capable of extensive hyphal development, and in the absence of the host, cease growth within 15-20 days of germination (Becard and Piche, 1989; Logi *et al.*, 1998). The elongating germ tubes give rise to a coenocytic mycelial network, containing many nuclei and total mycelial lengths range from 30-50mm in *Glomus caledonium* to 8mm in *Glomus clarum* and to 18-25mm in *Gigaspora margarita* (Louis and Lim, 1988; Gianinazzi-Pearson *et al.*, 1989).

Spore morphology: Dissecting, light and electron microscopes are used to determine spore size, shape, colour and wall structure of all known species of AM fungi. *Glomus tenue* is the smallest spores with an average 10-12µm in diameter. In

contrast, *Gigaspora gigantea* is the largest spores, the spore diameter ranged from 183-500 x 291-812 μ m. Morton (1986) suggested that the variation in spore shape may be the result of environmental stress. Thin walled spores produced in the root cortex are often ellipsoid as in *Glomus intraradices*, *Glomus diaphanum* and *Scutellospora pellucida*. Spores that are produced with a large thick wall are mostly globose or subglobose as in *Glomus clarum* and *Glomus mannihot*. Ovoid, obovoid, pyriform, irregular, reniform, pyriform and clavate spores occur in AM fungi. Spore colour ranged from hyaline to white to pale yellow, orange, red, brown, dark brown or black. The difference in colour could be due to pigmentation in the spore wall or in the spore content (Morton, 1988).

Traditionally, morphological characteristics of the spore walls of AM fungi are used in species identification (Mosse and Bowen, 1968; Gerdemann and Trappe, 1974; Trappe, 1982). Spore wall characteristics became the most important morphological characteristics after Walker (1983) suggested use of standardized terminology for wall micrographs. He originally proposed four wall types, viz., the unit, laminated, evanescent and membraneous wall and also introduced the concept of hyphal peridial wall. As new species were described, new categories of spore walls were added, i.e coriaceous wall (Walker, 1986), amorphous wall (Morton, 1986) expanding wall (Berch and Koske, 1986) and germinal wall (Spain *et al.*, 1989).

Subtending hyphal morphology: The spores of AM fungi are produced on one or more subtending hyphae. Most *Glomus* species have single subtending hyphae on their spore (Morton, 1988). Some species such as *G. glomerulatum*, *G. heterosporum* and *G. lacteum* produce spores on 1-3 subtending hyphae. *Glomus formosanum*, *G. multisubtensum* and *G. multicaule* form spores on 1-4 subtending hyphae.

The colour of the subtending hyphae in most species of *Glomus*, *Gigaspora* and *Scutellospora* is similar or lighter than spore wall colour. Subtending hyphal shape ranged from straight, recurved, cylindrical, flared, funnel shaped, constricted to irregular. The opening between the subtending hyphae and the spore content may remain open or may be closed by a plug or septum by spore wall thickening or by spore inner wall. Width and length measurement of the subtending hyphae are used in species identification.

Sporocarp morphology: Sporocarps are formed in peridial and possibly in glebal hyphae in some species of *Glomus*. Sporocarps are not known in *Entrophospora*, *Gigaspora* and *Scutellospora* (Berch, 1985; Walkers and Sanders, 1986). Sporocarps are typically absent in *Acaulospora*, the exceptions being *A. myriocarpa* in which spores are in cluster (Schenck *et al.*, 1986) and *A. sporocarpa* which has an aggregation of spores in a network of hyphae (Berch, 1985). External sporocarp colour ranges from white to brown. However, internal sporocarp colour ranges from white to black to brown in *Glomus* species. Sporocarp is irregular but is globose,

subglobose or ellipsoid in *Glomus* species (Morton, 1988). Two terms are used to describe hyphal arrangement in sporocarp: the term peridium is used when the hyphae form a loosely or tightly interwoven network on the surface of the sporocarp, and gleba is used when the hyphae form a matrix in the sporocarp.

Spore dispersal: Arbuscular mycorrhizal fungi depend on passive means of spore dispersal. Wind and animals are good vectors for spore dispersal (Friese, 1984; MacMohan and Warner, 1984). In arid ecosystems, wind might be the most important dispersal agent for AM inoculum (Warner *et al.*, 1987). However, animals are the major vectors for AM inoculum in mesic habitats (Maser *et al.*, 1978; Allen, 1987). In soil, spores of AM fungi are protected by tiller or rhizome leaves and scales, which carry spores with them to new sites. When roots arise at the node of the tillers or the rhizomes, hyphae of germinated spore penetrate the young roots and establish mycorrhizal association (Gemma, 1987).

The number of AM fungal spores in the soil is a good indicator of species abundance. Arbuscular mycorrhizal fungal spores are usually more numerous and diverse in cultivated soils than under natural vegetation (Hayman, 1978; Hayman and Stovold, 1979). Since AM fungal spores are not easily dispersed from the point of sporulation, the upper ten centimeters of soil is the best indicator of AM fungal populations (Friese and Koske, 1991).

Spore germination: Spore germination in AM fungi has been studied in only few species. Mosse (1970) studied spore germination in *Acaulospora laevis* where spore germination occurred by the growth of a germ tube from a peripheral chamber that has formed between the walls. Further studies showed that chamber was not formed before germination (Walker, 1987). Berch and Fortin (1983b) studied spore germination in three genera (*Endogone*, *Gigaspora* and *Acaulospora*) and suggested that there is a phylogenetic relationship between these genera based on the formation of an inner wall germ tube.

There is no published information on spore germination for *Entrophospora* (Walker, 1987). In *Glomus* species, spores typically germinate either by a germ tube that penetrate through the subtending hyphae or by germ tubes that emerge that directly through spore wall (Walker and Rhodes, 1981; Miller and Walker, 1986).

Morphological Characteristics: Spores, subtending hyphae and sporocarp morphology are used as taxonomical features in AM fungal identification. However, Hall (1977) and Morton (1985) concluded that the characteristics (spore size, spore colour and spore structure) and ontogenetic characteristics (production of arbuscules, vesicles and number of spores) were not influenced significantly by the host. Later, Morton (1988) apparently contradicted himself and hypothesized that either the host or environmental factors may cause variation in morphological structures. Abbott (1982) developed a key for 10 species of AM fungi that was based entirely on the

morphological anatomy of hyphal development in AM fungi and used twenty characteristics including hyphal diameter, mode of branching vesicles, arbuscules and staining reaction in the key and these characteristics are stable in different host and soil environment.

Molecular Biology: The biochemical and genetic characterization of AM fungi has been hindered by their biotrophic nature, which impedes laboratory culturing. The obstacle had recently been surpassed with the use of hairy root cultures. The first mycorrhizal gene to be sequenced was the small subunit SSU rRNA (ribosomal RNA) (Simon *et al.*, 1992). This gene is highly conserved and commonly used in phylogenetic studies. The SSU rRNA was isolated from spores of each taxonomic group and amplified using PCR techniques (Simon *et al.*, 1993). A molecular clock approach based on the substitution rates of SSU sequences was used to estimate the time of divergence of the AM fungi. The molecular analysis found that the AM fungi are between 353 and 462 million old (Simon *et al.*, 1993). More recent molecular clock analyses date back the origin of AM fungi and first land plants but all data known suggests that AM fungal symbiosis may have been instrumental in the colonization of land by plants.

SECTION IV- ROLE AND BENEFITS OF ARBUSCULAR

MYCORRHIZAL (AM) FUNGI:

In terms of ubiquity and partnerships throughout the plant kingdom, mycorrhizal relationships are the most significant plant-microbe symbiosis. It is well documented that elevation of soil P concentrations as a result of intensive agriculture can decrease the soil populations of AM fungi (Smith and Read, 1997). Thus, mycorrhizal technology becomes an important consideration in low-input, organic or soil less agriculture. Plant growth can also be inhibited as a result of the accumulation of phytotoxic levels of heavy metals and organic xenobiotics. Application of mycorrhizal technologies requires knowledge of biodiversity across and within the species involved. This may be achieved through bioaugmentation by inoculating soils with AM fungi or by using transplanted seedlings that already have the appropriate AM fungi in their roots.

Contribution of AM fungi to soil health: Arbuscular mycorrhizal fungi are essential components of soil biota, as they can be found in nearly all ecological situations, mainly in natural ecosystems, particularly those supporting plant communities with high species diversity and in normal cropping systems, especially with sustainable practices (Gianinazzi and Schuepp, 1994). Arbuscular mycorrhizal fungi are obligate symbionts and their life cycle depends on plant roots and in return they decrease disease in the host and reduce population levels of pathogenic

microorganisms in the soil, especially when the supply of P is limiting (Linderman, 1994).

Arbuscular mycorrhizal fungi develop intensively inside roots and within the soil by forming an extensive extraradical network and this help plants considerably in exploiting mineral nutrients and water from the soil. The important role of soil mycelium in the formation of water stable soil aggregates is well documented (Miller and Jastrow, 2000). Arbuscular Mycorrhizal fungi produce a very stable hydrophobic glycoprotein, glomalin, which is deposited on the outer hyphal walls of the extraradical mycelium and on adjacent soil particles, which appear to act as a long-term soil-binding agent (Wright and Upadhyaya, 1999).

Interaction of AM fungi and abiotic factors: A number of abiotic factors such as climate change, drought stress, pollution and heavy metal contamination can influence the development of mycorrhizal relationships. Inefficient mining processes, treatment of soil with sewage sludge or industrial effluents, overuse of heavy metal containing fertilizers or gas exhaust are other factors that contribute to the creation of large areas contaminated by heavy metals radionuclides and persistent organic pollutant (Jeffries *et al.*, 2003). Arbuscular mycorrhizal fungi also have a potential role in monitoring the site toxicity (Gucwa Przepiora and Turnau, 2001) and in restoration techniques (Orlowska *et al.*, 2002). A well developed mycorrhizal symbiosis enhance the survival of plants in polluted areas by better nutrient

acquisition, water relations, pathogenic resistance, phytohormone production, contribution to soil aggregation, amelioration of soil structure and also in bioremediation (Jeffries *et al.*, 2003).

Arbuscular mycorrhizal fungi have been found to decrease cesium uptake by plants (Berreck and Haselwandter, 2001) and this could be used in the establishment of plant vegetation on soil contaminated with radionuclides, and therefore have the potential to reduce environmental risks (Jeffries *et al.*, 2003).

Interactions of Arbuscular Mycorrhizal (AM) fungi and biotic factors:

Arbuscular mycorrhizal fungi are the key components of soil microbiota and interact with other microorganisms in the rhizosphere (Bowen and Rovira, 1999). Mycorrhizal colonization changes plant physiology and certain nutritional and physical properties of the rhizosphere soil. This in turn, affects colonization patterns by soil microorganisms by the so-called mycorrhizosphere effect (Gryndler, 2000). Arbuscular mycorrhizal fungi thus interact with natural and introduced microorganisms in the mycorrhizosphere hence affecting the soil properties and quality. Soil microorganisms can produce compounds that increase root cell permeability, thereby increasing the rates of root exudation. This in turn stimulates the growth of hyphae of AM fungi in the rhizosphere and facilitates root penetration by the fungus. Rhizosphere microorganisms are also known to affect the presymbiotic stages of AM fungal development (Giovannetti, 2000) such as spore germination and

germ tube growth (Azcon-Aguilar and Barea, 1995). Biologically active substances such as amino acids, plant hormones, vitamins, other organic compounds and volatile substances (CO₂) produced by soil microorganisms can stimulate the growth rates of AM fungi (Azcon-Aguilar and Barea, 1995).

In Horticulture and Agriculture: The use of AM fungi in agriculture could lead to a considerable decrease in the amount of chemical pollution in soil water, as recently demonstrated for maize (Giovannetti, 2001). This clearly indicates the potential of AM fungi for promoting a low chemical input agriculture. The recent development in molecular probes could differentiate AM fungi within roots and soils (Jacquot-Plumey *et al.*, 2001) and also opened new biotechnological perspectives for defining their population biology and management strategies in the use of these symbiotic microbes in agriculture. Successful inoculation is usually achieved when AM fungi are introduced very early in the plant developmental process followed by the use of low amount of phosphate fertilizers and selective use of pesticides (Guillemin *et al.*, 1993). By doing so, colonization by AM fungi will follow root development of the inoculated seedlings or cuttings with the consequence that plants will already be extensively mycorrhizal when transplanted into the field. Micro propagated plants inoculated with AM fungi can 1) reduce plant losses during the acclimatization phase, 2) subsequently stimulate plant development (induce flowering) and 3) increase productivity after transplantation to the field (Estaun *et al.*, 1999).

In alleviating desertification: As a result of the ecosystem degradation processes in desertification of threatened areas, disturbance of natural plant communities is often accompanied by loss of physico-chemical and biological properties, such as soil structure, plant nutrient availability, organic matter content and microbial activity. There is an increasing interest in using AM fungi to improve revegetation processes for desertified ecosystems as inoculants. Experiments carried out for assessing the long-term benefits of inoculation of shrub legumes with rhizobia and AM fungi include improving the establishment of target legume species as well as the benefits induced by symbiotically tailored seedlings in physico-chemical properties of soil (Requena *et al.*, 2001).

In bioremediation of soils containing pollutants: Phytostabilisation is a process in which pollutants are immobilized by plant activity, resulting in attenuation of wind and soil erosion and run off processes into ground water or air (Losi *et al.*, 1994). Phytodegradation covers the whole range of metabolic processes in which plants usually assisted by microorganisms, degrade organic compounds such as hydrocarbons, pesticides and explosives. Phytoextraction involves hyper metal accumulating plants, which contain more than 1% of metals in harvestable tissues. Mycorrhizal fungi were shown to be involved in the degradation of organic pollutants and thus may be potentially useful in phytodegradation. Although colonization by AM fungi was negatively affected by increasing polycyclic aromatic hydrocarbons

(PAHs) level in soil (Levyal and Binet, 1998) enhanced plant survival and growth by decreasing P deficiency (Joner and Levyal, 2001), water stress (Sanchez-Diaz and Honrubia, 1994), improving membrane integrity (Graham *et al.*, 1981) and stimulation of oxidative enzyme production (Salzer *et al.*, 1999).

Arbuscular mycorrhizal fungi can also be helpful in the management of constructed wetlands used for detoxification of a broad range of toxic substances. This plays an important role in the initial steps of the establishment of wetland places and subsequently influences plant biodiversity in later stages, encouraging the re-appearance of mycorrhizal species (Vangronsveld *et al.*, 1996). Recently, the presence of AM fungal symbiosis was also demonstrated in hyper accumulating plants, which is being used in phytoextraction. For successful bioremediation, symbionts must be selected that could withstand the hostile environment of polluted sites (Jeffries *et al.*, 2002).

Phytoremediation: The use of AM fungi in ecological restoration projects have enabled their host plant establishment on degraded soil and improved soil quality and health (Jeffries *et al.*, 2002). A relatively new approach to restore land and protection against desertification is to inoculate the soil with AM fungi with the reintroduction of vegetation. Jeffries *et al.* (2002) demonstrated that significantly long term improvement in soil quality parameters was attained when the soil was inoculated with a mixture of indigenous AM fungal species compared to uninoculated soil and

soil inoculated with a single exotic species of AM fungi. The benefits observed were an increased plant growth and soil nitrogen content, higher soil organic matter content and soil aggregation. The improvements were attributed to the higher legume nodulation in the presence of AM fungi, better water infiltration and soil aeration due to soil aggregation. Inoculation with native AM fungi increased plant uptake of P thereby improving plant growth and health, and also supported AM fungi as a biological tool in the restoration of self-sustaining ecosystems (Jeffries *et al.*, 2002).

Soil quality: Arbuscular mycorrhizal fungi enhance soil aggregate stability through the production of a soil protein known as glomalin. Glomalin related soil proteins (GRSP) have been identified using a monoclonal antibody (Mab32B11) raised against crushed mycorrhizal spores and is defined by its extraction conditions and reaction with the antibody Mab32B11. Glomalin is hypothesized to improve soil aggregate water stability and decrease soil erosion (Rillig, 2004).

The AM fungal diversity regulates patterns of plant diversity, if one AM fungal species or indigenous species becomes extinct in a habitat (Allen *et al.*, 1995). As AM fungal species are below ground organisms, they spread slowly over short distances, using plants as stepping-stones. When a plant establishes itself, the plant and soil dwelling mycorrhizal community coadapt to develop a symbiosis (Allen *et al.*, 1995). Moreover, plants in distributed areas of low fertility would benefit from a diverse mycorrhizal community, as there is greater chance of adaptation to

environmental changes (Abbott and Gazey, 1994). Additionally soil surface temperature above 60°C kill mycorrhizal spores which decreases the infectivity potential in the surface layer (Thompson, 1989).

Crop nutrition: Arbuscular Mycorrhizal (AM) fungi play a significant role in crop nutrition, by increasing total P uptake (Koide *et al.*, 2000), growth and yield (Ibibijen *et al.*, 1996; Koide *et al.*, 2000) but under conditions of high soil P concentrations, it may reduce crop growth (Kahiluoto *et al.*, 2001). Though P uptake usually dominates consideration of the AM fungal association, it has become increasingly apparent that mycorrhizae can be important in the uptake of other nutrients. Zinc is most commonly reported being influenced by the AM association, though uptake of Cu, Fe, N, K, Ca and Mg also being enhanced by AM fungal association (Smith and Read, 1997; Clark and Zeto, 2000).

Crop pests and diseases: Arbuscular mycorrhizal fungi play a major role in the suppression of crop pests and diseases, particularly soil-borne fungal diseases (Linderman, 1994; Borowicz, 2001). Other types of pest and disease causing organisms which may be suppressed by AM fungi include pathogenic nematodes (Talavera *et al.*, 2001) above ground fungal diseases (Feldmann and Boyle, 1998) and herbivores (Gange *et al.*, 2002). Though the mechanisms involved are complex,

change in nutritional status resulting in changes to leaf defensive chemicals, are likely to be involved in above ground interactions with herbivores (West, 1995).

Interaction with other soil microorganisms: Bacterial communities and specific bacterial strains promote germination of AM fungal spores and can increase the rate and extent of root colonization by AM fungi (Johansson *et al.*, 2004). The legume–*Rhizobium* symbiosis is strongly influenced by AM fungi and enhanced P nutrition arising from the AM fungal colonization resulted in an increase in nodulation and N₂ fixation (Vazquez *et al.*, 2002).

Crop water relations: Arbuscular mycorrhizal fungi increase the host plant's tolerance to water stress (Auge, 2004) and several mechanisms have been proposed to explain the effect including increased root hydraulic conductivity, improved stomatal regulation, osmotic adjustment of the host and improved contact with soil particles through the binding effect of hyphae, enabling water to be extracted from smaller pores (Auge, 2004).

Benefits of AM fungi: Arbuscular Mycorrhizal colonized plants exhibit an increased rate of photosynthesis (Dixon *et al.*, 1994) and tolerance to drought (Osnumi *et al.*, 1992) and salinity (Rosendahl and Rosendahl, 1991). Resistance to root pathogens also increases primarily due to AM fungi occupying the root niche and

increased plant vigour (Smith and Read, 1997). Another feature important in the establishment of seedlings is the transport of photo derivatives from an unshaded to a shaded plant via the AM fungal mycelium (Eissenstat and Newman, 1990).

As AM fungi is a mutualistic symbiont, it drains upto 20% photosynthetic carbon (Jakobsen and Rosendahl, 1990) and in return, provide plants with large amounts of nutrients (P, N, K, Zn) and water from the soil. Arbuscular Mycorrhizal fungi also produce glycoprotein extracellularly on the mycelia in the bulk soil, which together with the physical network of hyphae helps to aggregate soil thus improving aeration and water percolation (Wright and Upadhyaya, 1998). The carbon inflow to soil attracts soil microbes; altogether producing a functionally diverse and dynamic soil biota which are fundamental for plant nutrition in natural systems and sustainable agriculture (Schreiner *et al.*, 1997).

The benefit of AM fungal symbiosis depends on when in a plant life stage its roots are colonized by AM fungi (Solaiman and Hirta, 1996). The outcome is also affected by the growth rates of both fungi and plant. Moreover, the AM symbiosis is also influenced by the composition of plant and fungal species (Schreiner *et al.*, 1997) by the hierarchical structure of AM fungal species in the root niche, and by their inherited genetic and functional diversity (Smith and Gianinazzi Pearson, 1988). Additionally the outcome of an AM fungal symbiosis is affected by soil properties,

soil and plant treatments and the presence and amount of soil microbes being mutualists, commensalists and inhibitors or parasites.

Mycorrhizae also enhances plant growth and improves crop yield by essential nutrient absorption, improvement in plant photosynthesis, nutrients translocation and plant metabolism processes which reduces the use of chemical fertilizers and in turn increases income for the farmers. Mycorrhizae are endurable to several chemical substances; e.g. pesticide such as endrin, chlordane, methomyl carbofuran, herbicide such as glyphosate, fuazifopbutyl, chemical agents for plant disease elimination such as captan, benomyl, maneb triforine, mancozed and zineb.

Evaluation of biodiversity and conservation of AM Fungi: The diversity of AM fungi has significant ecological consequences as individual species or isolates vary in their potential to promote plant growth and adaptation to biotic and abiotic factors. Thus, the composition and dynamics of populations of AM fungi have marked impact on the structure and diversity of the associated plant communities, both in natural and agricultural ecosystems (van der Heijden *et al.*, 1998). An important pre requisite to the analysis of populations of AM fungi in ecological studies is the correct identification of individual isolates. Allozymes have been helpful in providing diagnostic biochemical markers to identify species of AM fungi, even in colonized roots. However, the most powerful tools to study the evolution and

population genetics of AM fungi are molecular techniques that analyze DNA sequences (Sen and Hepper, 1996).

A wide variety of techniques could be employed to detect DNA sequence variation in populations of AM fungi (Lanfranco *et al.*, 1998). PCR amplification of targeted genomic sequences followed by RFLP, allele-specific hybridization, direct sequencing or single strand conformation polymorphisms are increasingly used to detect AM fungi in natural ecosystems (Redecker *et al.*, 1997; Helgason *et al.*, 1999). DNA markers have been successfully employed to track specific AM fungi from agricultural and natural ecosystems (Jacquot-Plumey *et al.*, 2001).

SECTION V - INFLUENCE OF CLIMATIC FACTORS ON AM FUNGI:

Soil environmental conditions as well as plant nutrient level, light intensity and cropping systems affect the development of AM fungi and the formation of mycorrhizae (Evans and Miller, 1990; Furlan and Fortin, 1977; Jasper *et al.*, 1989, 1991; Reinharts *et al.*, 1994).

Temperature: Of the factors influencing mycorrhizal development and function, temperature is important (Fabig *et al.*, 1989). Temperature strongly influences the physiology of living organisms and low temperature influence plant roots and mycorrhizal fungal development. Mycorrhizal development is usually optimal; at least in plants of cool temperate climates at 20–25⁰C (Matsubara *et al.*, 2000; Zhang

et al., 1995) and maximal spore germination occurs between 20⁰C and 28⁰C depending on the species (Wang *et al.*, 1997). Charest *et al.* (1993) reported that mycorrhizae counteract chilling injury in maize (*Zea mays* L.). AM colonization increased nodule size and leaf N concentration in soybean grown at a root zone temperature of 15⁰C (Zhang *et al.*, 1995). Mycorrhizal leek plants exposed to the same root zone temperature were better able to absorb P³² from soil than non-mycorrhizal plants (Wang *et al.*, 2002).

There is data on the effect of temperature on spore germination and hyphal elongation under axenic conditions. Whereas *Glomus mosseae* and *Acaulospora laevis* can germinate between 10-18⁰C and 30⁰C with an optimum between 20⁰C and 30⁰C (Safir, 1986), and germination was best at 10-25⁰C for *G. caledonium* (Tommerup, 1983) and at 25⁰C for *G. epigaeum* (Graham, 1982). The optimal germination temperature seems to depend on the environment where the fungus has been isolated. *Scutellospora coralloidea* and *S. heterogama* both isolated in Florida germinated at 34⁰C whereas *G. mosseae* isolated from cooler regions showed maximal germination at 20⁰C and failed to germinate at 34⁰C (Schenck *et al.*, 1975).

Apart from the direct effect of temperature on AM fungal spore germination and hyphal growth, the temperature to which dormant spores are exposed can affect spore germination (Hepper and Smith, 1976; Gemma and Koske, 1988), spore mortality and hyphal growth pattern (Juge *et al.*, 2002). Cold storage (4⁰C) of spores

of *Glomus intraradices* for more than 14 days increased spore germination, reduced spore mortality and resulted in the growth of a clearly distinguishable, several centimeter long hypha with few branches, whereas hyphal growth of spores stored at a higher temperature (25⁰C) was without a viable dominant hyphae, and the hyphae emerging from the spores continuously curled and branched heavily (Juge *et al.*, 2002).

A number of studies reported inhibition of mycorrhizal development at temperatures allowing plant growth. Arbuscular mycorrhizal colonization of soybean was strongly repressed at a root zone temperature of 15⁰C (Zhang *et al.*, 1995). Colonization of barley was similarly repressed at 15⁰C and inhibited at 10⁰C (Baon *et al.*, 1994). Arbuscular mycorrhizal spores and mycelium from 20 glomalean isolates from soil and root debris survived in storage at 80⁰C (Kuszala *et al.*, 2001). The stimulation of AM spore germination by a period of exposure to low temperature suggests that some AM fungi possess mechanisms of dormancy that synchronize their activity to the seasonal cycle of cool climate ecosystems (Juge *et al.*, 2002). Sporulation by an isolate of *Glomus fasciculatum* was greatest at 30⁰C, which coincided with the optimum temperature for the host plant (Ferguson and Menge, 1982).

Root colonization by AM fungi often decreases when the temperature exceeds 30⁰C (Bowen 1987), and soil temperatures above 40⁰C which are generally lethal to

AM fungi (Bendavid-Val *et al.*, 1997). The germination of spores of *Scutellospora coralloidea* and *S. heterogama* was found to decrease above 34⁰C (Schenck *et al.*, 1975). The presence of AM fungal arbuscules in soybean roots were found to decrease above 30⁰C, while production of external hyphae outside soybean roots was found to decrease above 34⁰C (Schenck and Schroder, 1974). Haugen and Smith (1992) reported that colonization of cashew (*Anacardium occidentale*) roots by *Glomus intraradices* declined above 30⁰C and was severely reduced at 38⁰C.

Temperature also can influence secondary mycorrhizal colonization so that certain species might not achieve that minimum level of colonization necessary to trigger sporulation (Frank and Morton, 1994). Temperature is certainly one of the major environmental factors influencing AM fungal species distribution in the field. Schenck and Schroder (1974) observed that sporulation and mycelial growth of *Gigaspora* species was optimized at higher temperatures within the range of temperature tested and correlated the affinity of the fungus for high temperature with its presence and abundance in summer rather than spring or winter crops. Koske (1987) observed that some AM fungal species were more abundant in northern cooler regions than in southern warmer regions within a latitudinal temperature gradient and also found that frequency of some species such as *Scutellospora weresubiae* and *Glomus tortuosum* was correlated with higher temperatures.

Negative effects of mycorrhizae on plant growth at suboptimal temperatures have been attributed to the inability of an established fungus to take up (Bowen *et al.*, 1975) or transport P (Hayman, 1977) while still utilizing host carbon (Hetrick and Bloom, 1986).

pH: The efficiency of AM fungi is influenced by properties of the soil (Slankis, 1974). The AM fungi have been found in soils from pH 2.7 to 9.2, but different fungal isolates have varied pH tolerances (Siqueira *et al.*, 1984). Optimal pH conditions for spore germination differ between species and genera. The optimal pH for spore germination seems to be linked to the pH of the soil, where the AM fungi are isolated (Giovannetti, 2000). *Acaulospora* species germinates between pH 4 and 5 (Hepper, 1984), *Gigaspora* species at a pH from 4 to 6 and *Glomus* species between pH 6 and 9 (Green *et al.*, 1976).

CO₂: Although released by roots, CO₂ cannot be regarded as a plant specific signal for AM fungi, as CO₂ levels in the soil can also be increased from other sources such as the respiration of soil organisms. When initiating AM fungal monoaxenic cultures, an enriched CO₂ atmosphere developed in the petriplate due to root organ respiration is probably an activator of spore germination and of asymbiotic hyphal growth under these conditions (Vierheilig and Bago, 2005).

The CO₂ effect on AM fungi seems to be concentration dependent. In axenic systems, CO₂ levels ranging from 0.5 to 2.5% stimulate hyphal growth of *Gigaspora margarita* (Becard *et al.*, 1989; Becard *et al.*, 1992) CO₂ level of 0.1% showed no effect on hyphal growth (Poulin *et al.*, 1993) and high CO₂ levels (5%) irreversibly inhibited in vitro growth of *Glomus mosseae* (Le Tacon *et al.*, 1983). Hyphal growth of AM fungi was highest at CO₂ levels around 2% (Becard *et al.*, 1992; Poulin *et al.*, 1993) a concentration that usually is found in most soils. Becard and Piche (1989) suggested that during germination CO₂ may be a net source of carbon for anabolic processes of the spore. Bago *et al.* (1999) have demonstrated recently that a significant rate of trehalose, a short-term fungal storage carbohydrate was ¹³C-labelled when ¹³CO₂ was supplied to asymbiotic spores.

Light: In nature, the exposure of soil borne AM fungi to light is an extremely unlikely event as underground roots are colonized. Light treatments affect the growth pattern of axenically growing hyphae. Light induced hyphal branching was observed in developing germ tubes of *Gigaspora gigantea*, *Gi. rosea* and *Glomus intraradices* (Nagahashi *et al.*, 2000).

Light availability (presumably through its effects on plant carbon fixation) is positively correlated with AM fungal formation (Bethlenfalvay and Pacovsky, 1983; Tester *et al.*, 1986), P uptake (Son and Smith, 1988; Smith and Gianinazzi-Pearson, 1990), root soluble carbohydrate levels (Graham *et al.*, 1982), and plant growth

response (Hayman 1974; Bethlenfalvay and Pacovsky, 1983). Hayman (1974) observed that mycorrhizal colonization in onion roots was reduced and consisted of fewer arbuscules and external hyphae under lower light intensity.

Earlier studies demonstrated a decrease in growth response on inoculation with AM fungi as light intensity diminished (or as defoliation increased), but little or no change in the extent of intraradical AM fungal colonization (Bayne *et al.*, 1984; Pearson *et al.*, 1991) was observed. Tester *et al.* (1986) measured fewer AM fungal entry points under low irradiance, but no change in the rates of either root or AM hyphal extension. Hayman (1974) noted fewer and smaller arbuscules in AM plants grown at low light intensity compared to those grown at higher light (Pearson *et al.*, 1991). Similarly, decreased AM sporulation (Ferguson and Menge, 1982) and fewer vesicles (Bethlenfalvay and Pacovsky, 1983, Pearson *et al.*, 1991) have been observed in a variety of herbaceous plants under decreased photon irradiance.

As AM fungus is a significant carbon sink in the mycorrhizal plant system (Kucey and Paul, 1982; Harris *et al.*, 1985), the influence of light environment on mycorrhizal colonization is indirectly mediated by the carbon status of the host plant. Previous studies in AM systems have shown that light intensity decreases root to shoot ratio (Son and Smith, 1988), root length (Tester *et al.*, 1985) and initiation of lateral roots (Tester *et al.*, 1986). Several studies have documented lower levels of

soluble carbohydrates in roots (Hayman 1974) and diminished root exudation under low irradiance (Graham *et al.*, 1982).

CHAPTER 1

ARBUSCULAR MYCORRHIZAL ASSOCIATION IN MEDICINAL PLANTS

INTRODUCTION

India is endowed with a rich wealth of medicinal plants. Although a good proportion of the medicinal plant species do occur throughout the country, peninsular Indian forests and the Western Ghats are highly significant with respect to varietal richness (Parrota, 2001). Medicinal plants are important for pharmacological research and drug development, not only as plant constituents used directly as therapeutic agents, but also as starting materials for the synthesis of drugs or as models for pharmacologically active compounds (Mukherjee, 2003). It is reported that in India, 4,365 ethnic communities, including over one million folk healers, use around 8,000 species of medicinal plants. They are also becoming increasingly economically important due to the growing demand for herbal products in the domestic and global market.

Across the country, the forests are estimated to harbour 90% of the India's medicinal plant diversity and only about 10% of the known medicinal plants of the country are restricted to non-forest habitats. Demand for medicinal plants is increasing in both developing and developed countries due to growing recognition of natural products, being non-toxic, having no side effects and easily available at affordable prices. Due to an increasing demand for medicinal plants and a loss and fragmentation of natural habitats, close to 300 species of Indian medicinal plants have been so far assessed as under threat in the wild (based on International Union for Conservation for Nature (IUCN) (Red List Criteria). Around 1,000 species are

estimated to be facing various degrees of threat across different biogeographic regions in the country (Seth and Sharma, 2004).

Arbuscular mycorrhizal (AM) fungi are a major component of rhizosphere microflora in natural ecosystems and play a significant role in the re-establishment of nutrient cycling (Peterson *et al.*, 1985). Mycorrhizal fungi modify the structure and function of plant communities (Douds and Miller, 1999) and may be useful indicators of ecosystem change (McGonigle and Miller, 1996).

Earlier studies on the occurrence of AM fungi in medicinal plants are mostly on rhizomes (Taber and Trappe, 1982; Selvaraj *et al.*, 1986). Later, Nasim (1990); Udea *et al.* (1992); Gautam and Sharma (1996); Rani and Bhaduria (2001); Selvaraj *et al.* (2001), Muthukumar *et al.* (2001) and Pawar and Tarafdar (2006) reported the occurrence of AM fungal association in medicinal plants from India.

The Western Ghats, a valuable repository for biodiversity after the Himalayas, is one of the 34-mega diversity hot spots of the world. It contains 4000 (27%) of the country's plant species, of which 38% (1500 species) are endemic. The high biodiversity of the Western Ghats can be attributed to its varied habitat types ranging from semi-arid grasslands to tropical rainforests (<http://web.biodiversityhotspots.org/xp/hotspots/ghats/biodiversity.xml>).

Recently mycorrhizal association in several plant species from different habitat types of Western Ghats region of Southern India were reported (Appasamay and Ganapathi, 1995; Muthukumar *et al.*, 1996; Muthukumar and Udaiyan, 2000; Khade *et al.*, 2002 and Bukhari *et al.*, 2003). However, the species diversity and composition of AM fungal communities from medicinal plants of the Western Ghats of Goa region is largely unknown. Therefore, the present work was undertaken to study the AM fungal status in medicinal plant species of Western Ghats region of Goa state.

MATERIALS AND METHODS

Study sites:

Roots and rhizosphere soil samples of selected medicinal plant species were collected between January 2004 to March 2006 from different localities of both North and South Goa in Western Ghats region (**Fig. 1**). The climate is tropical, warm and humid with laterite, lateritic, clayey-loamy soil. Mean temperature ranged from 20°C-35°C with average rainfall of 2500mm.

North Goa: The North Goa District has an area of 1736 sq. kms and lies between 15° 48' 00" N to 14° 53' 54" N latitudes and 73° E to 75° E longitudes. The Western Ghat areas selected for the study include Valpoi, Ustae, Bhuipal, Mhadei and Sanquelim (**Fig. 1**).

South Goa: The South Goa District has an area of 1966 sq. kms situated between the latitudes 15°29'32" N and 14°53'57" N and longitudes 73°46'21" E and 74°20'11" E. The areas selected for the study include Tamdi Surla, Sadolxem, Codra, Neturlim, Sanguem, Uguem, Surla, Sancordem and Mollem (**Fig. 1**).

Sample collection:

Thirty-six medicinal plant species belonging to 25 families along with the rhizosphere soil samples were collected from different localities of both North and South Goa of Western Ghat region. Samples were collected in all the three seasons viz., monsoon (June-September), post-monsoon (October-January) and pre-monsoon (February- May). Both wild (19) and cultivated (17) plant species (**Plate 1 a- f & Plate II g-l**) were selected for the study. Identification of the plant species was carried out based on the floras of Rao (1985) and Mathew (1991).

Soil analysis:

For soil analysis, samples were collected from a depth of 0-25cm from different locations of North and South Goa and were brought to the laboratory in polythene bags. Samples were passed through a 2mm sieve to remove larger soil particles and were mixed thoroughly to obtain a homogenous sample.

Soil pH was measured in 1:2 soil water suspension using pH meter (LI 120 Elico, India). Electrical Conductivity (EC) was measured at room temperature in 1:5

Fig. 1: Map of Goa showing the study sites.

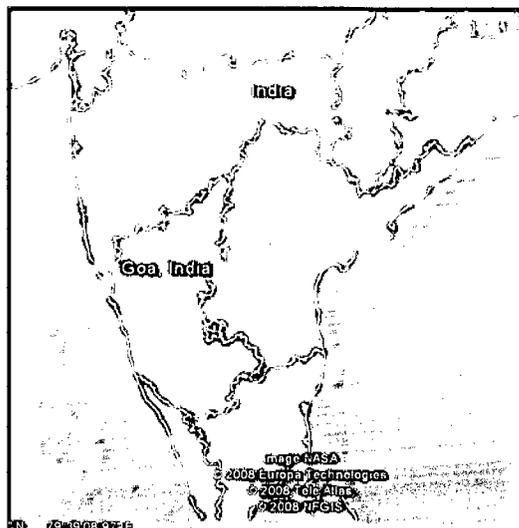
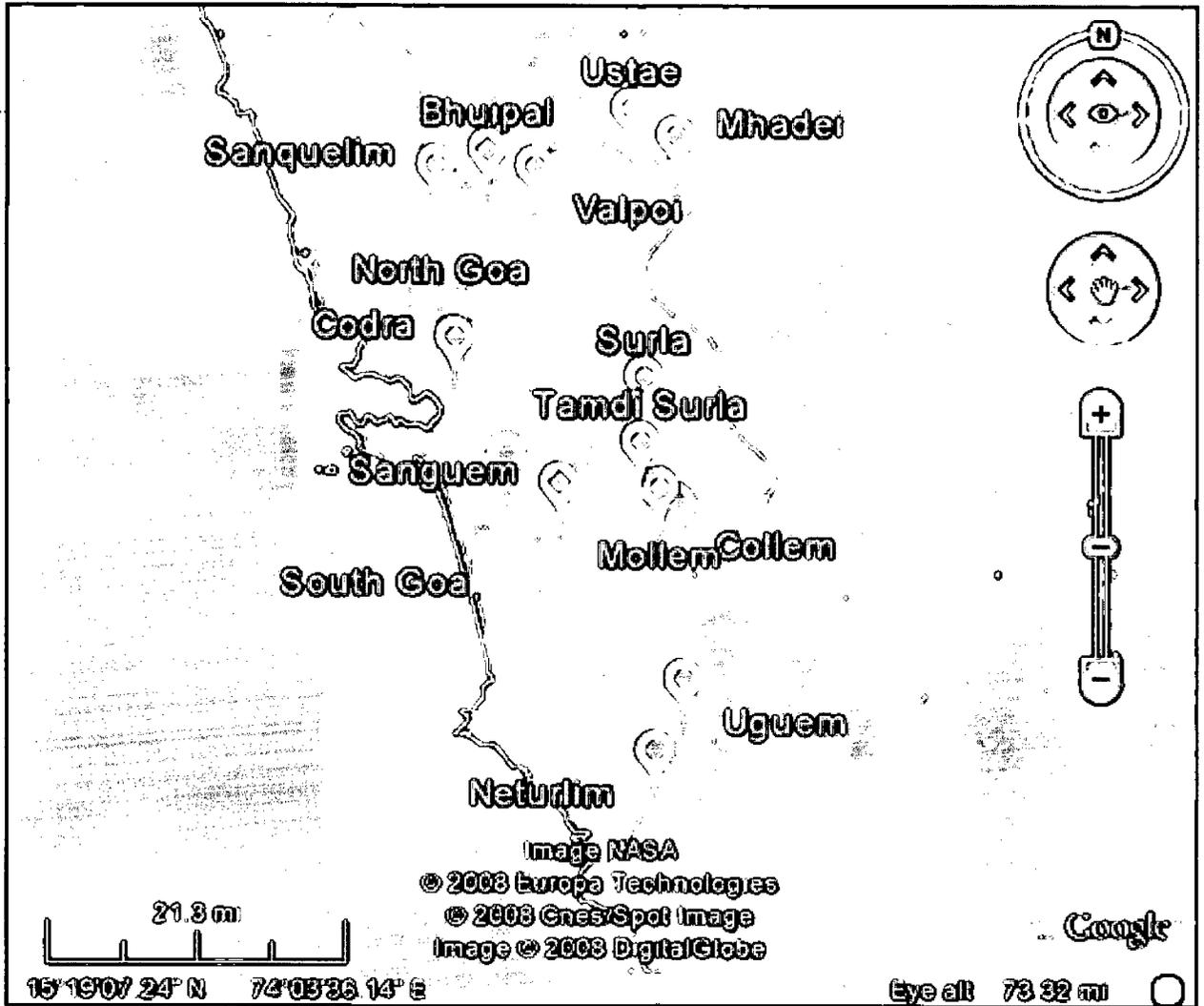


Plate I: Medicinal plant species

- a) *Aloe vera* L.
- b) *Andrographis paniculata* Nees.
- c) *Ocimum sanctum* L.
- d) *Leucas aspera* L.
- e) *Hemidesmus indicus* R. Br.
- f) *Ixora coccinea* L.

Plate I

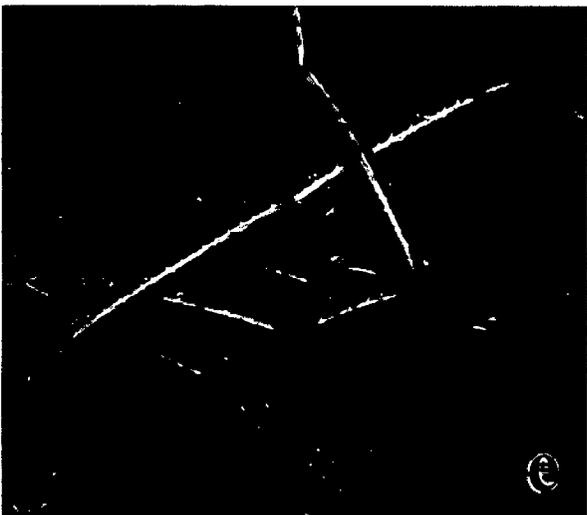
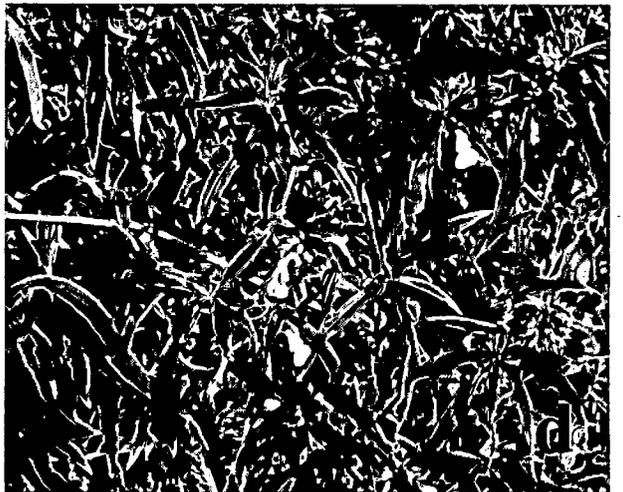
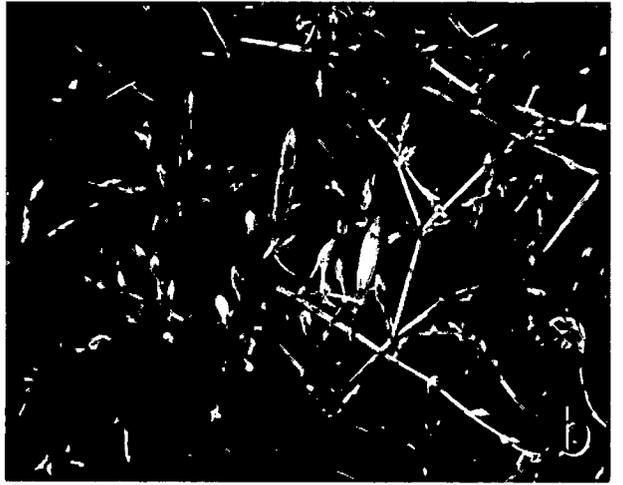


Plate II: Medicinal plant species

- g) *Piper nigrum* L.
- h) *Clitoria ternatea* L.
- i) *Bryophyllum pinnatum* (Lam.) Kurz.
- j) *Hibiscus rosa-sinensis* L.
- k) *Cymbopogon citrates* Stapf.
- l) *Rauwolfia serpentina* (L.) Benth.

Plate II



soil suspension using Conductivity meter (CM-180 Elico, India). Standard soil analysis techniques viz., Walkley and Black (1934) rapid titration method and Bray and Kurtz method (1945) were employed for determination of organic carbon and available phosphorus respectively. Available potassium was estimated by ammonium acetate method (Hanway and Heidel, 1952) using flame photometer (Systronic 3292). Available zinc, copper, manganese and iron were quantified by DTPA-CaCl₂-TEA method (Lindsay and Norvell, 1978) using Atomic Absorption Spectrophotometer (AAS 4139). Soil analysis was carried out in Ela Farm, ICAR, Old Goa.

Estimation of AM fungal colonization:

Homogenous rhizosphere soil samples prepared for each of the plant species were packed in polythene bags, labeled and brought to the laboratory. Root samples were freshly processed for AM fungal colonization whereas soil samples were stored in refrigerator at 4⁰C until analyzed. Fixed roots were placed in 2.5% KOH (Koske and Gemma, 1989) acidified with 1N HCl and stained with trypan blue. The stained roots were examined using a compound microscope (100X-1000X) for AM fungal structures and percentage root length colonization was estimated using slide method (Giovannetti and Mosse, 1980).

A segment was considered mycorrhizal when it showed the presence of hyphae and arbuscule or vesicle. Arbuscular mycorrhizal fungal spores were isolated by modified method of wet sieving and decanting technique (Muthukumar *et al.*,

1996). Intact and crushed spores in Polyvinyl-lacto glycerol (PVLG) (Koske and Tessier, 1983) were examined under Olympus BX41 compound microscope and were identified based on spore morphology and sub cellular characters.

RESULTS

Results of soil characteristics of North and South Goa are depicted in **Table 2**. Soil pH was found to be acidic and ranged from 5.7-5.8. Electrical conductivity ranged from 0.048 to 0.049 m/mhos. Organic carbon content was higher in the soils of South Goa (1.67%) as compared to those analyzed from North Goa (0.39%). Soils at both the sites were deficient in available P and available K which ranged from 268.8- 604.88Kg/Ha. Levels of micronutrients Cu, Zn, Fe and Mn varied at both the sites.

Arbuscular mycorrhizal colonization was recorded in 30 plants out of 36 medicinal plant species undertaken for the study. The AM colonization was characterized by arbuscules and/or vesicles and intraradical hyphae. Both arum and paris type of arbuscules were observed. In Arum type morphology, hyphae mostly extended intercellularly (longitudinal hyphae) and formed arbuscules (**Plate III b**) whereas in Paris type morphology, compound arbuscules (**Plate III d**) and arbusculate coils were observed. No colonization was recorded in *Commelina benghalensis*, *Physalis minima*, *Adathoda vasica*, *Murraya koenigii*, *Piper nigrum* and *Euphorbia pulcherrima* (**Table. 3& 4, Fig. 2 & 3**). Hyphal (**Plate III a**) and

Table 2: Soil sample analysis in North Goa and South Goa.

Parameters	North Goa	South Goa
pH	5.80±0.02	5.70±0.01
Electrical conductivity m/mhos	0.049±0.005	0.048±0.003
Organic carbon %	0.39±0.03	1.67 ±0.08
Phosphorus P ₂ O ₅ Kg/Ha	Traces	9.81±0.80
Potassium K ₂ O Kg/Ha	604.80±1.90	268.80±0.60
Micronutrients (ppm)		
Zinc (Zn)	0.72±0.07	4.20±0.49
Iron (Fe)	16.24±0.90	71.60±0.16
Manganese (Mn)	17.58±0.10	83.00±0.08
Copper (Cu)	0.48±0.08	9.02±1.07
Boron (B)	1.30±0.07	0.84±0.05

Legend: Values are means of three replicates, ± indicates Standard deviation.

Table 3: List of medicinal plants surveyed for AM fungal association from North Goa.

Sr. No.	Plant species & Family	Locality	Status	Type of AM fungal colonization	Percent (%) colonization
1.	<i>Adathoda vasica</i> Nees.(Acanthaceae)	Mhadei	Wild	–	–
2.	<i>Andrographis paniculata</i> Nees. (Acanthaceae)	Valpoi	Cultivated	H, V	40.00 ± 1.70
3.	<i>Azadirachta indica</i> A Juss. (Meliaceae)	Valpoi	Cultivated	H,V	100.00 ± 28.60
4.	<i>Catharanthus roseus</i> L. (Apocynaceae)	Valpoi	Cultivated	H, V	70.00± 1.80
5.	<i>Centella asiatica</i> L.(Apiaceae)	Ustae	Wild	H, V, A	90.00±5.89
6.	<i>Commelina benghalensis</i> L. (Commelinaceae)	Valpoi	Wild	–	–
7.	<i>Curcuma</i> sp. (Zingiberaceae)	Bhuipal	Wild	H,V	40.00 ± 1.15
8.	<i>Eclipta alba</i> Hassk. (Asteraceae)	Valpoi	Wild	H,V,A	95.00 ± 2.36
9.	<i>Garcinia indica</i> Choisy. (Clusiaceae)	Valpoi	Cultivated	H,V	95.00±14.40
10.	<i>Hibiscus rosa-sinensis</i> L. (Malvaceae)	Valpoi	Cultivated	H, V	20.00±3.60
11.	<i>Impatiens balsamina</i> L. (Balsaminaceae)	Sanquelim	Wild	H, V, A	85.00±9.90
12.	<i>Lawsonia inermis</i> L. (Lytharaceae)	Mhadei	Cultivated	H, V	20.00±7.80
13.	<i>Leucas aspera</i> L. (Apocynaceae)	Ustae	Wild	H, V	87.00±10.90
14.	<i>Physalis minima</i> L. (Solanaceae)	Valpoi	Cultivated	–	–

Legend: H= Hyphal colonization, V = Vesicular colonization, A= Arbuscular colonization, - = no colonization. Values are means of three replicates, ± indicates standard deviation.

Table 4: List of medicinal plants surveyed for AM fungal association from South Goa.

Sr. No.	Plant species & Family	Locality	Status	Type of AM fungal colonization	Percent (%) colonization
1.	<i>Aloe vera</i> L. (Liliaceae)	Uguem	Cultivated	H, V, A	21.00±0.50
2.	<i>Alpinia galanga</i> (L.) Sw. (Zingiberaceae)	Mollem	Wild	H, V	8.33 ±3.21
3.	<i>Artemisia vulgaris</i> L. (Asteraceae)	Sancordem	Cultivated	H, A	66.67 ±3.05
4.	<i>Asparagus officinalis</i> L. (Leguminosae)	Surla	Wild	H,V,A	40.00 ±3.78
5.	<i>Bryophyllum pinnatum</i> (Lam.) Kurz. (Crassulaceae)	Sadolxem	Cultivated	H,V	65.22 ± 9.90
6.	<i>Cajanus</i> sp. (Leguminosae)	Surla	Wild	H, A	100.00 ± 0.57
7.	<i>Clitoria ternatea</i> L. (Leguminosae)	Sadolxem	Cultivated	H, V, A	77.70 ±12.06
8.	<i>Curculigo orchidoides</i> Gaertn. (Amaryllidaceae)	Mollem	Wild	H, V	22.80±0.59
9.	<i>Curcuma decipiens</i> Dalz. (Zingiberaceae)	Sancordem	Wild	H, V	29.34±3.60
10.	<i>Cymbopogon citrates</i> Stapf. (Poaceae)	Sadolxem	Cultivated	H, V	20.00±5.68
11.	<i>Euphorbia hirta</i> L. (Euphorbiaceae)	Codra	Wild	H,V,A	33.50±2.90
12.	<i>Euphorbia pulcherrima</i> Willd. (Euphorbiaceae)	Codra	Wild	–	–
13.	<i>Hemidesmus indicus</i> R. Br. (Asclepiadaceae)	Sanguem	Wild	H, V, A	34.00±1.50
14.	<i>Ixora coccinea</i> L. (Rubiaceae)	Tamdi Surla	Cultivated	H, V	24.00±6.80
15.	<i>Mentha</i> sp. (Lamiaceae)	Sadolxem	Cultivated	H, V	30.00 ± 9.80
16.	<i>Mimosa pudica</i> L. (Leguminosae)	Codra	Wild	H, V, A	98.00 ± 21.59
17.	<i>Murraya koenigii</i> (L.) Spr. (Rutaceae)	Sadolxem	Cultivated	–	–
18.	<i>Naregamia alata</i> W. & A. (Meliaceae)	Uguem	Wild	H, A	60.00±17.34
19.	<i>Ocimum sanctum</i> L. (Lamiaceae)	Tamdisurla	Cultivated	H, V, A	66.00±27.64
20.	<i>Phyllanthus niruri</i> L. (Euphorbiaceae)	Tamdi Surla	Wild	H, A	50.00±11.53
21.	<i>Piper nigrum</i> L. (Piperaceae)	Neturlim	Cultivated	–	79.00±22.03
22.	<i>Rauwolfia serpentina</i> (L.) Benth. (Apocynaceae)	Tamdi Surla	Wild	H, V	40.00±11.80

Legend: H= Hyphal colonization, V = Vesicular colonization, A= Arbuscular colonization, - = no colonization. Values are means of three replicates, ± indicates standard deviation.

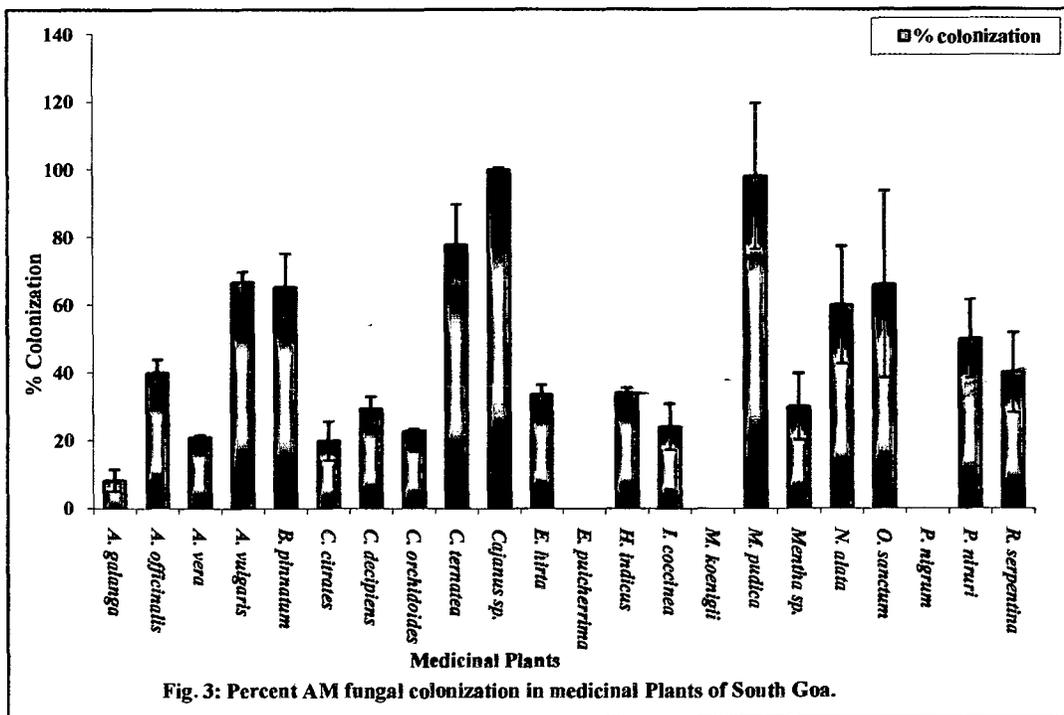
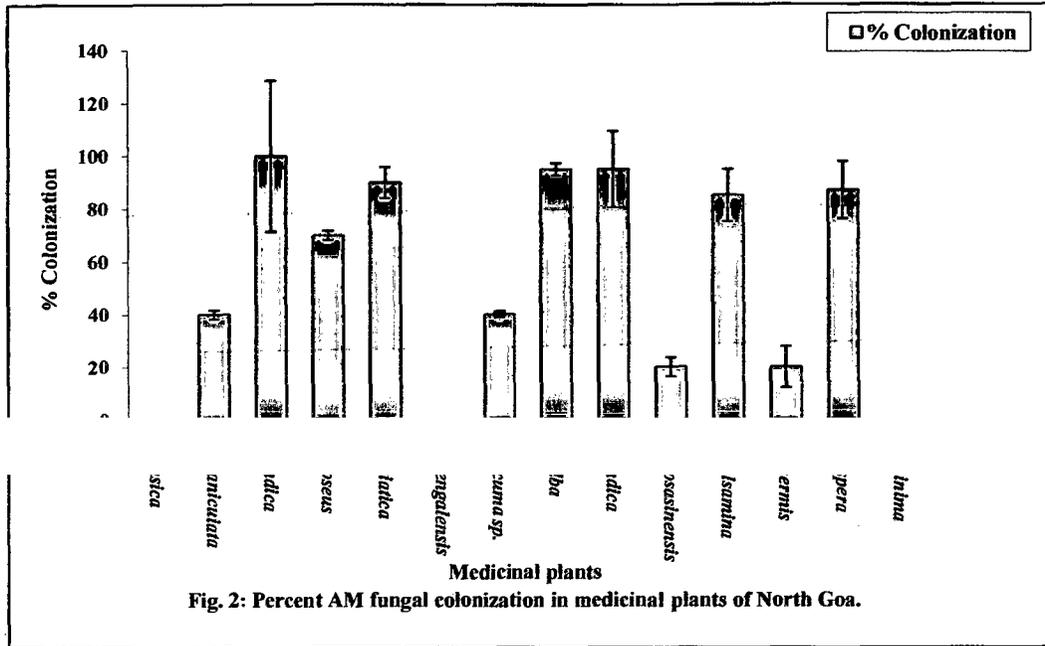
vesicular colonization (**Plate IV a, c d, e & f**) was observed in 16 plant species whereas four plant species exhibited arbuscular colonization (**Plate III c, d, e & f**). Hyphal, vesicular and arbuscular (**Plate IV b**) colonization were recorded in 10 plant species. Maximum percent colonization was found in *Azadirachta indica* and *Cajanus* sp. (100%) and minimum in *Alpinia galanga* (8.33%) (**Fig. 2 & 3**).

Medicinal plant species exhibited higher root colonization levels during pre monsoon and least during monsoon. The percent root colonization varied throughout the season with highest (83%) during April and least during June (38.9%). Arbuscules were relatively infrequent and were observed in few plant species in all the seasons, whereas vesicular colonization was observed in all the plant species (**Fig. 4**).

Correlation studies carried out in the study sites revealed that mean total root colonization exhibited a non significant negative correlation ($r=-1$, $P\leq 0.05$) with available P, EC, OC, Zn, Fe, Cu, Mn and, a non significant positive correlation ($r=1$, $P\leq 0.05$) with pH, K and Bo. A non significant positive correlation was observed between mean spore density and EC, OC, Zn, Fe, Cu and Bo ($r=1$) and, non significant negative correlation with pH, P, K and Mn ($r=-1$, $P\leq 0.05$)⁷

DISCUSSION

In the present study, rhizosphere soil at both sites showed less amount of available P. This could be due to the fact that tropical soils are P fixing and also due



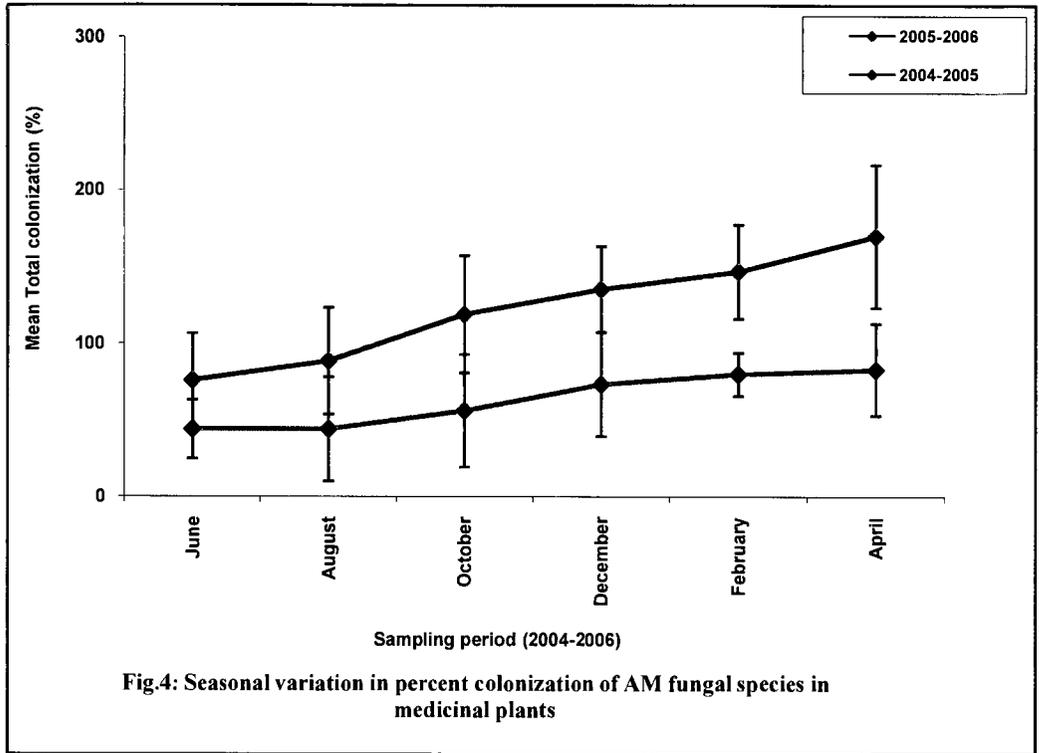


Plate III: Root colonization of arbuscular mycorrhizal fungi:

Hyphal and Arbuscular colonization (a-f).

- a) Hyphal colonization in *Curculigo orchidoides* Gaertn. (X100).
- b) Arum type of arbuscular colonization in *Eclipta alba* Hassk. (X 400).
- c) Arbuscular colonization in *Artemisia vulgaris* L. (X 400).
- d) Paris type of arbuscular colonization in *Aloe vera* L. (X 400).
- e) Arbuscular colonization in *Naregamia alata* W. & A. (X 400).
- f) Arbuscular colonization in *Mimosa pudica* L. (X 400)

Plate III

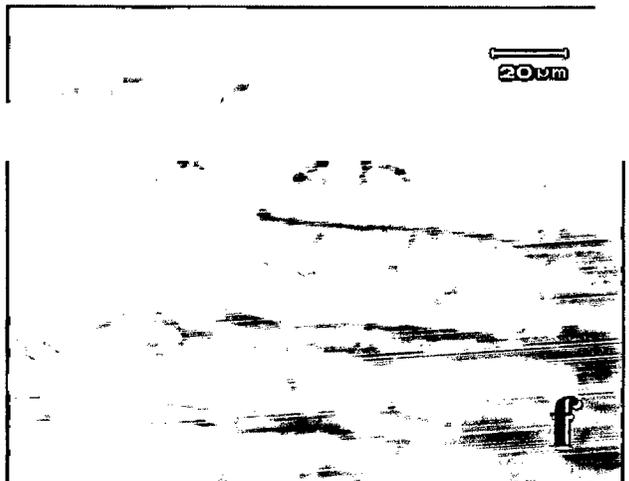
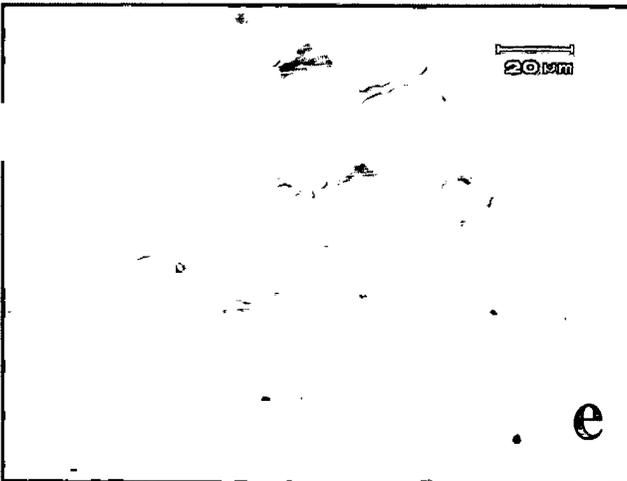
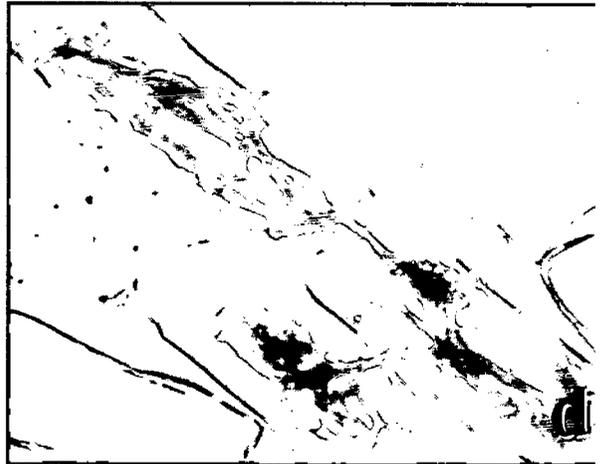
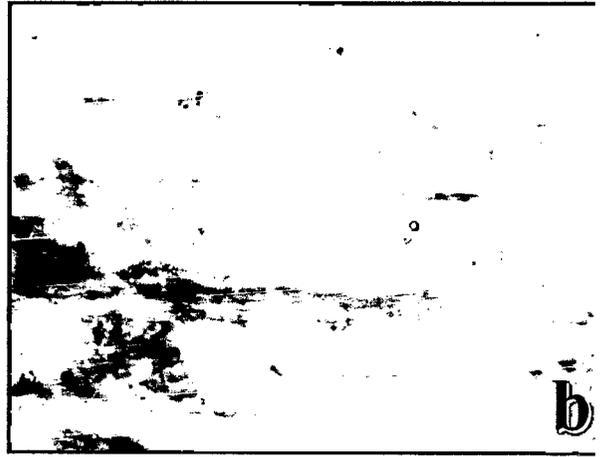
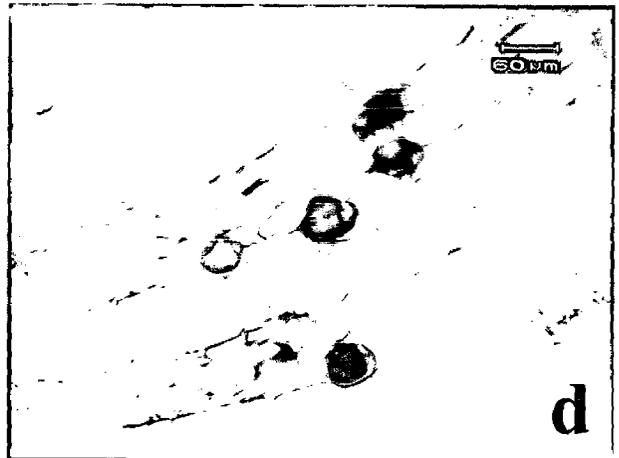
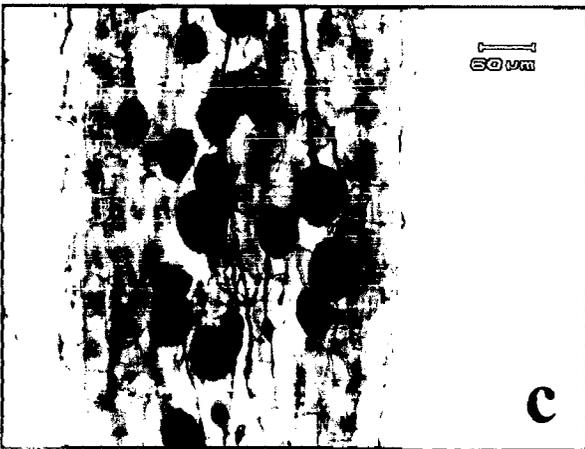
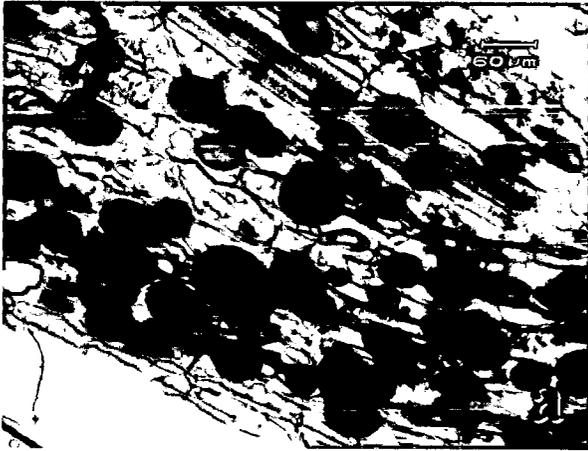


Plate IV: Root colonization of arbuscular mycorrhizal fungi:

Vesicular colonization (a-f).

- a) & c) Vesicles seen in cluster in *Garcinia indica* Choisy and *Cymbopogon citrates* Stapf. (X 100)
- b) Arbuscular and vesicular colonization in *Impatiens balsamina* L. (X 100).
- d) Globose vesicles in *Bryophyllum pinnatum* Salisb.(X 100).
- e) & f) Subglobose vesicles in *Rauwolfia serpentina* (L.) Benth. and *Lawsonia inermis* L. (X 400).

Plate IV



to long term exposure to optimal weathering conditions. It is reported that nearly 80-85% of P applied to the soil is made unavailable to plants because of fixation and immobilization (Rosalind Padma and Kandaswamy, 1990). If P is scarce, mycorrhizas are abundant but if P is readily available, colonization is reduced. Furthermore, organic carbon content and available K content of the rhizosphere soil was high and not influenced by management regimes. Earlier workers have reported that mycorrhizal response is greater in soil with lower amount of Zn, Cu, Fe and Mn (Sreenivasa and Bagyaraj, 1988). Except for Mn and Fe, other micronutrients were present in low concentrations.

The present study confirms the presence of AM colonization in medicinal plants. Similar observations were recorded earlier (Srivastava and Basu, 1995; Muthukumar and Udaiyan, 2000; Gorski, 2002). This study contradicts the earlier findings of Muthukumar and Udaiyan (2000) who reported that the proportion of non-mycorrhizal species in the Western Ghats is high compared with other vegetation world wide, whereas in the present study, 83.33% of the plant species were mycorrhizal. Two of the medicinal plants while lacked AM colonization belonged to non-mycorrhizal families viz., Commelinaceae and Euphorbiaceae (Tester *et al.*, 1987; Brundrett, 1991). Other plant species lacking mycorrhizas colonization belonged to families reported mycorrhizal viz., Solanaceae, Acanthaceae, Rutaceae and Piperaceae. Tester *et al.*, (1987) suggested that fungitoxic compounds present in

root cortical tissue or in root exudates may reduce susceptibility of plants to mycorrhization

Arbuscular mycorrhizal colonization is normally attributed to environmental factors while plant species and plant phenological events seem to influence the seasonal variation of AM colonization (Miller, 2000). Smith *et al.* (1979) reported that the extent to which typical AM fungi colonize root system varies with plant species and is known to be influenced by environmental conditions; the most important being the age of the plants, the level of P in the soil relative to the requirements of the plant and the capacity of the population of mycorrhizal propagules in the soil to form mycorrhiza. Brundrett and Kendrick (1990a & b) suggested that the presence or absence of continuous longitudinal airspaces in the root cortex and also differences in cell wall structure and modifications produced during fungal colonization could be the factor determining formation of either the Arum and Paris-types respectively (Bonfante-Fasolo and Fontana, 1985).

Seasonal patterns in the formation of mycorrhiza have also been said to vary considerably from year to year (Allen *et al.*, 1989). The ability of AM fungi to colonize the root system of the host plants has been studied previously (Graham and Abbott, 2000) and may be indicative of biological niche of each fungus in the rhizosphere, whereby some fungi may allocate more carbon to colonizing the root surface, others allocate more to develop external hyphae in the soil. Arbuscular

mycorrhizal symbiosis is characterized by short life cycle of arbuscules (Alexander *et al.*, 1988), rapid colonization of new roots and appearance of vesicles in the oldest colonization units (Smith and Read, 1997). Seasonal shifts in AM colonization have been found which indicates that the benefit of mycorrhizal symbiosis for the plant changes during the season (Fitter, 1986, 1991). Cavagnaro *et al.* (2001) observed that the presence of Arum and Paris type morphology is determined by both AM fungal and host plant genotype. The poor correlation of spore numbers with mycorrhiza formation has been pointed out by previous researchers (Abbott and Robson, 1991; Merryweather and Fitter, 1998).

Variation in percent root colonization has been reported to be affected by seasonal sporulation, seasonal variation in development of host plants (Sutton and Barron, 1972) and the nutrient availability in the soil (Louis and Lim, 1987). This variation may be the result of variable host susceptibility (Mehrotra, 1998), diverse type of AM fungi in the rhizosphere soils of individual plant species, host efficiency in soil resource capture and utilization (Koide, 1991; Clark and Zeto, 2000) soil types and quality (Raman and Gopinathan, 1992) and other edapho-climatic factors (Abbott and Robson, 1991). The variation in extent of mycorrhizal colonization among different plant species observed in the present study confirm earlier findings of Manjunath and Bagyaraj (1982), who stated that the extent to which plants respond to AM colonization varies with the plant species. Gerdemann (1965) has shown that the colonization pattern of AM fungal species can be distinctly different in various plant

species. According to Tommerup (1992), the variation in colonization patterns is due to the differences in the rate of intra-radical growth, amount of hyphae per entry point and growth of external mycelium along roots before entry points are formed. Muthukumar and Udaiyan (2000) reported variation in mycorrhizal colonization levels in wild plants growing in Western Ghat region of Southern India.

The presence of mycorrhizal colonization in all the seasons indicates that the plant species are dependent on mycorrhizae throughout the year. The studies that have reported seasonality of the mycorrhizal association generally assume a direct influence of environmental conditions such as temperature and moisture or phenology and physiological status of the plant (Siguenza *et al.*, 1996; Mohammad *et al.*, 1998, Brundrett, 2002). The relationship between mycorrhizal colonization and soil moisture may be associated with the development of the plant root systems due to the increase in the water content of soil with the formation of new roots, there will be a simultaneous increase in nutrients absorption and liberation of root exudates, stimulating mycorrhizal spore germination and subsequent colonization (Oliveira *et al.*, 1998). High values of root colonization in pre monsoon season could be attributed to a slow rate of root growth (Koide and Mooney, 1987; Cade-Menun *et al.*, 1990) as in rainy season there is a high rate of root production (Allen, 2001). Several other factors including environmental conditions, rate of root vs fungal growth, phenology and physiological status of the plant (Allen, 2001; Brundrett, 2002) are also responsible for the seasonal variation in percent colonization.

CHAPTER 2

ARBUSCULAR MYCORRHIZAL FUNGAL DIVERSITY IN MEDICINAL PLANTS

INTRODUCTION

The characteristics and dynamics of occurrence of AM fungi under natural conditions are important for the evaluation of the inoculum potential and root colonization in the process of understanding their behaviour in the soil and determining their symbiotic efficiency (Bethlenfalvay and Linderman, 1992). The presence and/or abundance of AM fungal spores show that these species may be active in the soil at a specific time, being capable of colonizing and multiplying themselves in the host roots. Arbuscular Mycorrhizal fungi are not host-specific, i.e. each species does not colonize only one plant species (McGonigle and Fitter, 1990), although there may be ecological specificity or selectiveness among symbionts (Van der Heijden *et al.*, 1998). Therefore, several AM fungi may colonize the same host plant and a specific fungus may be associated with different host plants. Recently, it has been shown that the growth rates (expressed as sporulation) of different AM fungi are host-specific, i.e. when inoculating a given AM fungal community onto different plant species, each host will selectively produce a differentiated spore composition. Moreover, host plants may produce positive or negative feedback on the mycorrhizal fungi, thereby also determining indirectly the diversity of the plant community (Bever *et al.*, 1996; Bever, 2002).

Different plant species have differential growth responses to AM fungi so that the composition and diversity of the AM fungi in a natural ecosystem could potentially affect the way plant species coexist, and therefore be a determinant of

plant community structure (Van der Heijden *et al.*, 1998). Tropical forests display high species diversity and complex community structure, and are a major distribution area of AM fungi in the world (Read, 1997). In tropical forests, where competition for light, nutrients and water may be high, the AM fungal association can influence the ability of plants to acquire nutrients and increase plant growth and fitness (Zobel *et al.*, 1997; Allen *et al.*, 2003).

In tropical systems studies on seasonal root colonization or density of spores in soil (Johnson and Wedin, 1997; Guadarrama and Álvarez-Sánchez, 1999; Picone, 2000) are scarce, and even fewer studies have evaluated these for the long term (Allen *et al.*, 1998, 2003). It is important to evaluate the dynamics of the AM association since the seasonality of host plants and their response to the symbiosis are related to their strategy for nutrient uptake and competition (Bethlenfalvay, 1992). Therefore, the present study has been carried out with the aim of studying the diversity and abundance of AM fungal species in the Western Ghat area of Goa.

MATERIALS AND METHODS

Isolation of arbuscular mycorrhizal spores:

Wet sieving and decanting method (Gerdemann and Nicolson, 1963) was employed for extraction of AM fungal spores and sporocarps from the rhizosphere soil sampled. The steps are as follows:

- 1) Hundred grams of rhizosphere soil was suspended in 1000ml of tap water. The mixture was stirred for 10-15 seconds and the coarse particles were allowed to settle in water for 1-2 minutes.
- 2) The soil water mixture was decanted through sieves arranged in descending order of mesh size (500 μ m-37 μ m).

The above two steps were repeated twice to ensure that the majority of spores were extracted from the rhizosphere soil.
- 3) Debris from each sieve was collected separately in beakers.
- 4) Debris was filtered through Whatman No.1 filter paper.
- 5) The filter paper was placed on petri-plate and care was taken to see that it remained moist.
- 6) The contents of the filter paper were examined for spores and sporocarps under stereomicroscope (Olympus BX41).

Quantification of spore density of AM fungi:

Quantification of AM fungal spores was carried out by using Gaur and Adholeya (1994) method as described below:

- 1) Whatman No.1 filter paper was given two folds.
- 2) The filter paper was opened and divided into four quadrates.
- 3) One-half of the paper was divided into approximately 15 columns by drawing lines that are 0.5cm apart. Each column is numbered and the direction was marked.

- 4) The filter was folded such that the marked surface received the sample during filtration. The spores were collected on the marked surface and the other portion of the filter paper remained free from spores.
- 5) The filter paper was spread on a petri-plate and examined under stereomicroscope (Olympus BX41).

While counting spores, two lines were focused and the space between them was followed by moving the petri-plate. Spore density was expressed as total number of spores recorded/100g of rhizosphere soil.

Taxonomic identification of spores:

Intact and unparasitized spores were used for the quantification of spore density and for identification of AM fungi. Arbuscular mycorrhizal fungi were identified based on their spore morphology and wall characteristics following various bibliographies (Schenck and Perez, 1990; Morton and Benny, 1990; Almeida and Schenck, 1990; Bentivenga and Morton, 1995; Walker and Vestberg, 1998; Redecker *et al.*, 2000; Morton and Redecker, 2001). Taxonomic identification of spores was also carried out by matching the descriptions provided by the International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (<http://invam.caf.wvu.edu>).

Diversity studies: Arbuscular mycorrhizal fungal species diversity and abundance studies were carried out in North and South Goa.

Species richness: The number of species present in an ecosystem and is calculated by counting the number of species.

Simpsons Diversity Index: $1 - D$ (Simpson, 1949) where $D = \sum (P_i)^2$ where $p_i = n_i/N$, (n_i) is the relative abundance of the species and is calculated as the proportion of individuals of a given species (n_i) to the total number of individuals in a community (N).

Shannon Wiener Index: The Shannon diversity index (H) is commonly used to characterize species diversity in a community, which accounts for both abundance and evenness of the species present.

$$H = -\sum (P_i \ln P_i) \text{ (Weaver and Shannon, 1949).}$$

Species Evenness: Shannon's equitability, $E_{(H)} = H/H_{\max}$ where $H_{\max} = \ln S$, S = total number of species in the community (richness).

Statistical analysis: Pearson correlation coefficient was performed to assess the relationship between root colonization and spore density. Statistical analysis for correlation coefficient was carried out using WASP (Web Based Agricultural Package) 0.2. For the analysis, difference were considered significant when $P \leq 0.05$.

RESULTS

Forty-two AM fungal species belonging to five genera viz., *Glomus* (24), *Acaulospora* (8), *Scutellospora* (7), *Gigaspora* (2) and *Ambispora* (1) were recovered from the rhizosphere soil samples with the species number given in parenthesis. Spore density varied from 1197 spores (*Hemidesmus indicus*) to 14 spores (*Eclipta alba*) per 100 gram of soil (Table 5 & 6). The study revealed that wild medicinal plant species showed higher spore diversity when compared with cultivated medicinal plants. The most dominant genus recorded in the present study was *Glomus* followed by *Acaulospora*, *Scutellospora*, *Gigaspora* and *Ambispora*. *Glomus fasciculatum* was found to be the most dominant AM fungal species followed by *A. scrobiculata*. Weak negative non-significant correlation was found between percent colonization and spore density ($r = -0.1$, $P \leq 0.05$). The spore density of AM fungi varied in the different seasons. Highest mean spore density was observed during the monsoon period (August) and the lowest during the post-monsoon period (January) (Fig. 5).

In North Goa, 27 AM fungal species belonging to five genera viz., *Glomus*, *Acaulospora*, *Scutellospora*, *Gigaspora* and *Ambispora* were recovered from the rhizosphere soil. Maximum spore density was recorded in *Curcuma* sp. (200 spores) and minimum in *Eclipta alba* (14 spores 100g⁻¹ soil) (Fig. 6). In South Goa, 26 AM fungal species belonging to five genera viz., *Glomus*, *Acaulospora*, *Scutellospora*, *Gigaspora* and *Ambispora* were recorded. Maximum spore density was observed in *Hemidesmus indicus* (1197 spores) and least in *Rauwolfia serpentina* (20 spores 100g⁻¹

Table 5: Spore density and AM fungal species identified from rhizosphere soil samples of selected medicinal plants from North Goa.

Sr. No	Plant species	Spore density*	AM Fungal spores
1.	<i>Adathoda vasica</i>	35.00±15.09	<i>A. scrobiculata</i> , <i>G. geosporum</i> , , <i>G. multicaule</i> , <i>G. fasciculatum</i>
2.	<i>Andrographis paniculata</i>	93.00±46.07	<i>A. scrobiculata</i> , <i>G. aggregatum</i>
3.	<i>Azadirachta indica</i>	72.00±18.10	<i>A. scrobiculata</i> , <i>G. fasciculatum</i> , <i>Gi. albida</i> , <i>S. calospora</i>
4.	<i>Catharanthus roseus</i>	52.00±15.07	<i>A. laevis</i> , <i>G. dimorphicum</i>
5.	<i>Centella asiatica</i>	80.00±11.09	<i>G. multicaule</i> , <i>G. clarum</i> , <i>G. fasciculatum</i> , <i>A. delicata</i> , <i>S. scutata</i>
6.	<i>Commelina benghalensis</i>	30.00±33.29	<i>A. myriocarpa</i> , <i>G. sp.</i>
7.	<i>Curcuma sp.</i>	200.00±50.0	<i>Am. leptoticha</i> , <i>G. rubiforme</i> , <i>G. geosporum</i> , <i>G. fasciculatum</i> , <i>G. aggregatum</i>
8.	<i>Eclipta alba</i>	14.00±5.50	<i>A. scrobiculata</i> , <i>Am. leptoticha</i> , <i>G. fasciculatum</i> , <i>S. heterogama</i>
9.	<i>Garcinia indica</i>	27.00±8.80	<i>A. scrobiculata</i> , <i>G. fasciculatum</i> , <i>Gi. sp.</i>
10.	<i>Hibiscus rosa- sinensis</i>	62.00±8.73	<i>G. maculosum</i> , <i>G. glomerulatum</i> , <i>A. scrobiculata</i>
11.	<i>Impatiens balsamina</i>	45.00±17.08	<i>G. microcarpum</i> , <i>G. fasciculatum</i>
12.	<i>Lawsonia inermis</i>	61.00±27.40	<i>A. scrobiculata</i> , <i>G. multicaule</i> , <i>G. intraradices</i>
13.	<i>Leucas aspera</i>	20.00±9.37	<i>G. rubiforme</i> , <i>G. formosanum</i> , <i>G. fasciculatum</i>
14.	<i>Physalis minima</i>	33.00±27.06	<i>A. rehmi</i> , <i>G. fasciculatum</i> , <i>G. multicaule</i> , <i>G. maculosum</i> , <i>G. geosporum</i> , <i>G. rubiforme</i>

Legend: *A*= *Acaulospora*, *Am*= *Ambispora*, *G*= *Glomus*, *Gi*= *Gigaspora*, *S*= *Scutellospora*

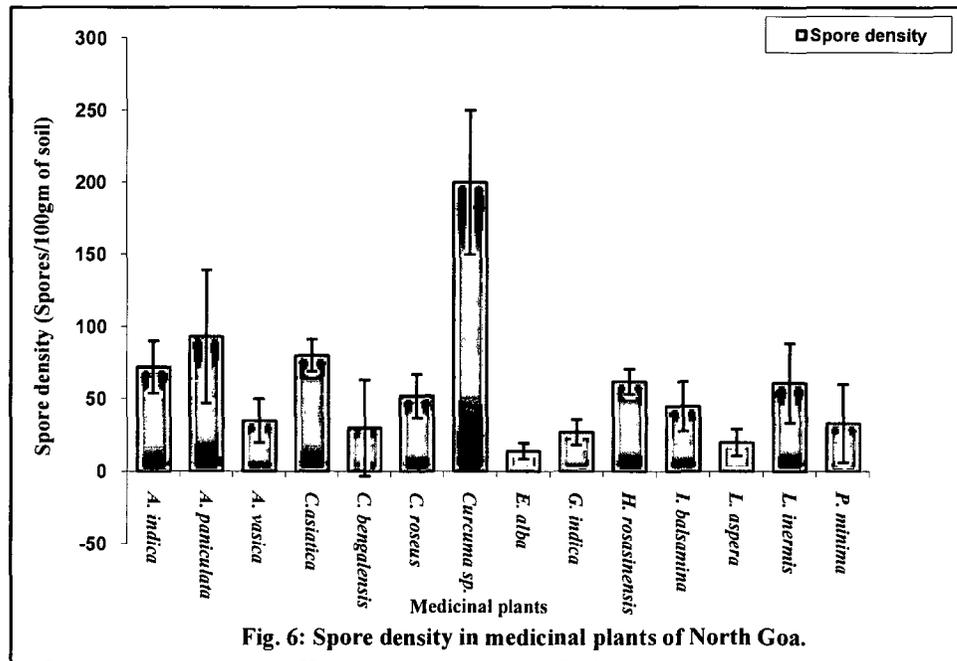
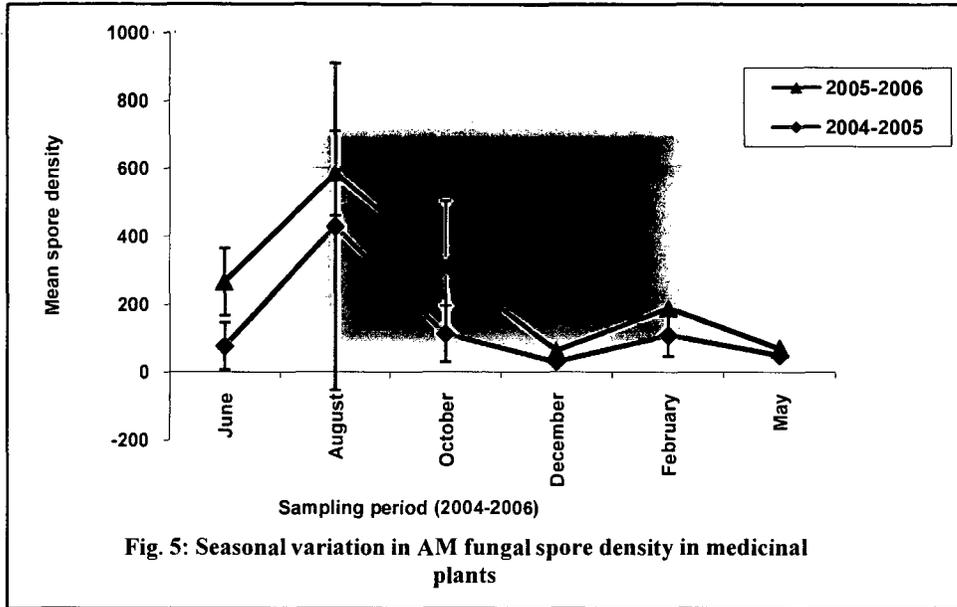
*Spores 100g⁻¹ of soil. Values are means of three replicates. ± indicates Standard deviation.

Table 6: Spore density and AM fungal species identified from rhizosphere soil samples of selected medicinal plants from South Goa.

Sr. No.	Plant species	Spore density*	AM Fungal spores
1.	<i>Aloe vera</i>	421.00±53.02	<i>G. maculosum</i> , <i>G. multicaule</i> , <i>G. geosporum</i>
2.	<i>Alpinia galanga</i>	76.00±12.09	<i>G. caledonium</i> , <i>G. mosseae</i> , <i>G. fasciculatum</i> , <i>G. geosporum</i> , <i>Am. leptoticha</i>
3.	<i>Artemisia vulgaris</i>	183.00±60.20	<i>G. fasciculatum</i> , <i>G. magnicaule</i> , <i>G. geosporum</i>
4.	<i>Asparagus officinalis</i>	149.00±28.58	<i>A. scrobiculata</i> , <i>G. rubiforme</i> , <i>G. fasciculatum</i> , <i>G. geosporum</i>
5.	<i>Bryophyllum pinnatum</i>	166.00±23.00	<i>A. laevis</i> , <i>G. multicaule</i> , <i>G. aggregatum</i> , <i>Gi. albida</i>
6.	<i>Cajanus</i> sp.	231.00±27.01	<i>A. nicolsonii</i> , <i>A. scrobiculata</i> , <i>G. constrictum</i> , <i>G. multicaule</i> , <i>G. fasciculatum</i>
7.	<i>Clitoria ternatea</i>	94.00±40.10	<i>A. spinosa</i> , <i>G. multicaule</i> , <i>G. glomerulatum</i> , <i>G. fasciculatum</i> , <i>Gi. albida</i>
8.	<i>Curculigo orchidoides</i>	256.00±15.00	<i>G. fasciculatum</i> , <i>G. intraradices</i>
9.	<i>Curcuma decipiens</i>	238.00±28.80	<i>Am. leptoticha</i> , <i>G. constrictum</i> , <i>G. fasciculatum</i> , <i>G. caledonium</i> , <i>G. geosporum</i> , <i>G. multicaule</i>
10.	<i>Cymbopogon citrates</i>	37.00±3.06	<i>G. fasciculatum</i> , <i>G. aggregatum</i> , <i>G. multicaule</i>
11.	<i>Euphorbia hirta</i>	120.00±18.98	<i>A. scrobiculata</i> , <i>G. geosporum</i> , <i>Gi. albida</i>
12.	<i>Euphorbia pulcherrima</i>	125.00±45.80	<i>A. scrobiculata</i> , <i>G. geosporum</i> , <i>G. albidum</i> , <i>G. mosseae</i>
13.	<i>Hemidesmus indicus</i>	1197.00±109.09	<i>Am. leptoticha</i> , <i>G. maculosum</i> , <i>G. geosporum</i> , <i>G. multicaule</i> , <i>G. fasciculatum</i>
14.	<i>Ixora coccinea</i>	40.00±33.50	<i>A. nicolsonii</i> , <i>A. scrobiculata</i> , <i>G. etunicatum</i> , <i>G. aggregatum</i> , <i>S. biornata</i>
15.	<i>Mentha</i> sp.	85.00±17.89	<i>A. scrobiculata</i> , <i>G. citricola</i> , <i>G. fasciculatum</i> , <i>G. multicaule</i>
16.	<i>Mimosa pudica</i>	50.00±13.06	<i>Am. leptoticha</i> , <i>G. aggregatum</i> , <i>G. glomerulatum</i> , <i>G. geosporum</i> , <i>G. intraradices</i>
17.	<i>Murraya koenigii</i>	91.00±32.00	<i>G. fasciculatum</i> , <i>G. multicaule</i> , <i>G. halon</i>
18.	<i>Naregamia alata</i>	614.00±80.90	<i>A. scrobiculata</i> , <i>Am. leptoticha</i> , <i>A. nicolsonii</i> , <i>G. rubiforme</i> , <i>G. maculosum</i> , <i>G. fasciculatum</i> , <i>S. verrucosa</i>
19.	<i>Ocimum sanctum</i>	50.00±14.00	<i>G. fasciculatum</i> , <i>G. macrocarpum</i>
20.	<i>Phyllanthus niruri</i>	40.00±1.77	<i>G. arborensense</i> , <i>A. tuberculata</i> , <i>S. gregaria</i>
21.	<i>Piper nigrum</i>	415.00±44.40	<i>Am. leptoticha</i> , <i>A. scrobiculata</i> , <i>G. multicaule</i> , <i>G. intraradices</i> , <i>G. geosporum</i> , <i>G. flavisporum</i> , <i>G. fasciculatum</i> , <i>S. pellucida</i>
22.	<i>Rauwolfia serpentina</i>	20.00±0.80	<i>G. maculosum</i> , <i>G. fasciculatum</i>

Legend: A= Acaulospora, Am= Ambispora, G= Glomus, Gi= Gigaspora, S= Scutellospora

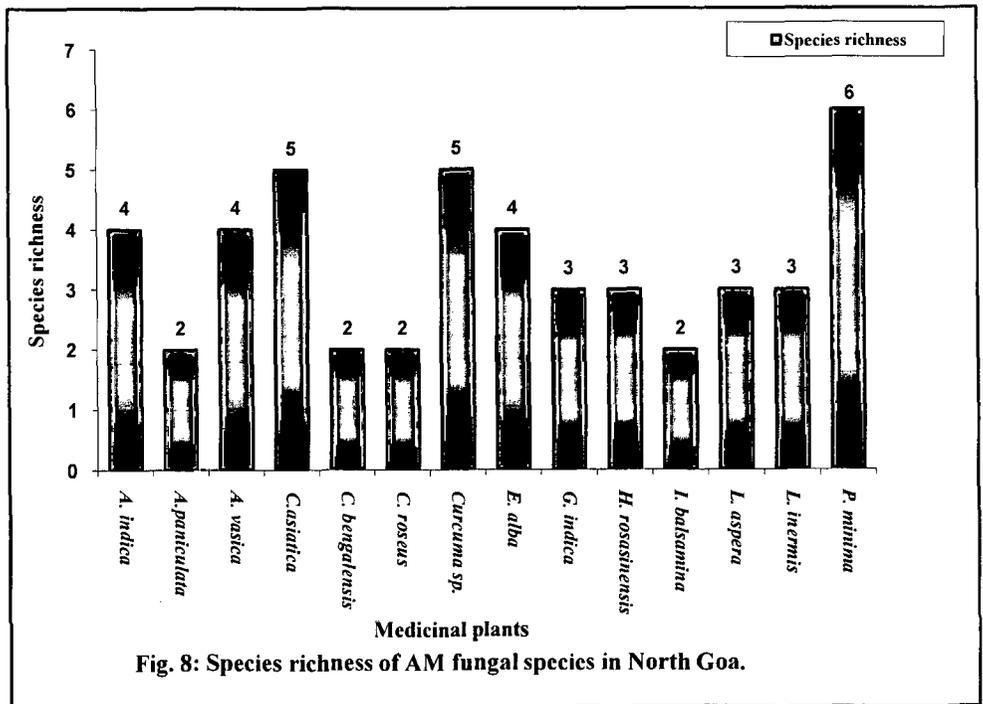
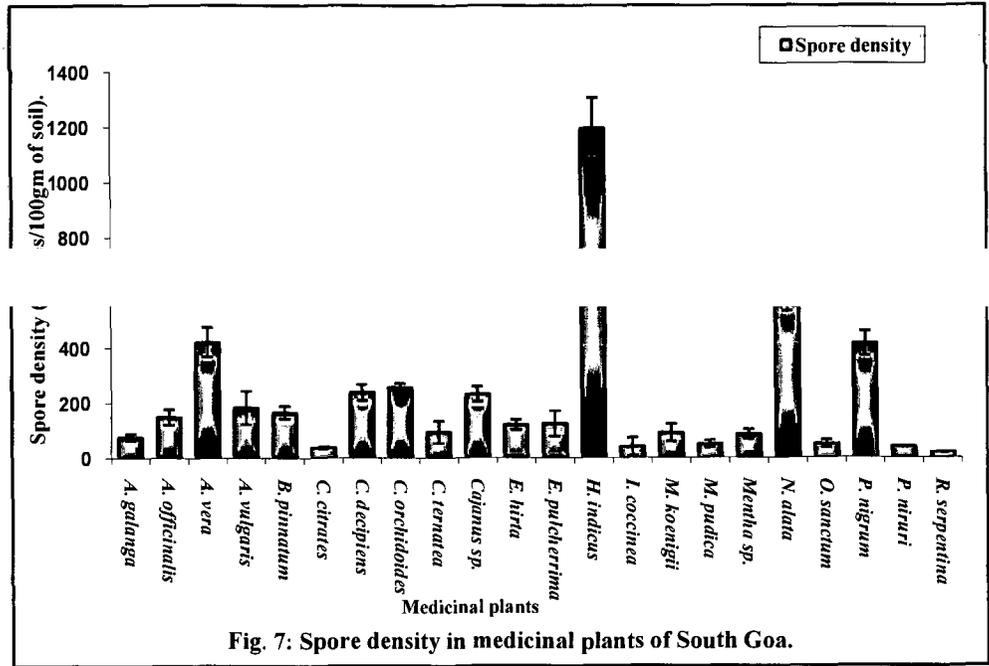
*Spores 100g⁻¹ of soil. Values are means of three replicates. ± indicates Standard deviation.



1 soil) (**Fig. 7**). Species richness of AM fungi was found more in South Goa (8 species/site) compared to North Goa (6 species/site) (**Fig. 8 & 9**). Diversity index studies viz., Simpsons Diversity Index and Shannon Wiener Diversity Index showed AM fungal species dominance in South Goa (0.95, 2.88) than in North Goa (0.9, 2.86) (**Fig. 10 & 11**). Complete evenness of AM fungal species was found in North Goa (1) when compared with South Goa (0.93) (**Fig. 12**).

DISCUSSION

The results of the present study revealed a rich diversity of AM fungal species associated with medicinal plants in Western Ghat, Goa. Arbuscular Mycorrhizal fungi displayed little or no host specificity in the present study. This could be due to the fact that AM fungi prefer certain habitats and earlier studies clearly demonstrated the role of environmental factors and vegetation on AM fungal community composition (Brundrett, 1991). The possible reason for the predominance of *Glomus* species is that spores of *Glomus* species have a wide range of temperature and pH preferences for germination (Wang *et al.*, 1997). Dominance of genus *Glomus* from medicinal plants has been reported earlier (Selvaraj *et al.*, 2001). Because of its dominance under semi-arid and arid habitats, species of *Glomus* have been considered the best-adapted genus for habitats subjected to drought and soil salinity stresses (Dalpe *et al.*, 2000; Muthukumar and Udaiyan, 2002; Haas and Menge, 1990; Blaszkowski *et al.*, 2002).



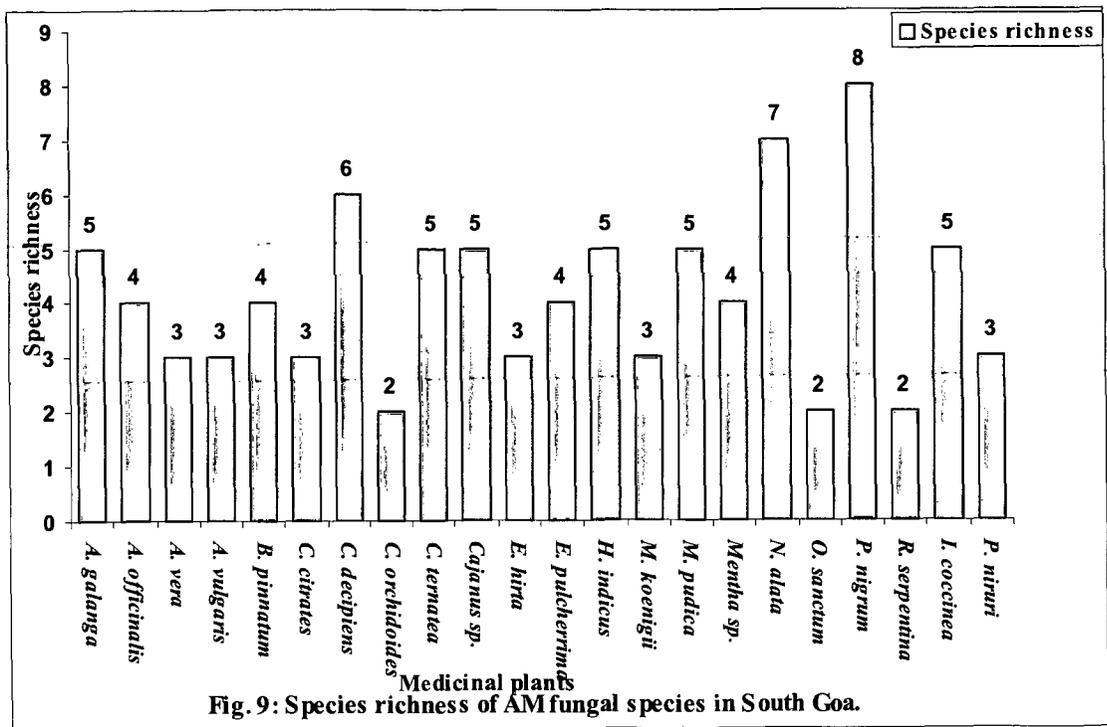
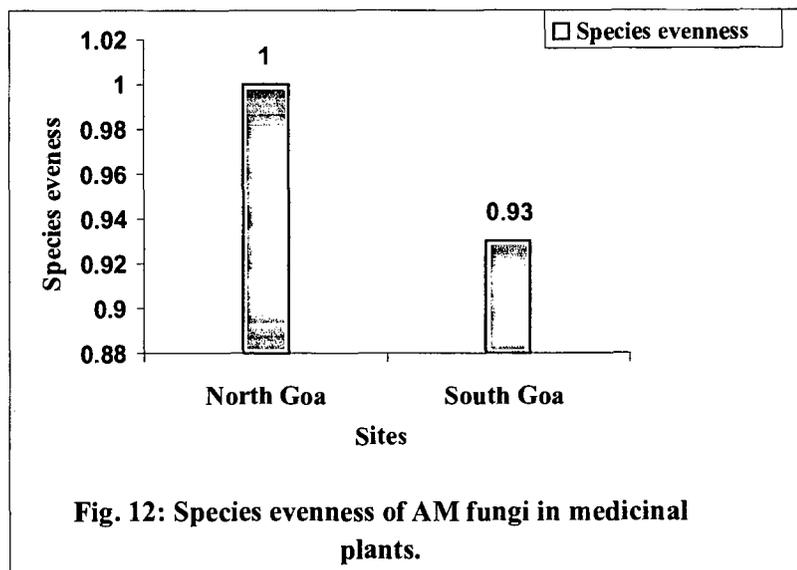
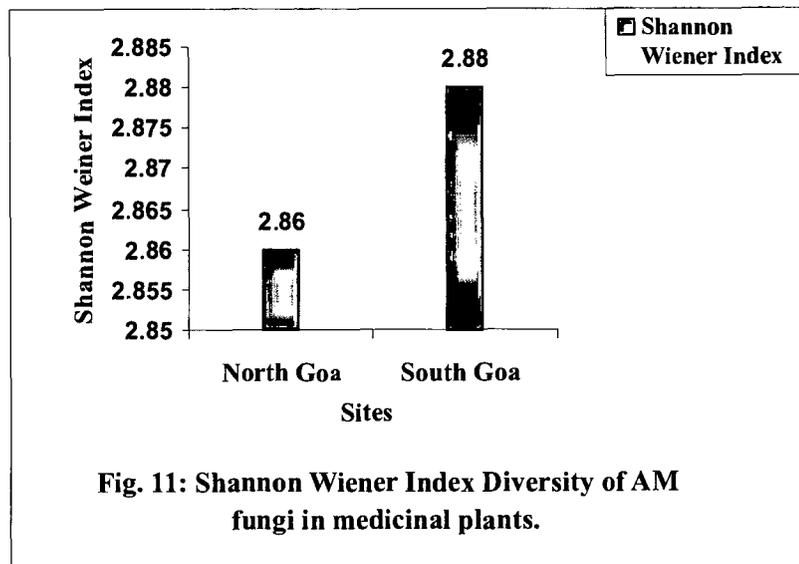
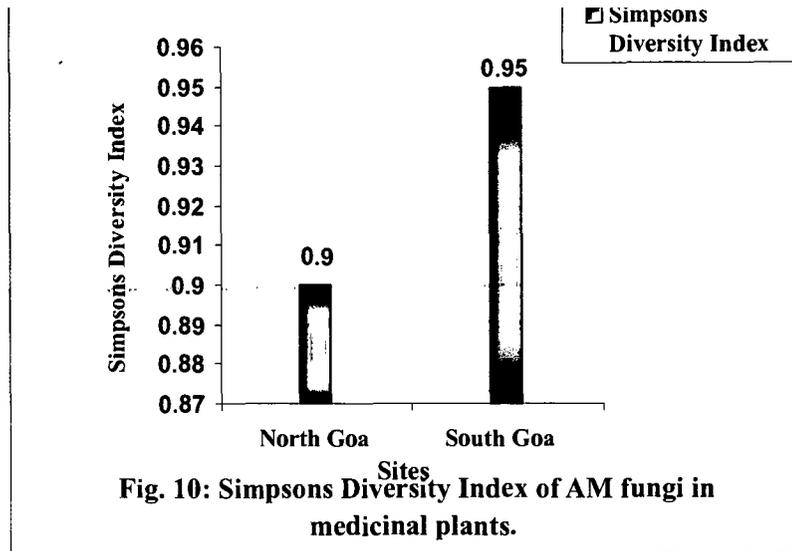


Fig. 9: Species richness of AM fungal species in South Goa.



Forty-two AM fungal species belonging to five genera were recovered in the present study. This is in agreement with the findings of Francis and Read (1994) and Allen *et al.* (1995) who reported high AM fungal species diversity in medicinal plants. However, Muthukumar *et al.* (2001) reported only 35 AM fungal species from 329 medicinal plant species from the Western Ghats.

No significant correlation could be established between root colonization and spore density of AM fungi, which is in agreement with the findings of Kalita *et al.* (2002). This could be due to the fact that when soil conditions are suitable for spore germination, mycorrhizal colonization increases and spore number decreases (He *et al.*, 2002; Ragupathy and Mahadevan, 1993) and also AM fungal sporulation is also dependent on a wide range of host, fungal and environmental factors and their germination potential varies at different times of the year (Tommerup, 1983; Gemma and Koske, 1988). Dhar and Mridha (2003) reported no significant relationship between AM colonization and spore population, which could be due to the different gradients of soil and the strong effect of plant factors on the formation, function and adaptation of the fungus to the respective soil conditions. However, Mutabaruka *et al.* (2002), Louis and Lim (1987), Muthukumar *et al.* (2001) reported a positive correlation between root colonization and spore density.

Spore quantification has been found useful for evaluating levels and diversity of mycorrhizae as spores are highly resistant to adverse conditions (Abbott and

Robson, 1991). Large variation in spore number (14-1197 spores 100g⁻¹ soil) recorded in the present study can be attributed to several factors. Firstly, the occurrence of several AM fungi in the soil or within roots suggest that interspecific competition between them is possible (Brundrett and Kendrick, 1990). Secondly, subsequent variation in spore production occurs among AM fungi associated with a host plant, suggesting that competition between fungi and environmental factors influence spore production in natural communities (Gemma and Koske, 1988). Peak period of spore production is generally thought to coincide with the period of fungal resource remobilization from senescing roots (Sutton and Barron, 1972) and is greatest in natural communities when root activity is interrupted by a long dry season (Janos, 1980). The variations in spore density between samples could also be due to microclimate (Koske, 1987), to physico-chemical and microbiological properties and the sampling season (Anderson *et al.*, 1984; Johnson *et al.*, 1991).

The factors favouring the higher spore population may either be conducive to edaphic conditions for sporulation such as low nutrient status, high aeration and optimum moisture or the undisturbed conditions of the soils, which allowed sufficient time for sporulation (Chulan and Omar, 1991).

Arbuscular Mycorrhizal spore numbers were strongly seasonal, increasing as the rainy season progressed. Similar seasonal patterns in spore number were observed in earlier studies resulting in a gradual increase in the spore numbers during the

rainfall period, followed by a decrease during the dry period (Martin *et al.*, 1999; Miranda *et al.*, 1997). Allen and Allen (1980) suggested that seasonal variations occur mainly because of fluctuation in rainfall pattern. Previous studies reported that seasonality of the mycorrhizal association is directly influenced by environmental conditions such as temperature and moisture (Sigüenza *et al.*, 1996; Mohammad *et al.*, 1998; Nehl *et al.*, 1998). Siqueira (1993) reported that cyclical changes in soil moisture stimulate AM fungal sporulation. According to Maschio *et al.* (1992) AM fungi have adapted to colonize areas that present adverse physical, chemical and biological conditions, and they depend on moisture for multiplication. Other studies affirm that mycorrhizal sporulation is linked to better soil moisture conditions as increase in the number of spores in the rhizosphere is a function of increased soil moisture (Braunberger *et al.*, 1994).

Seasonal variations in AM fungi are also influenced by plant phenological events, including new root growth (Van Duin *et al.*, 1989), flowering and fruiting (Stenlund and Charvat, 1994; Carvalho *et al.*, 2001), active plant growth (Miller, 2000), and seedling establishment (Carvalho *et al.*, 2001). Dodd *et al.* (1990) reported that the number of AM fungal spores in tropical savannahs decreased during the dry season due to wilt of the vegetation, which prevented carbon allocation to AM fungi and hence hindered growth and sporulation. Additionally, soil surface temperatures above 60°C kill AM fungal spores which decrease the infectivity potential in the surface layer (Thompson, 1989).

Arbuscular mycorrhizal spore density was found to be higher in wild medicinal plant species as compared to cultivated species. This observation could be attributed to the undisturbed nature of the ecosystem. Similar observations were recorded earlier (Moreira-Souza *et al.*, 2003; Sieverding, 1991). According to Moreira-Souza *et al.* (2003) differences in soil pH determined the distribution of AM fungal species. The genetic diversity of AM fungi might be responsible for the variation in their pattern of production and colonization (Dhar and Mridha, 2006).

Maximum species richness was recorded in *Piper nigrum* where eight AM fungal species belonging to three genera were recovered from rhizosphere soil samples. Species richness index is reported to be dependent on sample size, the greater the number of samples collected, the spores of more species are likely to be recovered (Sturmer and Bellei, 1994). Arbuscular mycorrhizal fungal species richness and diversity in plant roots can vary with the ecosystem type, with locality at landscape and community scale, host plant species and plant age (Helgason *et al.*, 1998; Wubet *et al.*, 2004).

Diversity indices showed less variation at both sites indicating a stable and a diverse AM fungal community in Western Ghat of Goa. Arbuscular mycorrhizal fungal species diversity exhibited at both the sites confirm the fact that species diversity and distribution of AM fungi are considered to be influenced by the environmental, physical and nutritional status of the soil rather than the identity of

their plant partner (Anderson *et al.*, 1984; Koske, 1987; Allen *et al.*, 1995). Arbuscular Mycorrhizal fungi do not show host specificity and are randomly distributed in natural communities. Multiple species of AM fungi can colonize a single host plant simultaneously (Harley and Smith, 1983; Sanders and Fitter, 1992). Varying life cycles of host plants have been reported to be the controlling factor of the species composition of AM fungi (Muthukumar and Udaiyan, 2000).

Dominance of AM fungal sporulation is related to the entanglement of roots of different plant species that AM fungi could bridge through their external mycelium in tropical environments where a stable microclimate at the soil surface can be found. Arbuscular mycorrhizal taxa have a specific multidimensional niche determined by the plant species that are present at the site and by edaphic factors such as pH, moisture content, phosphorus (P) and nitrogen (N) availability (Ahulu *et al.*, 2006). As a result there is a large variation between and within the site in the composition of AM fungal taxa (Burrows and Pflieger, 2002; Hart and Klironomos, 2002). Climatic seasons seem to be more influential on distribution and abundance of mycorrhizal spores. Mycorrhization of forest plants has recently been considered as a substitute for chemical fertilization reducing environment pollution and disease control for better management of tropical forests (Dhar and Mridha, 2006).

CHAPTER 3

*VARIATION IN PHOSPHORUS
CONCENTRATION IN WILD MEDICINAL
PLANTS DURING GROWTH
DEVELOPMENTAL STAGES*

INTRODUCTION

Arbuscular mycorrhizal fungi are ubiquitous and form symbiotic association with most terrestrial plant species. The characteristics and dynamics of occurrence of AM fungi under natural conditions are important for the evaluation of the inoculum potential and root colonization in the process of understanding their behaviour in the soil and determining their symbiotic efficiency (Bethlenfalvay and Linderman, 1992). The presence and/or abundance of AM fungal spores shows that these species may be active in the soil at a specific time, being capable of colonization and multiplying themselves in the host roots (Moriera *et al.*, 2007).

Phenology is the study of the timing of vegetative activities, flowering and fruiting and its relationship to environmental factors. A growing plant may experience different stages in mineral nutrition, based on the balance among internal and external nutrient supplies and crop demand for nutrients. Plants require adequate P from the very early stages of growth for optimum crop production (Grant *et al.*, 2001). The importance of P for plant survival has supported the development of plant adaptations to improve the access of the crop to P supply. It is estimated that on average, P could only diffuse approximately 0.5mm, so that only phosphate within 0.5mm of a plant root is positionally available for absorption (Grant *et al.*, 2001). The prevalent form of available P in the environment is the oxidized anion phosphate. More soluble minerals such as nitrogen (N) move through the soil via bulk flow and diffusion, whereas Pi moves by slow rate of diffusion in the soil solution creating a Pi-depletion

zone around the root (Jungk, 2001). Therefore, the low availability of Pi in the bulk soil affects its uptake into roots (Rausch and Bucher, 2002).

Phosphorus deficiency is one of the most widespread mineral nutrient stresses limiting crop production in the world (Holford, 1997; Sanchez and Salinas, 1981). It is well documented that AM fungi promote growth of the host plant through enhanced uptake of phosphate. According to Jones *et al.* (1998), the efficiency with which mycorrhizal plants take up Pi is 3.1 to 4.7 times higher than that of nonmycorrhizal plants. In soil not adequately supplied with P, uptake of the nutrient by plants far exceeds the rate at which it diffuses into the root zone, resulting in zones of Pi depletion surrounding roots. According to the most accepted mechanism, AM fungi help overcome this problem by extending their external hyphae from root surfaces to areas of soil beyond the Pi depletion zone, thereby exploring a greater volume of the soil than is accessible to the unaided root (Hayman, 1983; Jakobsen *et al.*, 1994; O'keefe and Sylvia, 1991). The external hyphae of some AM fungi may spread at least 10–12cm from the root surface (Jakobsen *et al.*, 1992; Li *et al.*, 1991).

Although mycorrhizal fungi have been shown to enhance the growth and P nutrition of plants in pots (Cooper and Tinker, 1978), few studies have shown a functional relationship of AM fungi to plants in the wild (Miller, 1987). Examination of the functional significance of AM fungi through studies of P uptake is also difficult because the need for P is not constant during the life cycle of most plants (Fitter,

1985). Mycorrhizae may benefit plants only during times of P demand, *i.e.* during flowering or seed development. Patterns and timing of AM development within plant roots vary and may be dependent on edaphic factors (Fitter, 1985; Sanders, 1990) or variation in plant nutrient levels (Sylvia and Neal, 1990).

No previous studies have reported the P concentration in wild medicinal plants in relation to phenology. Thus, the present study was undertaken to study the variation in P concentration in different developmental stages of three selected medicinal plant species growing in the wild.

MATERIALS AND METHODS

Study area and selection of plant species:

The site undertaken for the study is located in Sanguem taluka situated in South Goa. The Sanguem taluka has a geographical position marked at 15° 48' 00" N to 14° 53' 54" N latitude and 73° E to 75° E longitude. The climate of the tract is tropical with three main seasons viz., monsoon, winter and summer. The soil is moderately drained, gravelly with silty clay loam texture with pH ranging from 5.6 to 6.2 and low in nutrients especially P (16kg ha⁻¹) and total N (0.24%). Three medicinally important herbaceous plant species viz., *Rauwolfia serpentina* (L.) Benth. (Apocynaceae), *Catharanthus roseus* L. (Apocynaceae) and *Andrographis paniculata* Nees. (Acanthaceae) growing wild in the forest community were selected for the study

from the same locality. Of these, two plant species, *R. serpentina* and *C. roseus* are listed as endangered species by IUCN red data list.

Rauwolfia serpentina, an erect perennial shrub commonly known as Indian snakeroot or Sarpagandha contains a number of bioactive chemicals including ajmalicine, deserpidine, rescinnamine, serpentine and yohimbine. Reserpine is an alkaloid first isolated from *R. serpentina*, which is widely used as an antihypertensive drug (Lewis and Lewis, 2003).

Catharanthus roseus (Madagascar periwinkle), a perennial herb, has been cultivated for herbal medicine and as an ornamental plant. The substances vinblastine and vincristine extracted from the plant are used in the treatment of leukemia (Leveque and Jehl, 2007).

Andrographis paniculata an erect annual herb commonly known as “King of Bitters” has being used for centuries in Asia to treat upper respiratory infections, fever, Herpes, sore throat and other chronic and infectious diseases. Some of the extremely beneficial properties include analgesic, anti-inflammatory, antibacterial, antipyretic, cancerlytic, antiviral and vermicial. The primary medicinal component of *A. paniculata* is Andrographolide, which is a diterpene lactone. The other active components include 14 deoxy 11, 12- di dehydroandrographolide,

homoandrographolide, andrographan, andrographosterin and stigmasterol (Siripong *et al.*, 1992).

Sample collection:

Collections were made in different growing seasons of the plant viz., vegetative stage (June-August), flowering stage (September-December), and fruiting stage (January- April). Care was taken to choose plants of uniform size that appeared to be approximately the same age. Three plants of each species were collected from the same locality (radius of 100m) at different growth stages. For each plant species, three rhizosphere soil samples were mixed to form a composite sample, packed in polyethylene bags, labeled and brought to the laboratory. Root samples were freshly processed, whereas soil samples were stored in deep freezer at 4⁰C until analyzed.

Estimation of root colonization and spore density:

For processing of roots, trypan blue staining technique (Koske and Gemma, 1989) was used and percent colonization (proportion of root length colonized) was determined according to slide method (Giovannetti and Mosse, 1980). Isolation of AM fungal spores was carried out by wet sieving and decanting method (Gerdemann and Nicolson, 1963). Spore density was calculated by number of spores present per 100g of rhizosphere soil.

Taxonomic identification of spores:

Taxonomic identification of spores was carried by using the Manual for Identification of VAM Fungi by Schenck and Perez (1990) and various taxonomic papers viz., Morton and Benny (1990), Almeida and Schenck (1990), Bentivenga and Morton (1995), Walker and Vestberg (1998), Redecker *et al.* (2000), Morton and Redecker (2001). Taxonomic identification of spores was also carried out by matching the descriptions provided by the International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (<http://invam.caf.wvu.edu>).

Phosphorus estimation by Colorimetric method:

Root and shoot tissues of three medicinal plant species collected during different growth stages were previously analyzed by dry ash digestion procedure and assessed for the estimation of total P concentration (ppm) using Vanadomolybdate phosphoric yellow colour method (Chapman and Prat, 1961).

Dry ash-Digestion Procedure:

1. Portions of ground plant material (0.5-1.0g) were weighed in 30-50ml porcelain crucibles.
2. Porcelain crucibles were placed into a cool muffle furnace, and the temperature was increased gradually to 550⁰C.
3. Ashing was continued for 5 hours after attaining 550⁰C.
4. Porcelain crucibles carefully taken out after cooling.

5. Cooled ash was dissolved in 5ml portions of 2N hydrochloric acid (HCl) and mixed using a plastic rod.
6. After 15-20 minutes, volume was made up to 50ml using double distilled water.
7. After 30 minutes, the supernatant was filtered through Whatman No. 42 filter paper, discarding the first portion of the filtrate.
8. Aliquots were analyzed for P by Colorimetry method.

Ammonium Vanadate-Ammonium Molybdate yellow colour method:

1. 22.5g ammonium heptamolybdate $[(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}]$ was dissolved in 400ml double distilled water (A).
2. 1.25g ammonium metavanadate (NH_4VO_3) was dissolved in 300ml double distilled water (B).
3. (B) was added to (A) in a 1L volumetric flask, and mixture was cooled to room temperature.
4. 250ml concentrated nitric acid (HNO_3) was added to the mixture, made up to 1L volume with double distilled water.

Standard stock solution

- 2.5g Potassium dihydrogen phosphate (KH_2PO_4) was dried in an oven at 105°C for 1 hour, cooled in a desiccator and stored in a tightly stoppered bottle.

- 0.2197g dried Potassium dihydrogen phosphate was dissolved in double distilled water, and brought to 1-l volume with double distilled water. This solution contained 50ppm P (Stock solution).
- A series of standard solutions was prepared as follows:
1,2,3,4 and 5ml stock solutions were diluted to 100ml final volume by adding double distilled water. These solutions were contained 0.5, 1.0, 1.5, 2.0 and 2.5 ppm P respectively.

Procedure

1. Digest filtrate or aliquot (10ml) was pipetted out from the dissolved ash into a 100ml volumetric flask and 10ml of Ammonium-Vanadomolybdate reagent was added and diluted the solution to volume with double distilled water.
2. A standard curve was prepared as follows:
 - 1, 2, 3, 4 and 5ml was pipetted out from standard stock solution and proceeded for the samples.
 - Blank was prepared with 10ml Ammonium-Vanadomolybdate reagent, and proceeded as for the samples.
 - Absorbance of the blank, standard and the samples were read after 30 minutes at 410-nm wavelength using UV Visible Spectrophotometer (UV-2450UV-Visible Spectrophotometer).
3. A calibration curve for standard was prepared for plotting absorbance against the respective P concentrations.

4. Phosphorus concentrations in the unknown samples was read from the calibration curve.

Calculation

Percent total P in plant

$$\% P = \text{ppm P (from calibration curve)} \times \frac{R}{Wt} \times \frac{100}{10000}$$

R = Ratio between total volume of the digest/aliquot and the digest/aliquot volume used for the measurement.

Wt = Weight of dry plant (g).

Relative abundance and Frequency of occurrence:

Relative abundance and Frequency of occurrence of AM fungi was calculated in each plant species at different growth stages using the following formulae (Beena *et al.*, 2000).

$$\text{Relative abundance (\%)} = \frac{\text{Number of AM fungal spores of particular species}}{\text{Total number of AM fungal spores of all species}} \times 100$$

Frequency of occurrence (%) =

$$\frac{\text{Number of soil samples that possess spores of particular species}}{\text{Total number of soil samples screened}} \times 100$$

Statistical analysis:

Pearson correlation analysis was carried out to assess the relationship between colonization and spore density. Results of P concentration in roots and shoots of each plant species at different growth stages was analyzed using One Factorial Analysis of Variance (ANOVA) using WASP 1.0 (Web based Agricultural Statistical Package). For all the analysis, differences were considered significant when $P \leq 0.05$.

RESULTS

Mycorrhizal colonization was observed in all the plant species studied (**Table 7**). Hyphal and vesicular colonization (**Plate V a**) was observed in all the three medicinal plant species at different growth stages whereas arbuscular colonization was observed only during the flowering stage in *C. roseus* and *A. paniculata* (**Plate V b c & d**). Percent root colonization differed in all the plant species at different growth stages (**Table 7**).

In *R. serpentina* and *C. roseus* maximum percent AM colonization was observed during the vegetative stage (58.33% and 70%) followed by flowering stage (50% and 44.44%) and least in fruiting stage (35.71% and 30.7%) respectively whereas in *A. paniculata* flowering stage showed maximum colonization (55.50%) followed by fruiting stage (42.50%) and least in vegetative stage (38.50%) (**Table 7**).

Plate V: Mycorrhizal colonization in medicinal plants during growth stages.

- a) Heart shaped vesicles in roots of *Rauwolfia serpentina* (L.) Benth. during vegetative stage (X 400).
- b) Arbuscular colonization in *Andrographis paniculata* Nees. during flowering stage (X 400).
- c) Arbuscular colonization in *Catharanthus roseus* L. during flowering stage (X 100).
- d) Arbuscular and vesicular colonization in *C. roseus* during flowering stage (X 400).

Plate V

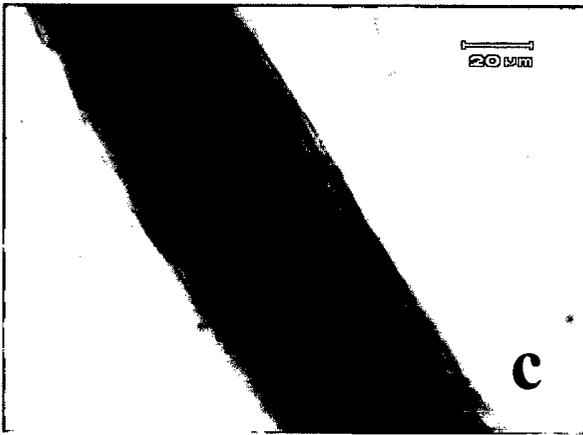
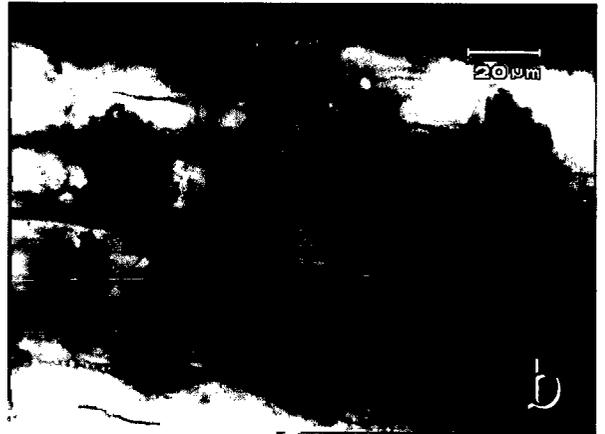


Table 7: Arbuscular Mycorrhizal colonization and spore density in medicinal plants at different growth stages.

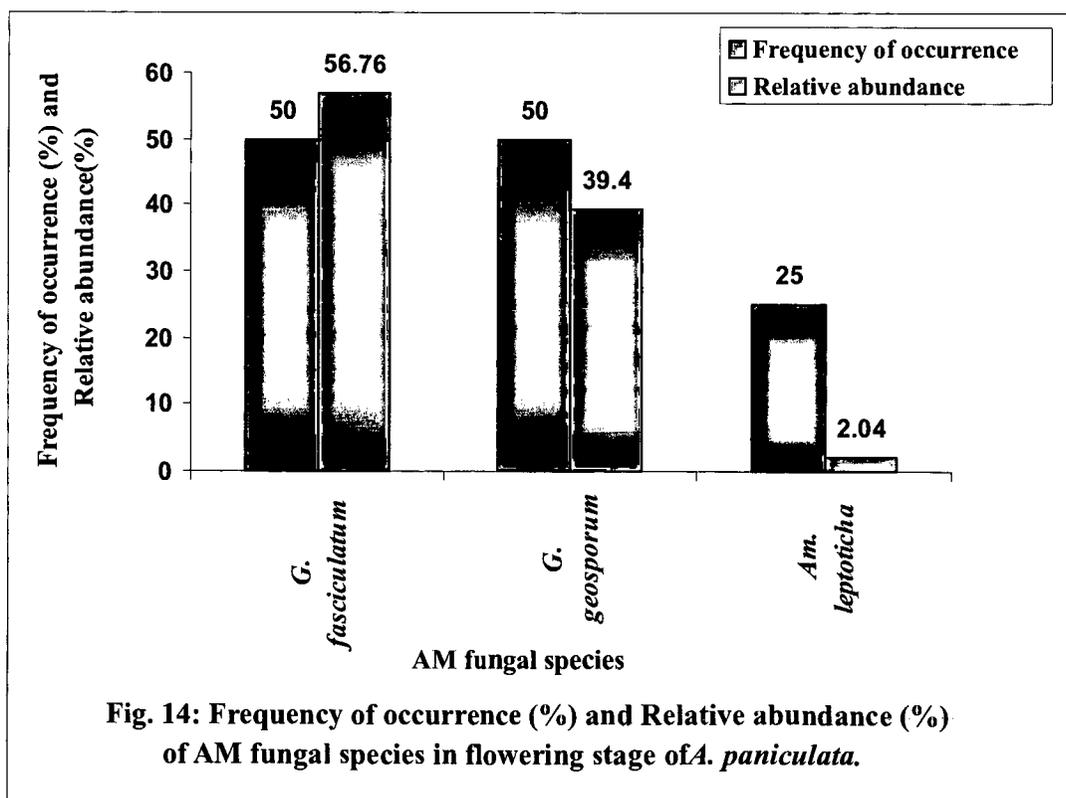
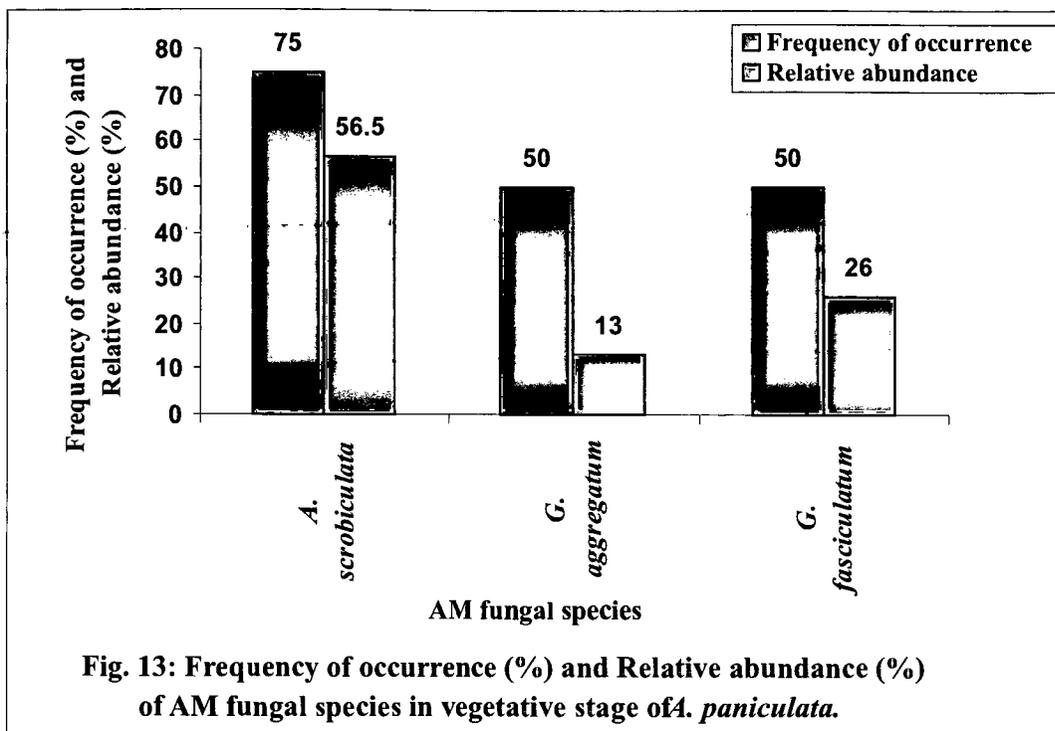
Sr. No.	Plant species & growth stages	% colonization	Type of colonization	Spore density*
1.	<i>Rauwolfia serpentina</i>			
	a) Vegetative stage	58.33±1.00	H,V	124.00±48.30
	b) Flowering stage	50.00±1.73	H,V	8.00±1.50
	c) Fruiting stage	35.71±1.00	H,V	251.00±62.60
2.	<i>Andrographis paniculata</i>			
	a) Vegetative stage	38.50± 2.08	H,V	23.00±1.06
	b) Flowering stage	55.50± 2.64	H,V,A	147.00±15.09
	c) Fruiting stage	42.40±1.15	H,V	52.00±20.60
3.	<i>Catharanthus roseus</i>			
	a) Vegetative stage	70.00±1.00	H,V	47.00±10.69
	b) Flowering stage	44.44±0.57	H,V,A	50.00±19.60
	c) Fruiting stage	30.70±0.50	H,V	56.00±7.50

Legend: H=Hyphal colonization, V=Vesicular colonization, A=Arbuscular colonization.
 Values are mean of three replicates. * Spores 100g⁻¹ of soil. ± indicates standard deviation.

Spore density showed variation in different growth stages of all the plant species studied. In *R. serpentina* and *C. roseus* maximum spore density was recorded in the fruiting stage (251 and 56 spores 100g⁻¹ soil) whereas in *A. paniculata* higher spore density was observed during the flowering stage (147 spores 100g⁻¹ soil). Among the plant species, least spore density was recorded in *R. serpentina* (8 spores 100g⁻¹ soil) in the flowering stage (**Table 7**). A non-significant negative correlation was observed between percentage colonization and spore density ($r = -0.1$, $P < 0.05$).

Twenty AM fungal species belonging to five genera viz., *Glomus*, *Acaulospora*, *Scutellospora*, *Gigaspora* and *Ambispora* were identified from the rhizosphere soil samples. *Glomus* was the most dominant genus and *Glomus fasciculatum*, the most dominant species, was recorded in all stages of development in the three plant species.

In *A. paniculata*, five AM fungal species belonging to three genera viz., *Acaulospora*, *Glomus* and *Ambispora* were recorded in different growth stages. *G. fasciculatum* was the most commonly occurring AM fungal species in all the growth stages and was more abundant during flowering and fruiting stages of the plant whereas *A. scrobiculata* was more abundant during the vegetative stage. Out of five AM fungal species, *Am. leptoticha* recorded the least in relative abundance (2.04%) and frequency of occurrence (25%), both observations made during flowering stage of the plant (**Fig. 13, 14 & 15**).

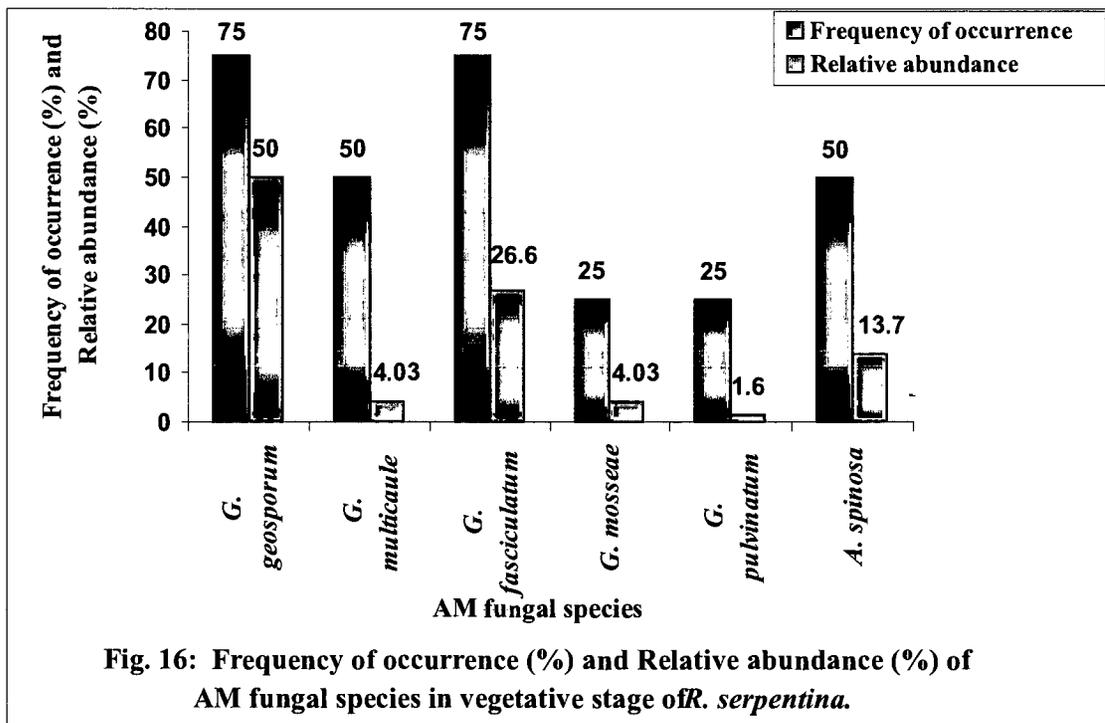
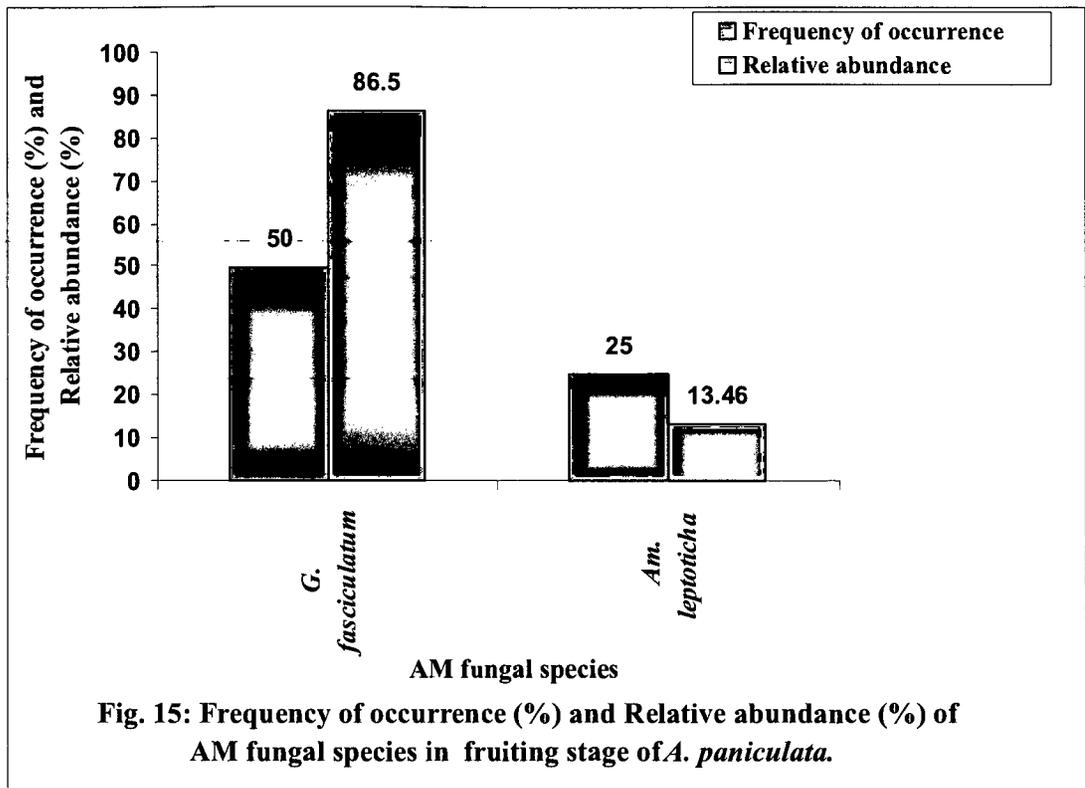


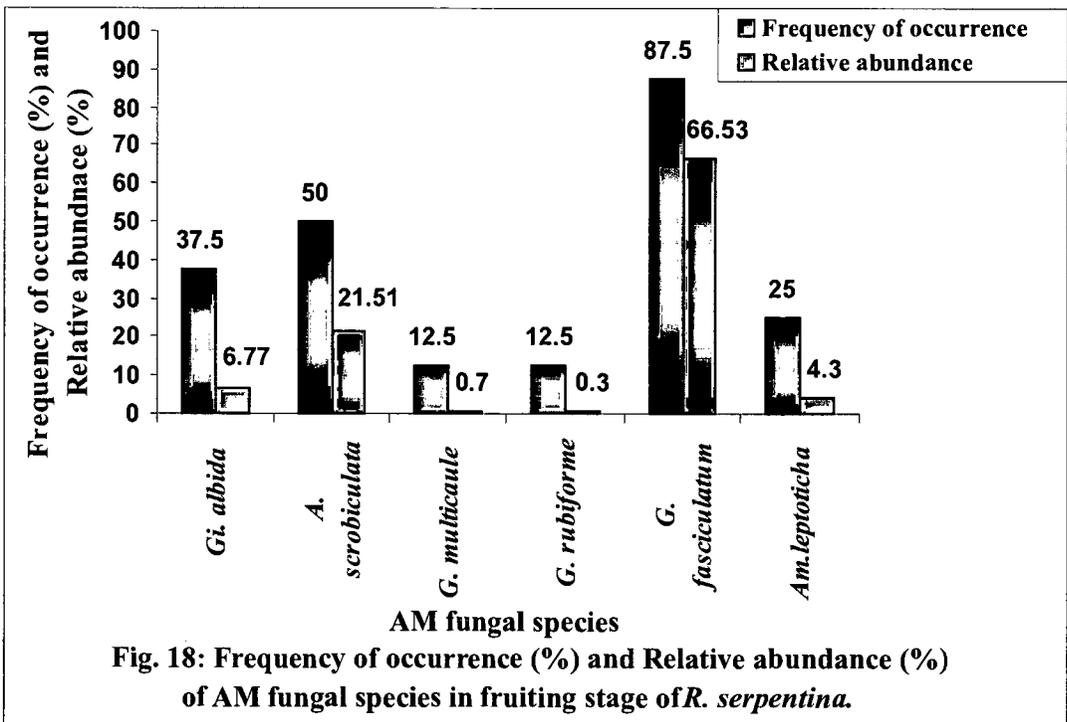
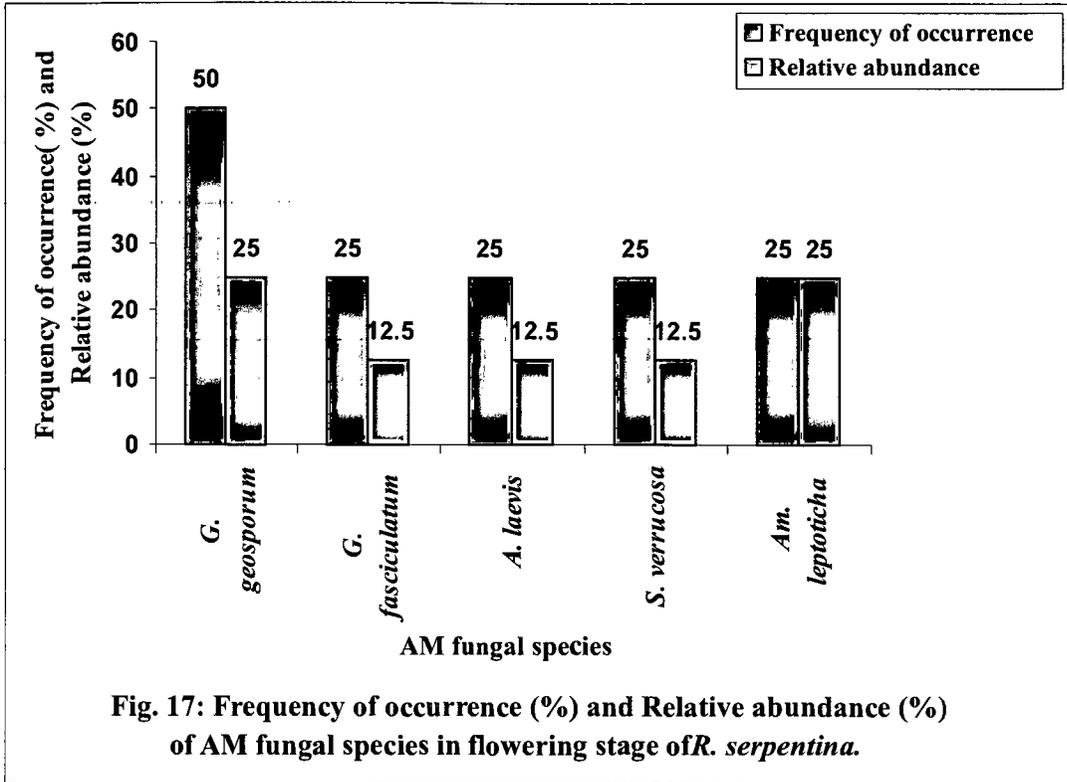
Thirteen AM fungal species belonging to five genera viz., *Acaulospora*, *Ambispora*, *Glomus*, *Gigaspora* and *Scutellospora* were recorded in all the growth stages of *R. serpentina*. *Glomus fasciculatum* was recovered in all the growth stages and found to be the most dominant species in terms of relative abundance (66.53%) and frequency of occurrence (87.5%) recorded during the fruiting stage whereas *G. geosporum* was the most abundant and frequently occurring AM fungal species during the vegetative and flowering stages (**Fig. 16, 17 & 18**).

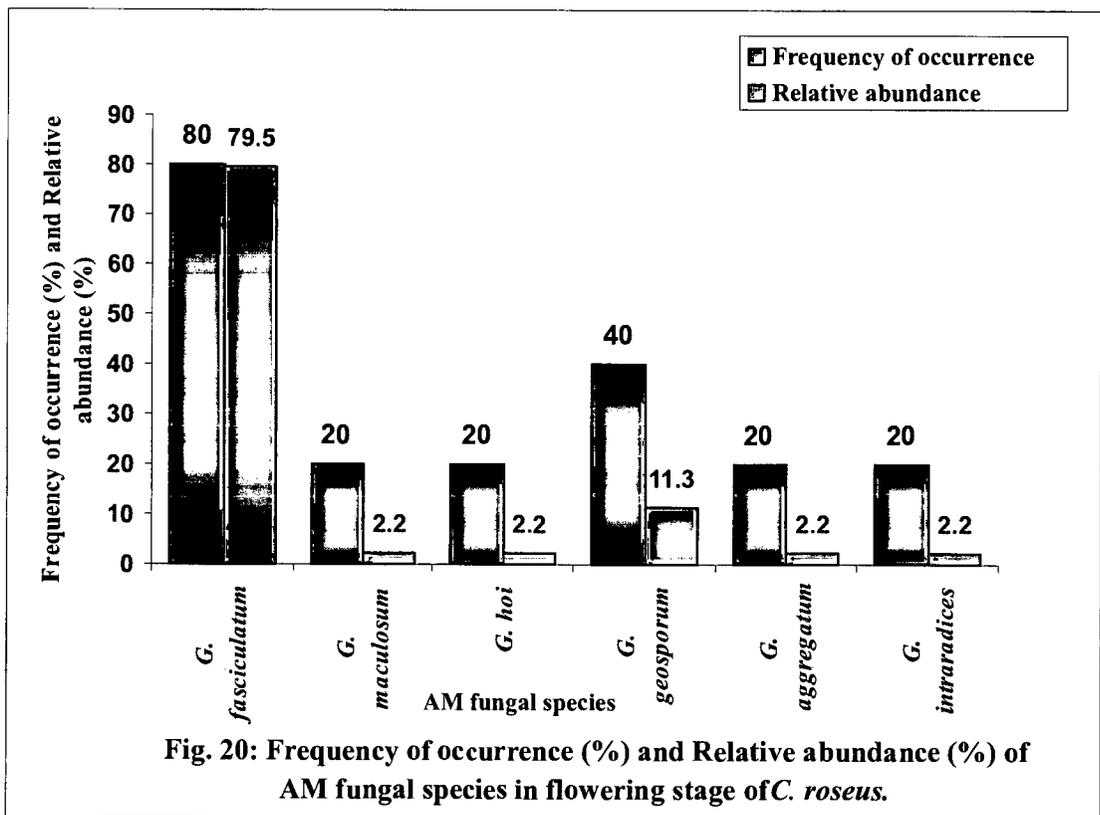
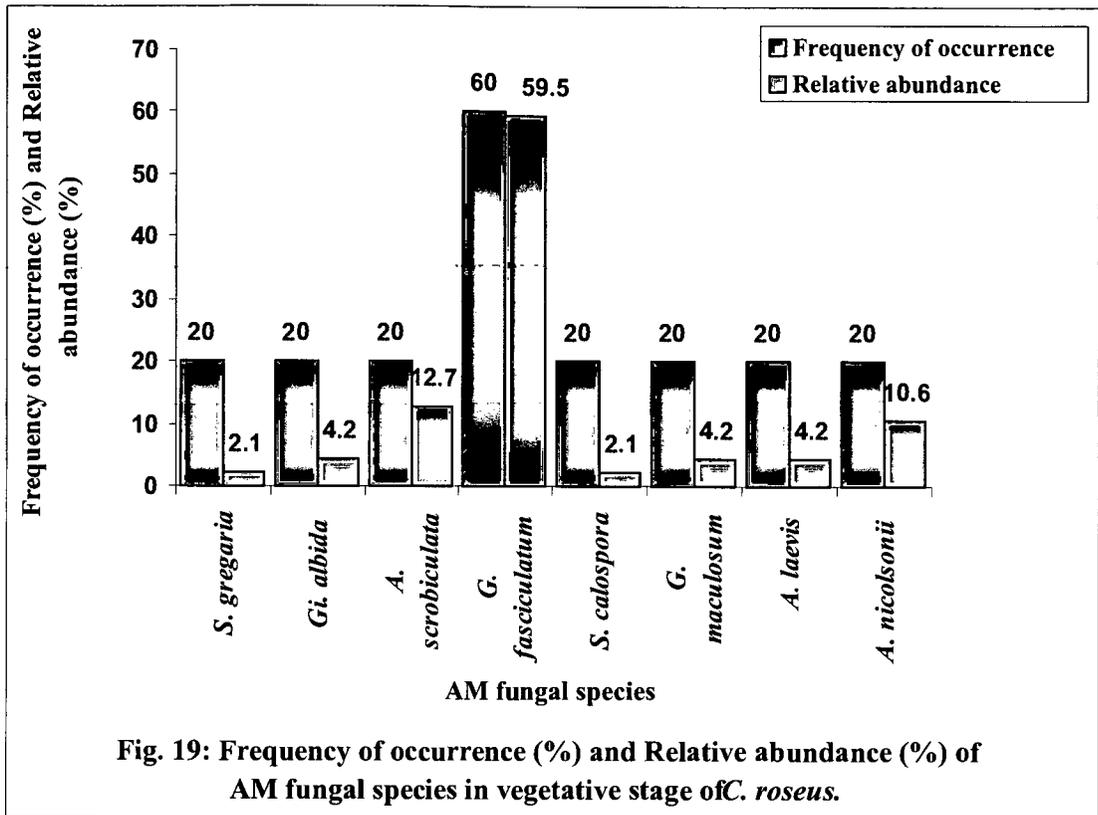
In *C. roseus*, 13 AM fungal species belonging to four genera viz., *Acaulospora*, *Glomus*, *Gigaspora* and *Scutellospora* were recorded in the rhizosphere soil samples in all the three growth stages. *Glomus fasciculatum* and *G. maculosum* occurred in all the growth stages. *G. fasciculatum* was the most abundant (79.5%) and frequently occurring (80%) species and was recorded during the flowering stage whereas *G. maculosum* was the most abundant and frequently occurring during the vegetative and fruiting stages (**Fig. 19, 20 & 21**).

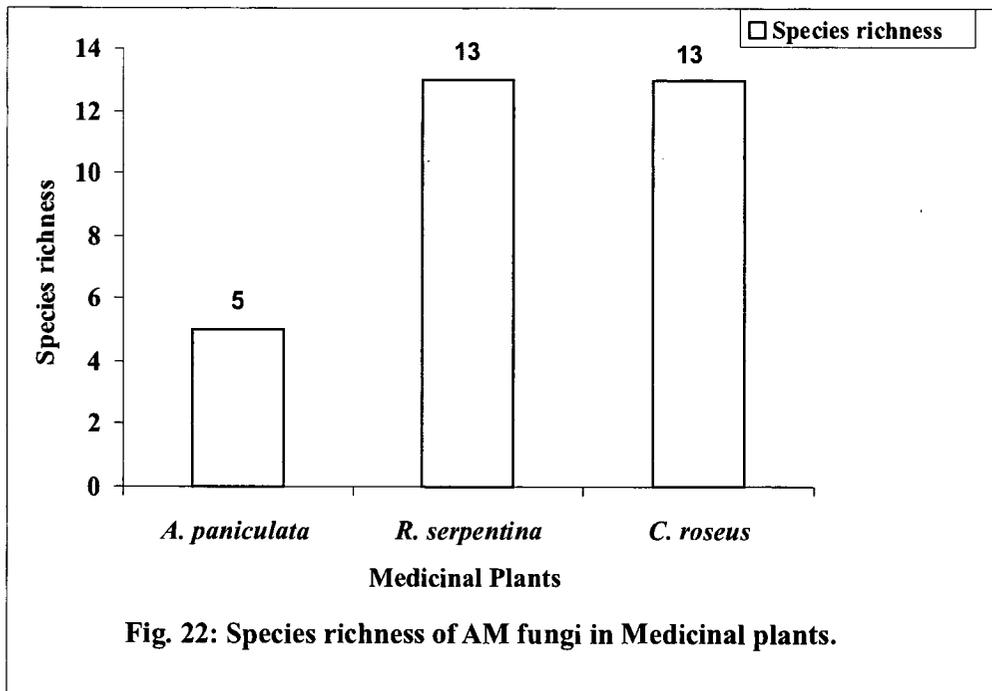
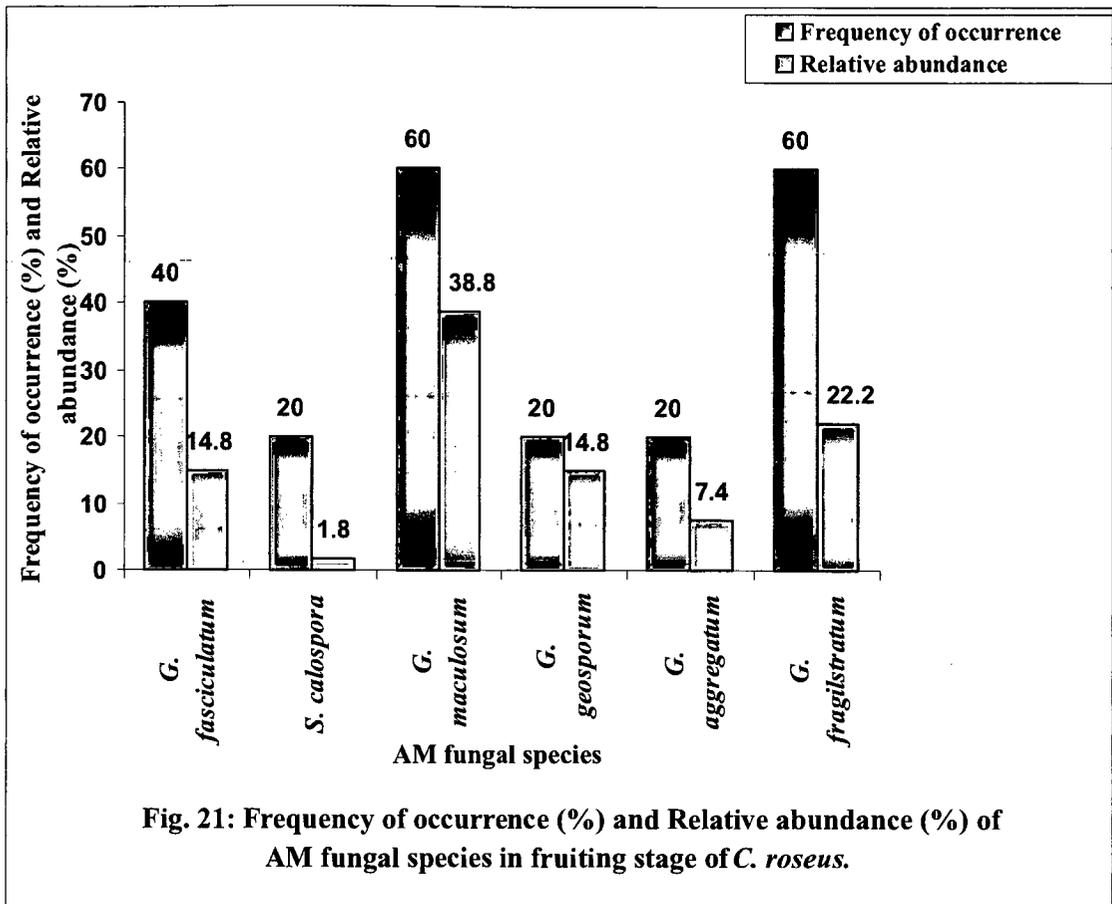
Maximum species richness (13) was recorded in the rhizosphere soil samples of *R. serpentina* and *C. roseus* and least in *A. paniculata* (4) (**Fig. 22**).

The P concentration studies revealed variation in both root and shoot tissues of the three medicinal plant species at different growth stages. In *R. serpentina*, maximum P concentration in root (39.3ppm) and shoot tissues (53.8ppm) was









recorded during the vegetative stage of the plant (**Fig. 25**) whereas in *A. paniculata* and *C. roseus* maximum P concentration was recorded during the flowering stage which suggests the presence of arbuscules. An increase in shoot P concentration was also observed after the formation of arbuscules (**Fig. 23 & 24**) (**Table 8**).

Results of P concentration in roots and shoots were analyzed using ANOVA. Root and shoot tissues of *R. serpentina* showed a significant difference in P concentration observed between the growth stages ($F=0.000$, $df=5$, $P\leq 0.05$) (**Table 8**). Here, a significant decrease in P concentration from vegetative to flowering stage followed by an increase in fruiting stage was observed (**Fig. 25**). Roots and shoots of *A. paniculata* showed a significant increase in P concentration from vegetative to flowering stage of the plant, and then showed a gradual decrease in the fruiting stage ($F=0.004$, $df=5$, $P\leq 0.05$) (**Table 8**) (**Fig. 23**). In *C. roseus*, a significant increase from vegetative to flowering stage was observed in the shoots followed by a decrease in the fruiting stage, whereas in roots, P concentration increased significantly from flowering stage onwards ($F=0.003$, $df=5$, $P\leq 0.05$) (**Table 8**) (**Fig. 24**). Negative correlation was observed between percentage colonization and P concentration in roots and shoots ($r = -0.1, -0.2, P\leq 0.05$).

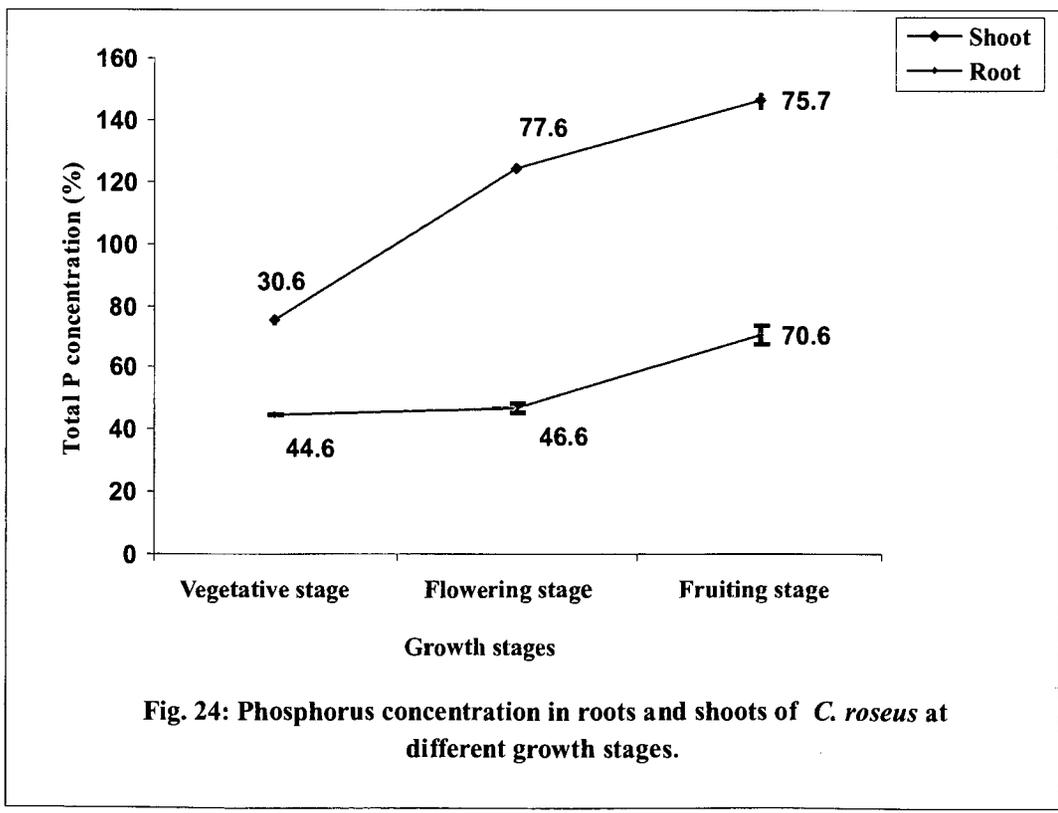
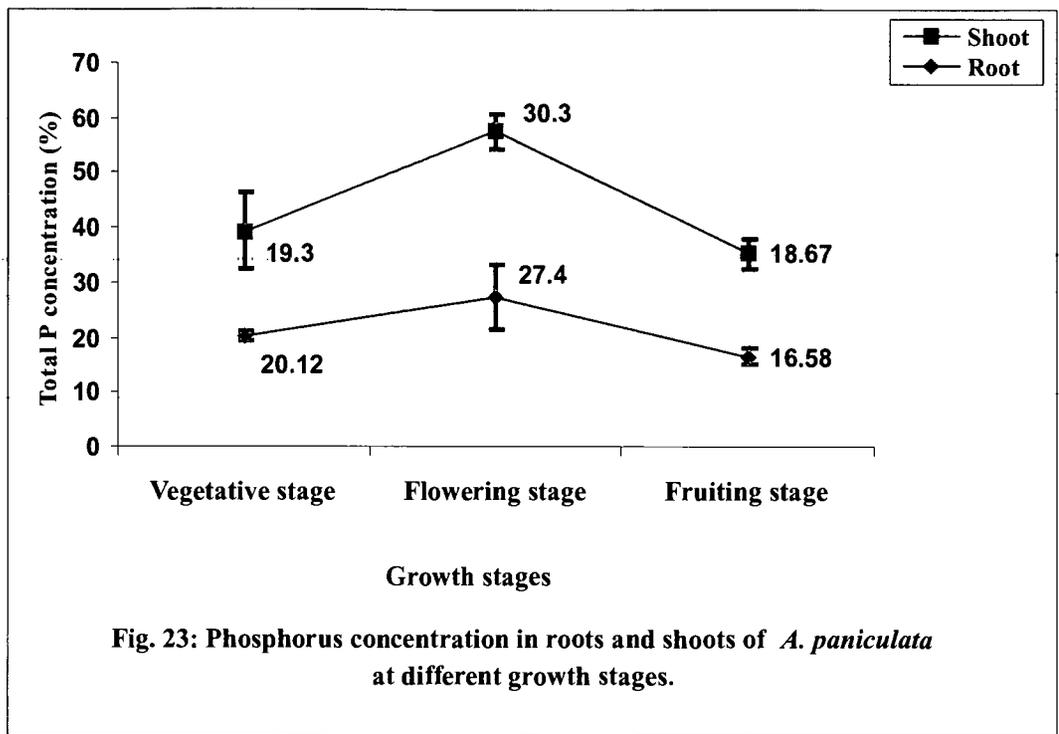
DISCUSSION

The present study indicates that AM fungi could play an important role in growth of medicinal plants. Muthukumar and Udaiyan (2000) reported the presence

Table 8: Phosphorus concentration in roots and shoots of medicinal plants at different growth stages.

Sr. No.	Plant species & growth stages	Total P concentration	
		Root (ppm)	Shoot (ppm)
1.	<i>Rauwolfia serpentina</i>		
	a) Vegetative stage	39.3 ^b ± 0.8	53.8 ^a ± 6.8
	b) Flowering stage	21.6 ^c ± 6.1	21.7 ^c ± 3.1
	c) Fruiting stage	38.7 ^b ± 1.5	37.6 ^b ± 2.7
2.	<i>Andrographis paniculata</i>		
	a) Vegetative stage	20.12 ^b ± 0.17	19.3 ^b ± 1.5
	b) Flowering stage	27.4 ^a ± 1.5	30.3 ^a ± 0.28
	c) Fruiting stage	16.58 ^b ± 2.8	18.67 ^b ± 2.4
3.	<i>Catharanthus roseus</i>		
	a) Vegetative stage	44.6 ^b ± 1.2	30.6 ^b ± 5.6
	b) Flowering stage	46.6 ^b ± 4.6	77.6 ^a ± 4.4
	c) Fruiting stage	70.6 ^a ± 2.1	75.70 ^a ± 2.3

Legend: Values are means of three replicates. Columns with different letters indicate that values are significantly different ($P \leq 0.05$).



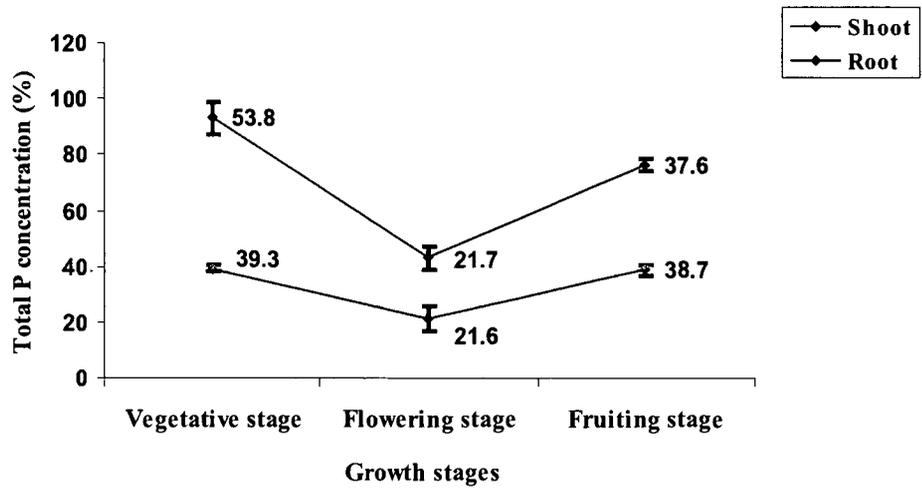


Fig. 25: Phosphorus concentration in roots and shoots of *R. serpentina* at different growth stages.

of AM fungal colonization in *A. paniculata* and *R. serpentina* but absence of colonization in *C. roseus*. However, in the present study AM fungal colonization was observed in all the growth stages of *C. roseus*. The study also revealed that *Glomus* was the most dominant genus in the rhizosphere soil of medicinal plants. The predominance of *Glomus* in tropical soils has been reported by other workers (Thapar and Khan, 1985; Ragupathy and Mahadevan, 1993). The study also recorded the presence of 20 AM fungal species from the rhizosphere soil of three medicinal plant species in different growth stages. Johnson *et al.* (1991) reported similar findings in their study on plant and soil controls of mycorrhizal communities. They reported the presence of 12 to 22 different species of AM fungi per study site. Muthukumar *et al.* (2001) however reported only 35 AM fungal species from the rhizosphere soil of about 329 plant species from the Western Ghats of Southern India. Recovery of relatively high number of species in the present study is in agreement with Francis and Read (1994) who reported that high species diversity, characteristic of phosphorus-deficient grassland ecosystem dominated by plant species with AM fungi, may be attributed to a low level of host specificity.

Large variations in the spore numbers (8 to 251 spores 100g⁻¹ rhizosphere soil) recorded in plant species at different growth stages in the present study can be attributed mainly to the interspecific competition (Brundrett and Kendrick, 1990) and by the subsequent variation in the timing of spore production associated with host plants, suggesting that the competition between fungi and environmental factors

probably influences spore production in natural communities (Gemma and Koske, 1988). Seasonality, edaphic factors, host dependence, age of the host plants and dormancy and distribution patterns of AM fungal spores in soil are factors which also affect sporulation in a given rhizosphere (Bever *et al.*, 1996; Greipson and El-Mayas, 2000; Zhao, 1999). The non-uniform distribution of spores in the present study may be due to various factors, the most obvious being the threshold of mycorrhizal biomass needed to induce spore production (Sturmer and Bellei, 1994).

In the present study, increase in the P concentration during flowering stage of *A. paniculata* and *C. roseus* is directly related to the presence of arbuscules as flower initiation demands extra uptake of P. An increase in the shoot P concentration was observed after the formation of arbuscules. Similar observations have been reported in *Ranunculus adoneus* (Mullen and Schmidt, 1993). Although the relationship between P accumulation and AM fungal development has not been previously documented in wild plant population, there have been several studies in agro ecosystems (Dodd and Jeffries, 1986; Dunne and Fitter, 1989; Jakobsen, 1986).

Sanders (1990) studied AM fungal development in wild grassland species but did not record nutrient accumulation and found no relationship from one year to the next in developmental patterns. Other studies of mycorrhizal development in wild populations (Brundrett and Kendrick, 1988; Sanders, 1990) have reported only mycorrhizal colonization. Reinhardt and Miller (1990), working on temperate

grasslands, found that AM colonization reached a peak in the growing season (April) and they assessed the levels of arbuscules from root cores containing roots of the entire plant community, not relating colonization to phenology. However, the presence of arbuscules recorded during the flowering stages in two plant species viz., *A. paniculata* and *C. roseus* indicated that arbuscules are essential for P uptake. In *R. serpentina* no arbuscules were observed in any of the growth stages, maximum P concentration being observed during the vegetative stage indicating that this species require P during early growth stage for optimum yield. A similar observation was made earlier (Grant *et al.*, 2001) and the study supports the fact that the need for P is not constant during the life cycle of the plant (Fitter, 1985). In *A. paniculata* a significant decrease in P concentration was observed during the fruiting stage as P concentration in tissue of annual plants declines with advancing plant age or stage of growth because as the plant matures an increasing proportion of its dry weight is composed of low P in structural and storage tissues. Similar observations were recorded earlier (Belanger and Richards, 1999).

The benefits gained by a root in association with mycorrhizal fungi should be directly related to its life span and the proportion of colonized roots occupied by vesicles increase over the growing season (Reinhardt and Miller, 1990). However, in this study which vesicles were observed in all the growth stages of the plants. The predominance of vesicles in the roots indicated that conditions were favourable for their formation. Dunne and Fitter (1989) who examined the P budget of strawberry

plants in the field, found that flower initiation required extra uptake of P. However, Dodd and Jeffries (1986) in the study of mycorrhizal development in winter wheat quantified arbuscule production and found that levels were highest immediately before seed formation, presumably a time of high P demand. Fitter (1991) suggested that wild plants may have different patterns of P uptake than crop plants.

In the present study, *C. roseus* showed high P concentration in plant tissues, but recorded fewer number of spores in the rhizosphere soil. This observation supports the earlier study that higher tissue P in the plant reduces the production of spores (De Miranda and Harris, 1994), and secondary external hyphae (Bruce *et al.*, 1994). Root exudates from host plants secrete signal molecules that are known to enhance hyphal branching when there is P limitation in host roots (Nagahashi and Douds, 2000). Therefore, increasing P status in the root may reduce the secretion of these signal molecules, thus reducing hyphal branching and mycorrhizal association. Phosphorus status of the roots may affect membrane phospholipids, thus influencing membrane permeability and the release of carbohydrates from the roots which nourish the fungi (Graham *et al.*, 1981; Schwab *et al.*, 1991). Therefore, when P concentration in the plant is low, carbohydrate exudation will encourage mycorrhizal association, which will then enhance the uptake of P from the soil (Grant *et al.*, 2005).

Increase in P concentration during flowering stage indicates that presence of arbuscules corresponds to active P accumulation in wild medicinal plant species. An increase in shoot P concentration was observed after the arbuscule formation which could be due to the fact that when P_i is readily available more P was transported to shoots leading to P luxury consumption (Chapin, 1980) or P_i storage that could be used in the future to support long term growth (Aerts and Chapin, 2000). Further studies pertaining to the beneficial effects on growth in medicinal plant species and understanding the importance of the relationships to each of the symbionts needs to be undertaken.

CHAPTER 4

*HISTOCHEMICAL LOCALIZATION OF
POLYPHOSPHATE (POLY P) GRANULES
AND LIPID BODIES IN TWO
ARBUSCULAR MYCORRHIZAL FUNGI*

INTRODUCTION

Plants and their AM fungal symbionts have coexisted for more than 400 million years (Redecker *et al.*, 2000) and the association is widespread in terrestrial ecosystems. Arbuscular mycorrhizal symbiosis provides multiple benefits for the plants, not only enhanced P and nitrogen nutrition but also resistance against pathogen and abiotic stresses (Smith *et al.*, 2003; Newsham *et al.*, 1995). Nutrition in AM fungi is based on the acquisition of soil nutrients by the fungus (George *et al.*, 1995; Jakobsen, 1999) and fixation of atmospheric carbon by the plant (Ho and Trappe, 1973) and on the exchange of these nutrients at specially adapted symbiotic interfaces (Gianainezzi-Pearson *et al.*, 1991; Smith and Read, 1997).

The enhanced growth rates of plants colonized by AM fungi are due to improved mineral nutrition, particularly for P (Tinker, 1975). Nutrients taken up by the fungus are transferred to the host and high P fluxes between $3.8 \times 10^{-8} \text{ mol P cm}^{-2} \text{ s}^{-1}$ and $10^{-9} \text{ mol P cm}^{-2} \text{ s}^{-1}$ have been calculated and experimentally determined (Sanders and Tinker, 1973; Pearson and Tinker, 1975). Such high rates of translocation require mechanisms based upon bulk flow and cytoplasmic streaming (Tinker, 1975) and it has been suggested that P may be translocated in a condensed form as polyP granules by cyclosis (Cox *et al.*, 1975).

Inorganic polyP is a linear polymer of phosphate linked by high-energy bonds and a wide range of microorganisms store P by accumulation of poly P (Kornberg *et*

al., 1999). It has been suggested that AM fungi accumulate poly P for long distance translocation along hyphae (Cox *et al.*, 1980; Solaiman *et al.*, 1999). High affinity type Pi-transporter genes have been isolated from AM fungi, and gene products are responsible for uptake of Pi from the soil solution and into the fungal cytosol (Maldonado-Mendoza *et al.*, 2001).

Recent attention has been given particularly to the mechanism of P uptake and activity of selected enzymes in this symbiotic interaction. Callow *et al.* (1978) suggested that AM fungi synthesize polyP vacuolar granules from soil phosphate and that the granules are broken down in the arbuscules to inorganic phosphate for release to the host. Alkaline phosphatase activity specific to AM has been reported in Onion and Tobacco (Bertheau, 1977). Gianiniazzi-Pearson and Gianiniazzi (1978) determined that this activity is closely linked to both the mycorrhizal growth stimulation and the arbuscular phase of colonization. They proposed that this enzyme could play a role in the assimilation of P by mycorrhizal roots.

Arbuscular mycorrhizal fungi are oleogenic fungi and store large amount of lipids as triacylglycerides (TAGs) (Gaspar *et al.*, 1994; Jagabaji-Hare, 1998). The synthesis of TAG is a substantial sink for carbon in the intraradical hyphae (Pfeffer *et al.*, 1999; Bago *et al.*, 2000). Synthesis of lipids by the fungal endophyte has been suggested as an alternate storage sink for the plant photosynthates (Cox *et al.*, 1975).

This chapter reports histochemical staining of polyphosphate granules and lipid bodies in the roots of *Coleus* sp. inoculated with two AM fungal species.

MATERIALS AND METHODS

Plant and Fungus material:

Spores of two AM fungal species viz., *Gigaspora albida* and *Glomus clarum* isolated from monospecific cultures using *Coleus* sp. as a host were used for the study. Intact spores mounted in PVLG (Poly-vinyl Lacto Glycerol) (Koske and Tessier, 1983) with or without Melzers reagent were examined under Leica compound microscope, identified based on spore morphology, subcellular characters and compared with original descriptions (Schenck and Perez, 1990; Almeida and Schenck, 1990; Redecker *et al.*, 2000; Morton and Redecker, 2001; Schubler *et al.*, 2001). Spore morphology was also compared with the culture data established by International Collection of Vesicular Arbuscular Fungi (INVAM) (<http://invam.cag.wvu.edu>).

The inoculum (spores) was mixed with autoclaved soil sand at a ratio of 1:3 and filled in to 15 cm diameter pots. Pots were planted with *Coleus* cuttings and watered regularly with sterile distilled water. Hoagland solution (Hoagland and Arnon, 1938) was added every 15 days. After 40 days, watering was stopped and on the 45th day, roots of *Coleus* sp. were checked for colonization using 0.5% trypan blue (Koske and Gemma, 1989).

Histochemical assessment for PolyP and Lipid droplets:

Coleus root (45 days old) pieces were also stained for polyP granules using Toluidine blue O (TBO) (Kumble and Kornberg, 1996), and Sudan Black for staining lipid bodies (McGee-Russell and Smale, 1963).

Preparation of Toluidine blue O: 1g of Toluidine blue O was dissolved in distilled water and 1N HCl added to adjust the pH to 1. For polyP staining, roots were gently washed in tap water and cut into 1cm root pieces, cleared in 2% KOH at 90°C for 30-45min, thoroughly rinsed in water, acidified with 5N HCl (Koske and Gemmae, 1989), stained with Toluidine blue for 20 minutes and then rinsed with distilled water.

Preparation of Sudan Black: For staining of lipid droplets, 100mg of Sudan black in 10ml of 70% saturated solution of ethyl alcohol was prepared and then filtered before staining (Baker, 1960). Root pieces were stained in Sudan black for 1-5 minutes after 1N HCl treatment (Koske and Gemma, 1989).

Photomicrographs of the stained root pieces under different magnifications were taken using Olympus DP12-Olympus BX41 compound microscope. Staining reactions were examined in the intraradical hyphae, arbuscules and vesicles of AM fungi.

RESULTS

Mycorrhizal colonization was observed in *Coleus* sp. (stained with trypan blue) inoculated with *Gi. albida* and *G. clarum*. Arbuscules, vesicles, intercellular hyphae, intraradical spores (**Plate VIII b**) and extraradical spores (**Plate VIII a, c & d**) were observed in roots which were stained dark blue in trypan blue stain. In *Coleus* sp. inoculated with *Gi. albida* Arum type of arbuscules were observed in all the root pieces examined. Arum type was characterized by the formation of intracellular arbuscules surrounded by a layer of the host cytoplasm within the cortical cells. At an early stage of development, the entire arbuscular unit (arbuscular branches), trunk hyphae and intercellular hyphae were stained blue in trypan blue (**Plate VI a**). As the colonization progressed, fine dichotomously branched arbuscules were observed. Cross wall structures were observed in the trunk of hyphae (**Plate VI b & c**). Matured arbuscules consisted of finer branches which were emptied and collapsed. Degeneration of arbuscules involved the collapse of branches apparently from finest branches, the arbuscule trunk being the last to collapse and eventually disappear and is found associated with the loss of metabolic activity of the branches. Deteriorated arbuscular branches were observed in almost every invaded cortical cell (**Plate VI d**). The intercellular hyphae contained abundant lipid droplets (**Plate VII a**).

Vesicle formation was observed in roots of *Coleus* sp. inoculated with *G. clarum* by the swelling of intercellular hyphae followed by the continuous movement

Plate VI: Arbuscular colonization in *Gigaspora albida*

- a) Early stage of arbuscule formation in roots of *Coleus* sp. inoculated with *Gi. albida* (X 400).

- b) & c) Dichotomously branched arbuscules (X 400).

- d) Degenerating arbuscules (X 400).

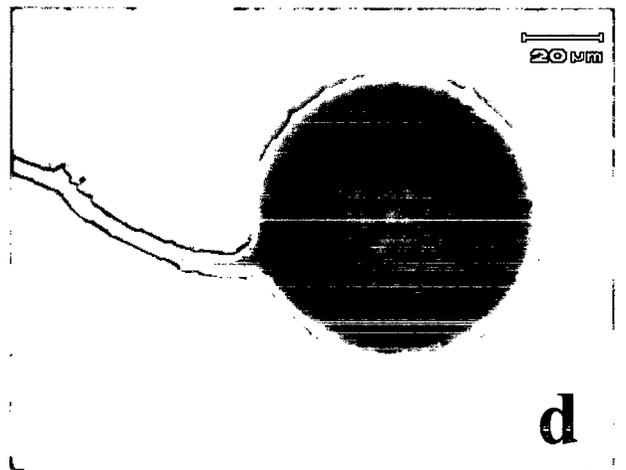
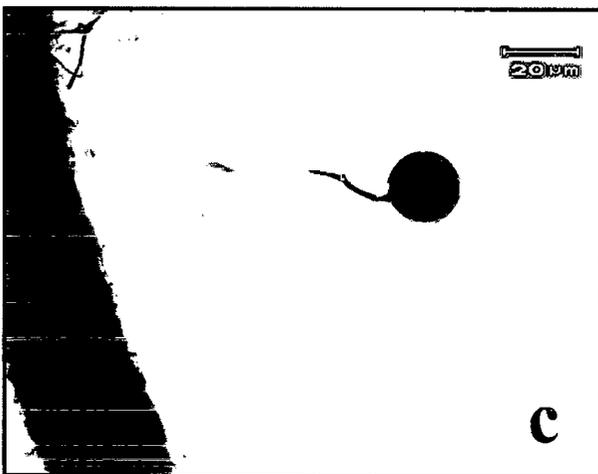
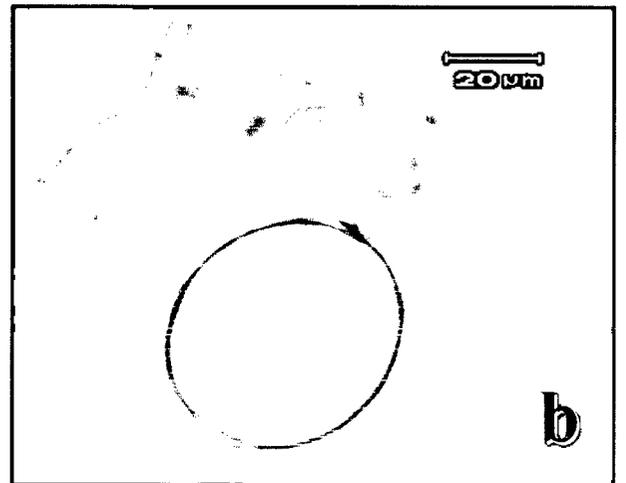
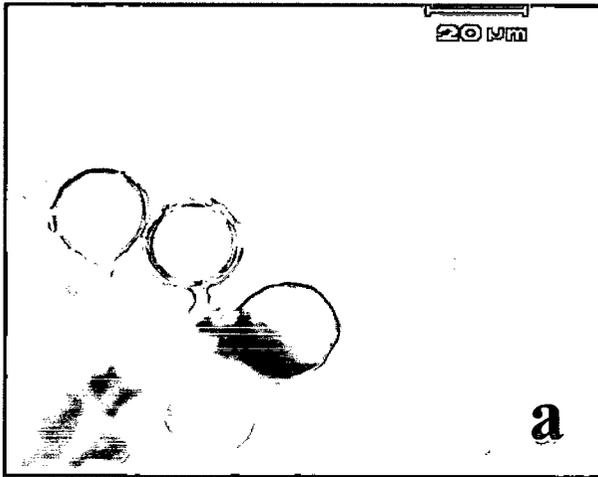
Plate VI



Plate VIII: Intraradical spores and Extraradical spores

- a) Extraradical spores of *Glomus clarum* in roots of *Coleus* sp. stained in trypan blue (X 100).
- b) Intraradical spores of *G. clarum* unstained in roots (X 400).
- c) Extraradical spore of *Gigaspora albida* stained in trypan blue (X100).
- d) Closer view of extraradical spore of *Gi. albida* (X 400).

Plate VIII



of lipid bodies until it filled the entire cell to form a mature vesicle (**Plate VII b-f**).

The matured vesicles later developed into intraradical spores by wall thickenings.

Histochemical observations revealed that intercellular hyphae contained polyP granules that stained metachromatically with Toluidine blue O. Accumulation of polyP granules was located in the intercellular hyphae in roots inoculated with *G. albida* which were stained pinkish purple and there was a net transport of polyP granules into the arbuscules by protoplasmic streaming (**Plate IX a-b**).

In *G. clarum* lipid bodies were present in the form of droplets of varying sizes in the intercellular hyphae, stained blue black. Vesicles developing into intraradical spores in the root cortex were also observed in the root, which stained blue-black. Accumulation of lipid bodies in the spores through intercellular hyphae was observed in the roots colonized by *G. clarum*. Some intracellular vesicles appeared to be almost completely filled by a large lipid droplet. The movement of such lipid bodies may be carried out possibly by cytoplasmic streaming. Vesicles formed were oval to elliptical when formed intercellularly whose wall thicken to produce spores were also observed in the roots of *G. clarum* (**Plate IX c-f**).

DISCUSSION

The presence of arbuscules in *Gi. albida* and vesicles and arbuscules in *G. clarum* stained in trypan blue were recorded in the present study. Arbuscules are

Plate VII: Vesicular colonization in *Glomus clarum*

- a) Lipid droplets in intercellular hyphae of *Coleus* sp. inoculated with *G. clarum* (X 400).
- b) Swelling (s) of intercellular hyphae (X 400).
- c) d) & e) Movement of lipid bodies in intercellular hyphae (X 400).
- f) Mature vesicle in the roots of *Coleus* sp. (X 400).

Plate VII

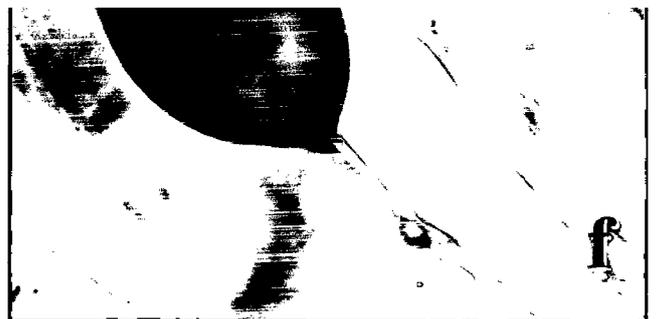
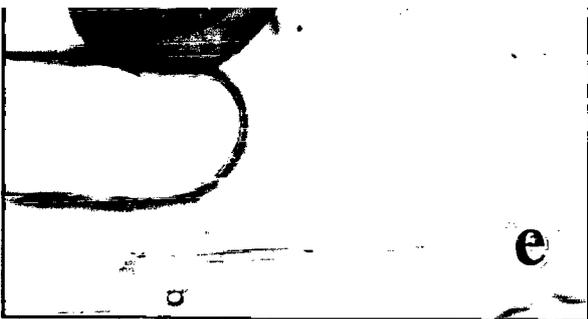
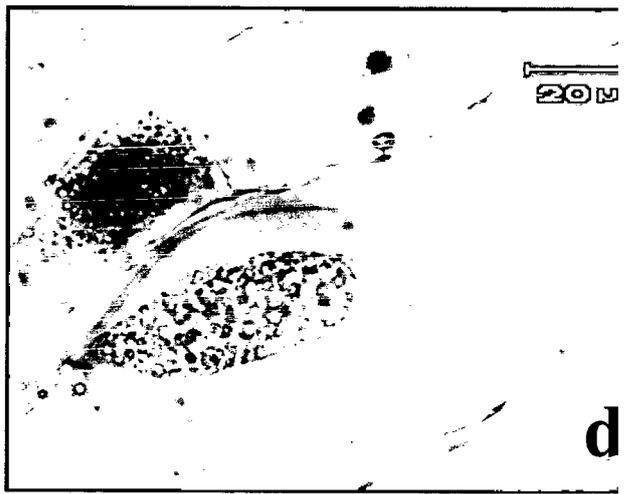
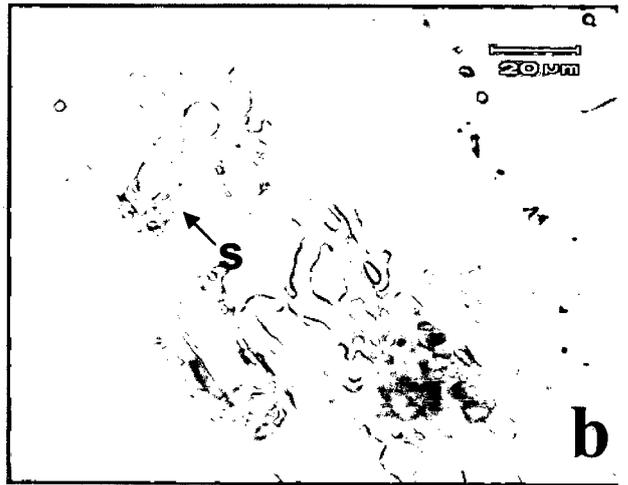
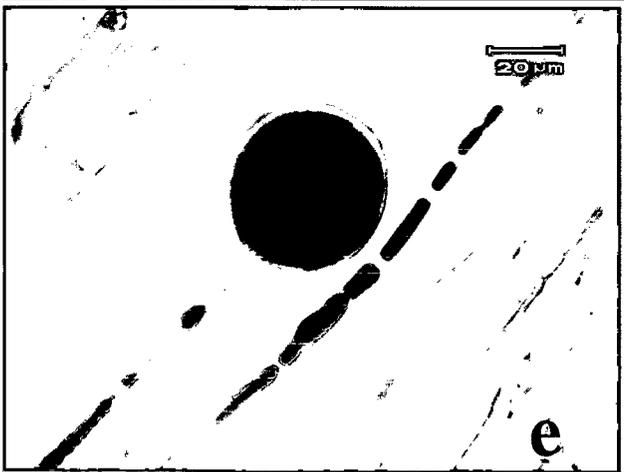
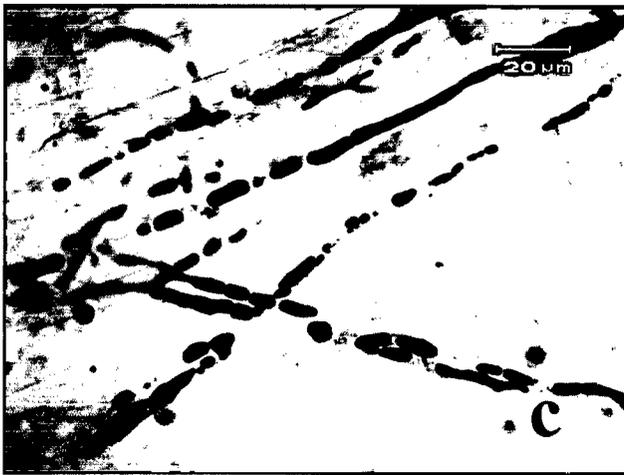
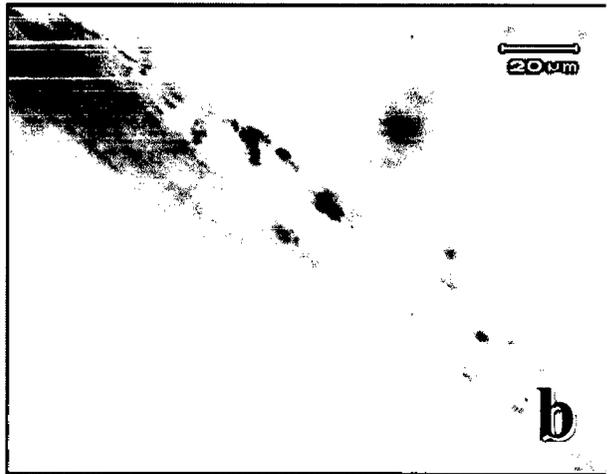


Plate IX: Polyphosphate granules and lipid bodies

- a) & b) Toluidine blue O stained Polyphosphate granules in intercellular hyphae of *Coleus* sp. inoculated with *Gigaspora albida* (X 400).
- c) Sudan black stained lipid bodies in intercellular hyphae (X 400).
- d), e & f) Sudan black stained intraradical spores of *Glarum clarum* (X 400).

Plate IX



thought to be the major sites of nutrient transfer. One of the major nutrients involved is P and it is generally considered that the pathway of transfer is from soil to plant, through the uptake by external mycelia, then into intercellular hyphae (in arum-type mycorrhizas), arbuscular trunk hyphae via the arbuscule branches to the arbuscular interface and then to the plant cell (Smith and Read, 1997). There have been studies on arbuscule development using both light and electron microscopy. The developmental cycle from the formation of the first arbuscule branches to final collapse has been estimated to be between 4 and 12d in different plant/fungal combinations (Toth and Miller, 1984; Alexander *et al.*, 1989). The activity of the arbuscules at different stages of development has usually been assessed on the basis of the physical appearance of the branches in electron microscopy or by light microscopy after nonvital staining (with trypan blue). The electron microscopy studies indicated the presence of cross walls within fine arbuscular branches (Kaspari, 1973; Bonfante-Fasolo *et al.*, 1990). The presence of cross walls is highly correlated with loss of activity of arbuscules and is often formed at sites between metabolically active and inactive fungal regions. It has been reported that P could be transported to the arbuscules when both intercellular hyphae and arbuscules are active and before the formation of the crosswall (Dickson and Smith, 2001). High metabolic activity was reported during early stage of arbuscule formation (Dickson and Smith, 2001).

Metachromatically stained polyP granules observed in the intercellular hyphae of *Gi. albida* have an essential role of transitory storage of P in the fungus vacuoles before its transfer to the host plant. Cox *et al.* (1975) observed cytochemical

identification of polyP granules in vacuoles within inter- and intra-cellular hyphae of *G. mosseae* Gerd & Trappe (Nicol. & Gerd.) in Onion roots. Ling Lee *et al.* (1975) also reported the occurrence of polyP granules in a wide range of ectotrophic and endotrophic mycorrhizas. Polyphosphate granules occupy a total volume fraction 0.008 of the fungal structures within the root (Cox *et al.*, 1980).

Localization of polyP granules stained with Toluidine blue O (TBO) has been observed earlier in roots of *Medicago truncatula* inoculated with *G. versiforme* (Javot *et al.*, 2007). Histochemical observations on AM fungi reported that hyphae contained small granules which stain with lead and react metachromatically with Toluidine Blue (Cox *et al.*, 1975). These staining reactions suggest that the granules contain polyphosphate in common with similar structures in other micro-organisms (Harold, 1966). These observations suggest that polyphosphates are of fundamental importance to the phosphorus nutrition of mycorrhizal associations.

Inorganic P is translocated through the AM fungal hyphae as polyP granules and after hydrolysis in the arbuscule, Pi is exported from the AM fungus to the periarbuscular space (Ezawa *et al.*, 2003, Kohijima and Saito, 2004). Alternatively, arbuscule death could be triggered by the plant either actively by acceleration of the normal mechanism of arbuscular turnover or simply as a consequence of inadequate carbon flow from the plant (Javot *et al.*, 2007). PolyP accumulated in the vacuolar compartment can be translocated from the extraradical hyphae to intraradical hyphae

possibly *via* cytoplasmic streaming or along a motile tubular vacuolar system (Uetake *et al.*, 2002). Once Pi has been released in the intraradical hyphae, it is assumed to be transferred to the periarbuscular apoplastic compartment and from there it is available to the plant. In the arbuscule, the polyphosphate may then be broken down by an enzyme of polyphosphate kinase type (producing ATP) or polyphosphatase (liberating Pi) (Harold, 1966). The products of break down may then be utilized in Pi transfer across the arbuscule into the host. Alternatively, polyphosphate may serve in phosphorylation mechanisms for the active transport of carbon skeleton into the arbuscule from the host (Woolhouse, 1975) either through ATP produced by degradative polyphosphate kinase action, or through a direct phosphorylation of sugars by an enzyme of the polyphosphate glucokinase type (Szymona, 1962).

Results of the present study revealed the presence of lipid droplets in intercellular hyphae and vesicles of *G. clarum*. Lipid staining of AM fungus colonized roots has demonstrated abundant lipid in the vesicles and intercellular and external hyphae (Cox *et al.*, 1975). Formation of spores from vesicles filled with small lipid droplets, which later become a globule, was observed. Similar observations were recorded earlier in spores of *Glomus* species (Maia and Kimbrough, 1998). This supports the fact that AM fungi need a compact form of carbon for translocation and storage, which could be the reason for abundant lipid bodies (Bago *et al.*, 2002). The major fluxes of carbon in intraradical mycelium thus appear to be efficient uptake of host derived hexose conversion to trehalose and

glycogen as storage forms and the synthesis of large amount of storage lipids. One of the most crucial changes is the differentiation of the fungal partner in two metabolically different but interconnected parts: the intraradical and the extra radical mycelium, with the former being the only location for hexose acquisition. Such hexose acquisition fuels metabolism and growth of intraradical mycelium, and it is transferred into typical fungal molecules such as trehalose, glycogen and chitin. In order to fuel extra radical fungal development, glycogen is translocated along newly developed runner hyphae, which would extend the extraradical hyphal fungal colony within the soil. These lipid bodies also play key roles in fungal morphogenic and reproductive events such as sporulation (Bago *et al.*, 2002).

CHAPTER 5

TAXONOMY OF AM FUNGI

INTRODUCTION

The diversity of AM fungi in ecosystems has been measured with respect to host plants, seasonal variation, soil nutritional gradients, crop rotation and plant successional stages (Koske, 1987; Gemma *et al.*, 1989; Anderson *et al.*, 1984; Johnson *et al.*, 1991). The most widespread approach to measure and analyze species diversity of AM fungi is to recover, count and identify spores from the field. However, spores collected directly from the field soil can be problematic: a) they appear healthy but are not viable, usually parasitized thereby hampering accurate species identification of *Gigaspora* and *Glomus* species b) they lose or change appearance in their structural characters in response to root pigments, soil chemistry, temperature, moisture and microbial activity, c) they represent only those colonizing mycorrhizal fungi with enough activity and biomass to trigger sporulation, and d) they are low in number and only those fungi sporulating in the rhizosphere of a given plant at the time of sampling are recovered (Brundrett *et al.*, 1999).

Trap cultures provide a non-molecular approach for baiting cryptic species of AM fungi present in plant communities. Trapping is necessary to obtain many healthy spores of colonizing fungi required for identification and also as an inoculum to establish monospecific cultures. Trap culture yield different results, depending on the biotic and abiotic factors that go into the growth, reproduction and partitioning of colonization and sporulation. Trap cultures, using host plants grown in sterilized soil: sand is the most commonly used method to isolate AM fungi (Brundrett *et al.*, 1999).

Pot culture method usually results in the isolation of greater number of species than other methods (Watson and Milner, 1996). It provides additional information on fungal diversity that complements spore occurrence data obtained from the same soil samples and may provide valuable new information about the biology of AM fungi (Brundrett *et al.*, 1999).

MATERIALS AND METHODS

Arbuscular mycorrhizal fungal spores isolated from rhizosphere soil using wet sieving and decanting technique (Muthukumar *et al.*, 1996) were used for the preparation of trap cultures and monospecific cultures. About 100g of each composite soil sample was dispersed in 1:1 water and the suspension left undisturbed for 15 min to allow soil particles to settle. The suspension was then decanted through 710 to 38 μ m sieves and the residues on the sieves washed and poured into beakers. After settlement of the heavier particles, the supernatant was filtered through gridded filter papers. Each filter paper was spread onto a glass plate and scanned under a dissection microscope at different magnifications (4X-100X). Intact AM fungal spores were transferred using a wet needle to polyvinyl-lactophenol (PVLG) with or without Melzer's reagent on a glass slide for identification.

Taxonomic identification of AM species:

Arbuscular mycorrhizal fungi were identified according to their spore morphology and wall characteristics using various bibliographies (Schenck and Perez,

1990; Morton and Benny, 1990; Almeida and Schenck, 1990; Bentivenga and Morton, 1995; Walker and Vestberg, 1998; Redecker *et al.*, 2000; Morton and Redecker, 2001). Identification of spores was also carried out by matching the descriptions provided by the International culture collection of Vesicular Arbuscular Mycorrhizal fungi (INVAM) (<http://invam.caf.wvu.edu>) and Glomeromycota phylogeny <http://www.lrz-muenchen.de/~schuessler/amphylo/>.

Preparation of Trap cultures:

Trap cultures were established using AM fungal spores isolated from the rhizosphere soil of the plant species collected by the wet sieving and decanting method. Freshly isolated AM fungal spores were stored at 4⁰C prior to use (Chabot *et al.*, 1992). Dominant spore types, having similar characters, were selected for AM culture development. Arbuscular mycorrhizal fungal spores obtained from trap cultures were further used for preparation of monospecific cultures.

<http://invam.caf.wvu.edu/methods/cultures/cultiindex.htm>).

Pots (15cm diam.) were filled with sterile sand soil (1:3) mix in which spores were placed along with the Whatman filter paper. The number of spores placed varied with fungal species. Pots were evenly planted with cuttings of *Coleus* sp., maintained in the glasshouse at 28⁰C (12h daylight) and Relative Humidity (RH) 62% and were watered regularly with sterile distilled water. Hoagland solution (Hoagland and

Arnon, 1938) (minus P) was added every 15 days. After 90 days of growth, watering was stopped and pots were harvested and analyzed for recovery of spores.

RESULTS

The present study recorded a rich diversity of AM fungal species associated with the medicinal plants from Western Ghats of Goa. Forty-five AM fungal species belonging to five genera viz., *Glomus*, *Acaulospora*, *Scutellospora*, *Gigaspora* and *Ambispora* were recovered. *Glomus* (27) was the most dominant species followed by *Acaulospora* (8), *Scutellospora* (7), *Gigaspora* (2) and *Ambispora* (1). *Glomus fasciculatum* was the most dominant species in the present study. Two out of 45 AM fungal species recorded in the present study could not be identified as they did not fit into known descriptions. Seven out of 42 AM fungal species were successfully multiplied using *Coleus* sp. as host plant (**Plate X a & b**). These include *Gigaspora albida*, *Acaulospora laevis*, *A. scrobiculata*, *Glomus aggregatum*, *G. fasciculatum*, *G. geosporum* and *Scutellospora calospora* which were further used for preparation of monospecific cultures.

Taxonomical descriptions of the identified AM fungal species are given below:

Plate X: Trap cultures of arbuscular mycorrhizal fungi

- a) With *Coleus* sp. as host plant in polyhouse.
- b) Monospecific culture of *Glomus fasciculatum* with *Coleus* sp. as host plant.

Plate X



Acaulospora laevis Gerdemann & Trappe. *Mycologia Memoir* No. 5:76, 1974 (Plate XIV a & b).

Spores 119-500 μ m in diam., dull yellow in colour. Spore wall with three layers; yellow brown outer wall, 2-4 μ m thick and inner two hyaline membranes. Spore contents dense, white in colour, globose to polygonal.

Acaulospora scrobiculata Trappe. *Mycotaxon* 6:359-366, 1977 (Plate XIV c & d).

Spore hyaline to light brown, globose, 100-240 μ m in diam. Spore surface evenly pitted with depressions 1-1.5 x 1-3 μ m, separated by ridges 2-4 μ m thick at the mouth of depressions, circular to elliptical or occasionally linear to Y-shaped. Spore wall consisting of four layers: 1) sub hyaline to light greenish yellow outer layer 3-6 μ m thick 2) an adhering smooth, hyaline layer, 0.2-0.5 μ m thick 3) Hyaline layer, 0.5-1.0 μ m thick and 4) roughened, hyaline inner layer of 0.2-1.0 μ m thick.

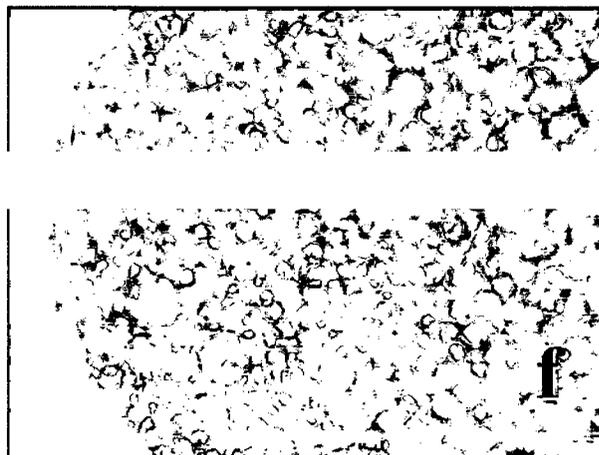
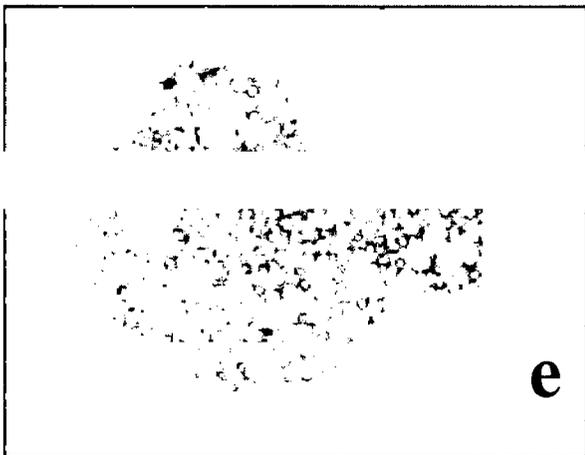
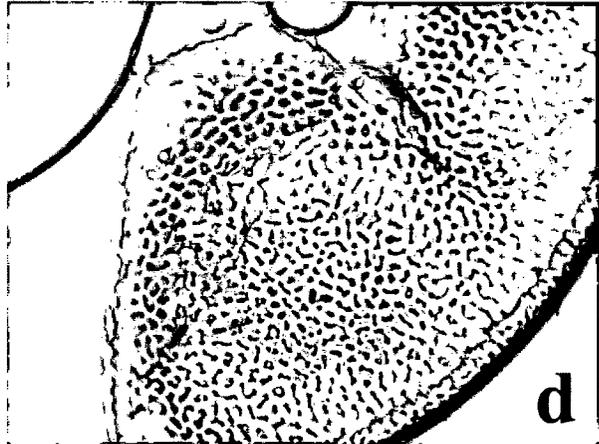
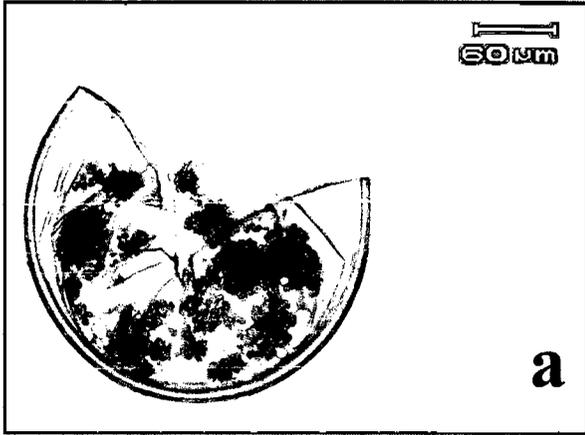
Acaulospora myriocarpa Spain, Sieverding & Schenck. *Mycotaxon* 25: 111-117, 1986 (Plate XIV e & f).

Spores hyaline, globose 22-90 μ m diam. Spore contents hyaline and granular. Spore wall consists of three wall layers of 1.5-3.5 μ m thick. Outer wall 0.75-2 μ m thick, middle layer 0.3-1.5 μ m thick, innermost wall layer <0.3 μ m thick, closely appressed to middle wall layer.

Plate XIV: Spores of *Acaulospora* species

- a) Crushed spore of *A. laevis* (X100).
- b) A portion of spore showing globose spore contents (X 400).
- c) Broken spore of *A. scrobiculata* (X 100).
- d) A portion of spore showing linear or 'Y' shaped ornamentation on spore surface (X 400).
- e) Crushed spore of *A. myriocarpa* comprising of oil droplets (X 100).
- f) A portion of spore showing hyaline granular oil droplets (X 400).

Plate XIV



Acaulospora nicolsonii Walker, Reed & Sanders. *Trans. Br. Mycol. Soc.* **83**: 360-364, 1984.

Spores pale yellow-brown, 93-218 μ m diam. Spore wall consists of 3 wall groups enclosing an inner membranous wall (wall 4). Outer wall, thin hyaline, 0.5-1 μ m thick, adherent to pale yellow-brown laminated wall (wall 2), 3-10 μ m thick, enclosing a loosely adherent, pale yellow, brittle unit wall, 0.5-1.5 μ m thick. Inner wall thin, 0.5 μ m thick. Spore content appearing vacuolate, due to the presence of many oil droplets, but later becoming reticulate as the droplets apparently coalesce.

Acaulospora delicata Walker, Pfeiffer & Bloss. *Mycotaxon* **25**: 621-628, 1986.

Spores hyaline to pale yellow, globose, 80-150 μ m, with four wall layers. Outer thin hyaline wall 1 μ m thick, closely attached to wall 2 laminated with 6 subequal laminations of 5-3.5 μ m thick. Wall 3 hyaline, 0.5 μ m thick, covered by minute granules. Wall 4 thin, hyaline, 0.75-1 μ m thick.

Acaulospora tuberculata Janos & Trappe. *Mycotaxon* **15**: 515- 522, 1982 (**Plate XIII d**).

Spores 255-340 μ m, yellowish brown to honey brown. Spore surface uniformly covered with tubercles to 0.7-1.1 μ m at the rounded tip. Spore wall consists of three layers, outer layer yellow in colour, 7-12 μ m thick, middle layer yellowish brown, 1.5 μ m thick, innermost hyaline layer, 1.5-3 μ m thick. Spore contents globose to hyaline, guttules of 8-19 μ m long.

Acaulospora rehmi Sieverding & Toro. *Angewandte Botanik* **61**: 217-223, 1987

(Plate XIII c).

Spores light yellow in colour, 82-175 μ m in diam. Spore wall consists of 4 wall layers. Outer wall, 3-13 μ m thick includes the ornamentation of labyrinth form folds with depressions between ridges, 1-4.5 μ m thick, middle wall layer hyaline, 0.5-2.0 μ m thick, inner wall layer 0.5-1.5 μ m attached to innermost wall layer of 0.5 μ m thick.

Acaulospora spinosa Walker & Trappe. *Mycotaxon* **12**: 515-521, 1981.

Spores dull yellowish brown, 100-335 μ m diam., ellipsoid to reniform. Spore surface ornamented with crowded blunt spines 1-4 μ m high, 1 μ m diam. at the polygonal base, tapering to 0.5 μ m at the tip. Patches of hyaline to sub hyaline amorphous material upto 2 μ m thick, irregularly encrusting the spines and covering the whole spore surface. Spore wall continuous except for the occluded openings, three layered. Outer layer, light yellowish brown, 4-10 μ m thick including spines and encrustations enclosing two membranous hyaline walls of 0.2-1 μ m thick.

Gigaspora albida Schenck & Smith. *Mycologia* **74**: 77-92, 1982 (Plate XIII a & b).

Spores dull white to light greenish yellow, spherical 143-350 μ m diam. Spore wall continuous 4-12 μ m thick with 1 to 6 walls. Outer wall smooth, 1-2 μ m thick with 4 to 5 inner laminated walls. Germ tube produced directly through the spore wall near

Plate XIII: Spores of *Gigaspora* (a & b) and *Acaulospora* (c & d) species.

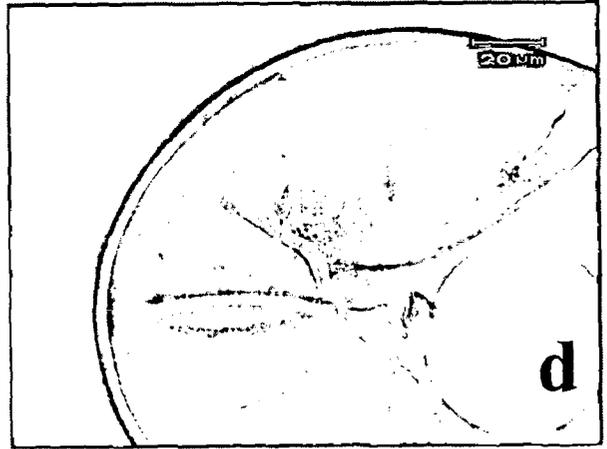
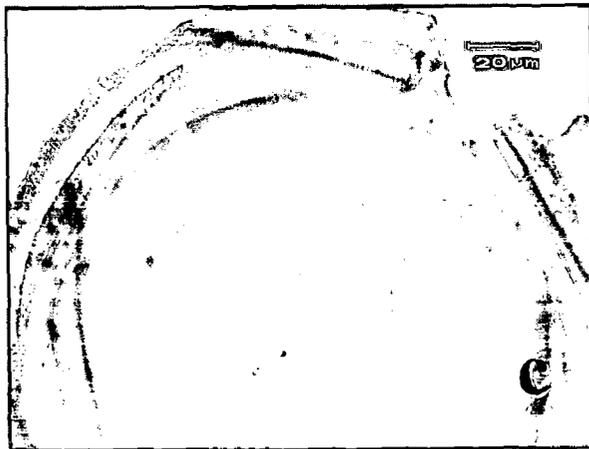
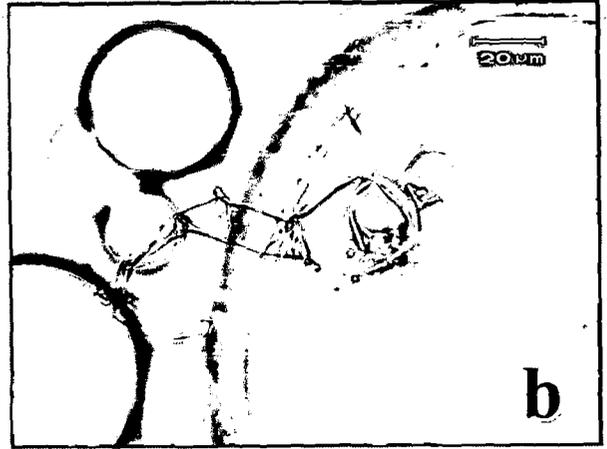
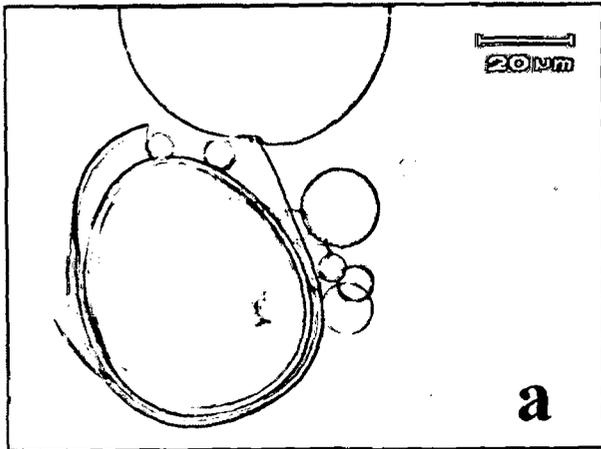
a) Broken spore of *Gi. albida* (X100).

b) Bulbous suspensor with septate hyphal branches in *Gi. albida* (X 400).

c) Crushed spore of *A. rehmi* showing ornamentation on the spore surface (X 400).

d) Broken spore of *A. tuberculata* with tubercles on spore surface (X 400).

Plate XIII



the bulbous suspensor separating it from the spore contents. Hyaline to yellow, bulbous suspensor, 24-36 μ m diam., attached to septate hyphae with fine hyphal branches.

***Gigaspora* sp. (Unidentified)**

Spores white in colour, 210-283.2 μ m diam. Spore wall 8.7 μ m-14.5 μ m thick, suspensor cell 29 μ m thick. Spore contents consist of oil globules of varying sizes.

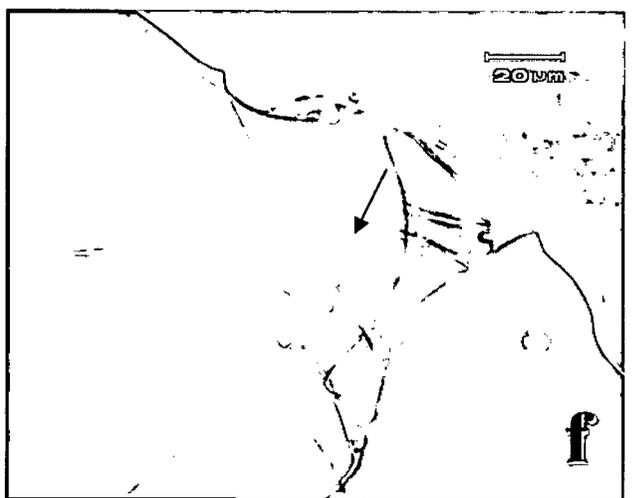
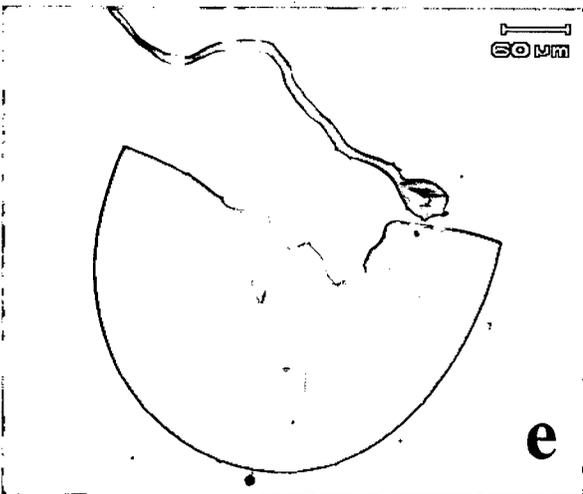
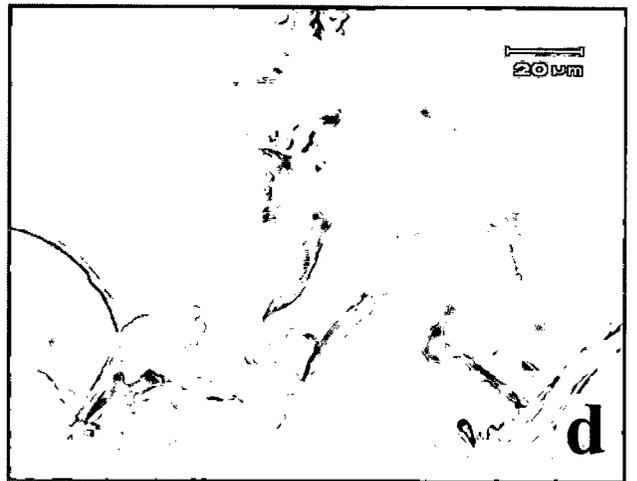
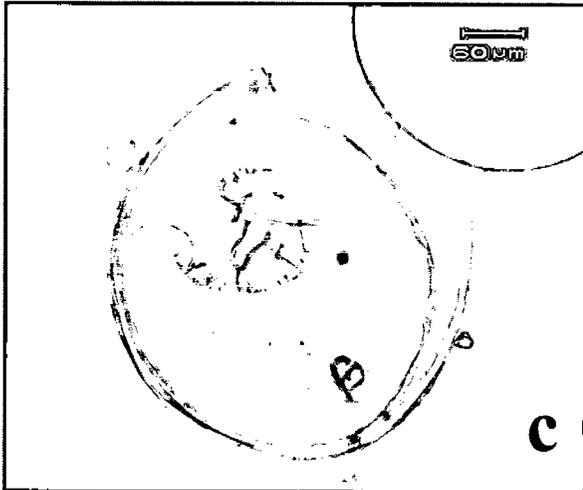
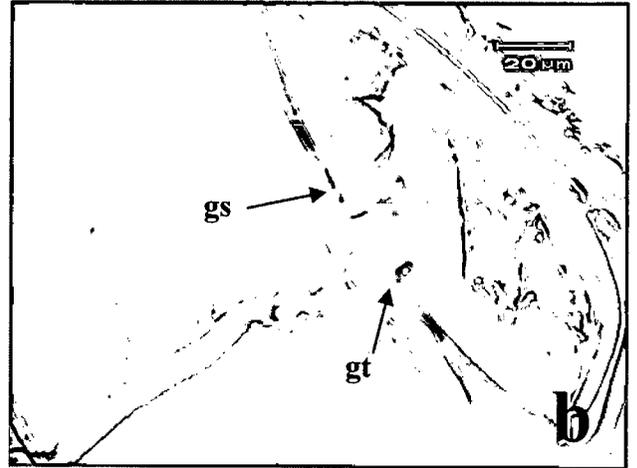
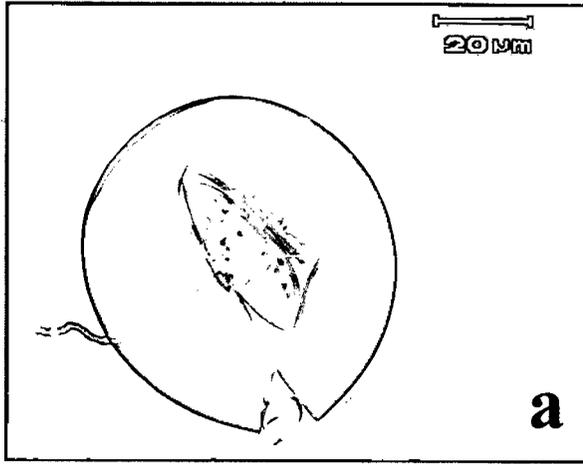
Scutellospora heterogama (Nicolson & Gerdemann) Walker & Sanders. *Mycologia* 77: 702-720, 1985 (Plate XI e & f).

Spores borne on a suspensor cell, 150-230 μ m in diam., pale yellow-brown to red-brown. Spore structure of four walls (1-4) into two groups (A and B). Group A ornamented unit wall 1 tightly adherent to an inner laminated wall 2, pale yellow to pale brown, 1-1.5 μ m thick. Warts densely crowded, 0.5-1 μ m diam. Wall 2, 4-7 μ m diam., yellow-brown in colour. Group B of two membranous walls (3 and 4) hyaline, <1 μ m thick. Suspensor cell borne on coenocytic septate subtending hypha, 21-42 μ m wide. Wall of suspensor cell 1-2.5 μ m thick, distally thickening at the spore base. One or two hyphal peg projections present, 5-9 μ m, arising from the suspensor cell and towards the spore. Germination shield 120 μ m diam., pale yellow to brown with smooth margins, with only a few folds. Shape of the shield resembles that of a violin.

Plate XI: Spores of Scutellospora species

- a) Spore of *S. verrucosa* (X 100).
- b) Germination shield (Gs) and Germination tube (Gt) of *S. verrucosa* (X 400).
- c) Broken spore of *S. scutata* (X 100).
- d) Germination shield of *S. scutata* (X 400).
- e) Broken spore of *S. heterogama* with bulbous suspensor(X 100).
- f) Violin shaped germination shield of *S. heterogama* (X 400).

Plate XI



Scutellospora scutata Walker & Diederichs. *Mycotaxon* **35**: 357-361, 1989 (**Plate XI c & d**).

Spores borne laterally on a brown pyriform suspensor cell, 350-713 μ m diam., transparent to dirty white. Spore wall structure of six walls (1-6) in two to three groups. Group A hyaline smooth, outermost unit wall (1) 0.2-0.8 μ m thick. Group B consists of four walls (3-6). Wall 3, 2.3-8.4 μ m thick, loosely associated with a thin membranous wall (4), 0.2-0.8 μ m thick. Wall 5, 2.2-8.2 μ m, tightly adherent to wall 6 (0.5 μ m). Germination shield circular to oval, 240-302 μ m, present in wall group B. Suspensor cell 119 μ m, produced terminally on recurved septate brown subtending hyphae. Wall of suspensor cell 1-5 μ m thick distally, thickening to 3-12 μ m near the spore base bearing one or more stout peg like hyphal protrusions. Germination shield oval to heart shaped, light yellow in colour, 208-323 μ m, usually armed with a shield.

Scutellospora verrucosa (Koske and Walker) Walker & Sanders. *Mycologia* **77**: 702-720, 1985 (**Plate XI a & b**).

Spores formed on a bulbous suspensor cell, 220-476 μ m, pale straw to yellow in colour. Spore wall structure of three walls (1-3) in two groups (A and B). Group A has as an outer, ornamented unit wall (1) tightly adherent to an inner laminated wall (2). Wall 1 brittle, hyaline to pale yellow, 2-3.5 μ m thick including crowded low rounded warts, 0.5-1.5 \times 0.5-1 μ m at the base. Wall 2 laminated pale yellow, 3-12.5 μ m thick. Group B membranous inner wall (3), hyaline 0.5-1 μ m thick. Ovoid germination shield 80-210 μ m diam. from which germ tubes emerge. Bulbous

suspensor cell borne terminally on a coenocytic to sparsely septate subtending hypha, 55-70 μ m, dumb bell-shaped, hyaline.

Scutellospora biornata Spain, Sieverding & Toro. *Mycotaxon* **35**: 219-227, 1989
(Plate XII g & h).

Spores 282-415 μ m in diam., yellowish brown to brown in colour. Spore wall structure consists of 6 walls, 8-15 μ m thick, in two groups. Group A, 0-12 μ m thick, with three walls. Outer wall, 0.5-1 μ m thick, brown, ornamented on outer surface with blunt tapering projections of 1-3 μ m diam. at the base fused to wall 2. Wall 2 hyaline, laminated, 8-10 μ m thick, adherent to wall 3. Wall 3 hyaline, membranous, 0.5-1 μ m thick ornamented on inside with blunt projections of 0.5-1 μ m diam. Wall 4 membranous, <0.5 μ m thick. Germination shield brown, 113-275 μ m, crescent shape formed between wall 5 and 6. Greatest pigment concentration around germ tube initials aperture, Y and U configuration and other fissures. Germ tube initials (6-17), 6-7 μ m diam., separated from each other by a long fissure.

Scutellospora calospora (Nicolson & Gerdemann) Walker & Sanders. *Mycotaxon* **27**: 219-235, 1986 (Plate XII I).

Spores formed terminally on a bulbous suspensor like cell, hyaline to pale greenish yellow, globose, 114-511 μ m diam. Wall structure of four walls (walls 1-4) in two groups. Group A consisting of an inner brittle, hyaline to pale yellow, laminated wall (wall 2) 3-5 μ m thick, surrounded by thin, hyaline unit wall (wall 1), 0.5-1 μ m

thick. Group B of two hyaline walls (wall 3 and 4). Wall 3, 0.5-1 μ m diam., wall 4, 1-1.5 μ m thick. Germination shield oval, 35-90 μ m diam. with invaginations along the margin. Suspensor cell 33-48 μ m, borne terminally on a septate subtending hypha, broad, concolorous with the spore base.

Scutellospora pellucida Nicolson & Schenck *Mycotaxon* 6: 29-32, 1986 (Plate XII k).

Spores light yellowish in colour, 58-212 μ m in diam. Spore wall 6-12 μ m thick, composed of two walls. Outer wall laminated 3-8 μ m thick, inner wall 1.5-5.0 μ m thick. Spore contents hyaline consists of small globules. Suspensor cell hyaline, 10-29 μ m thick. Subtending hyphae 7-12 μ m diam., septate, hyaline below the suspensor cell.

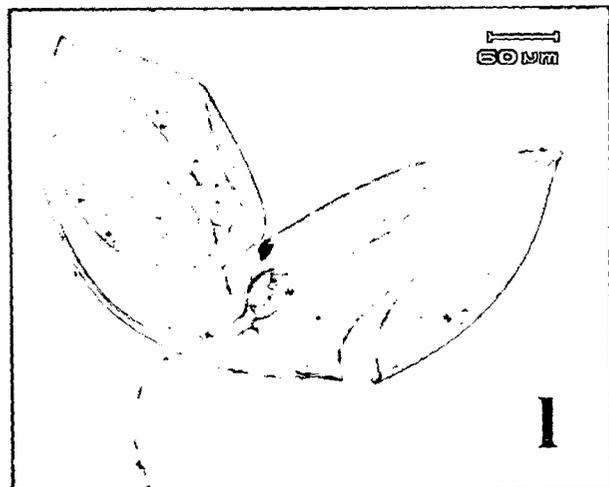
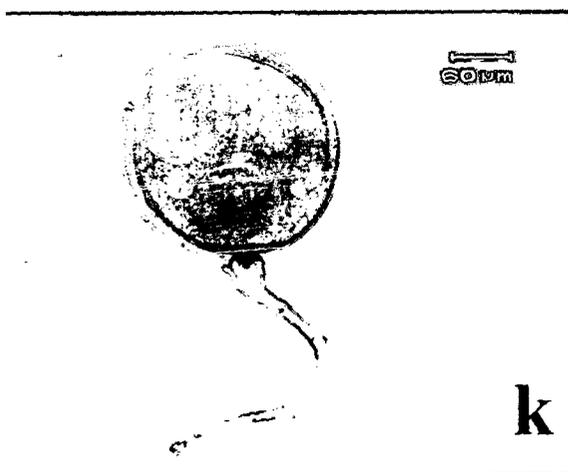
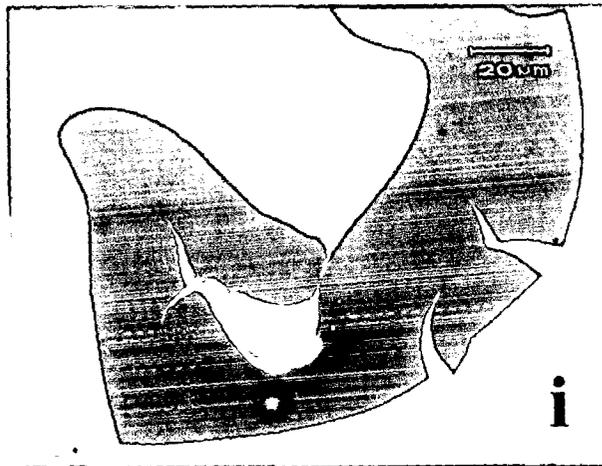
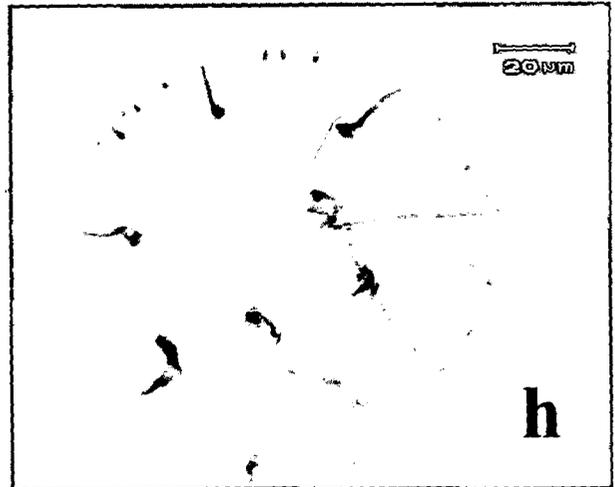
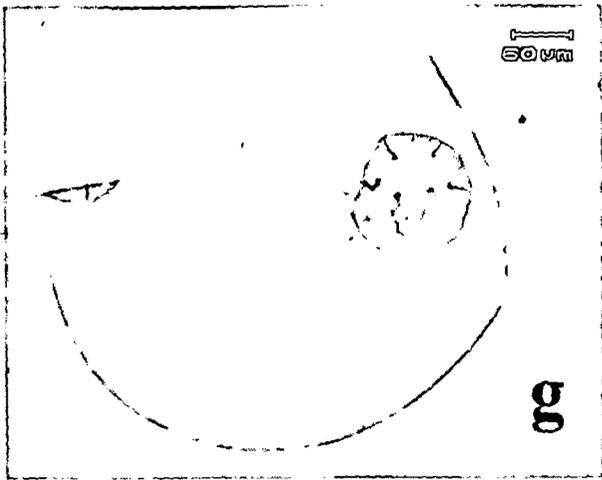
Scutellospora gregaria (Schenck & Nicolson) Walker & Sanders. *Mycologia* 77: 702-720, 1979 (Plate XII i & j).

Spores reddish brown in colour, 250-448 μ m in diam. Spore wall composed of four walls (1-4) in two groups. Group A composed of three closely appressed walls, an outer wall (1) and two laminated walls (2 and 3). Wall 1 brown, 1-5 μ m including the closely packed warts on its outer surface. Warts are brown 1-2 μ m high with rounded tips, 2-7(-10) μ m. Wall 2, yellow, 3 μ m thick. Wall 3 pale yellow, 5-13 μ m thick. Group B, hyaline, membranous. Wall (4) 1-2 μ m thick, enclosing the contents. Suspensor cell 2-4 μ m thick, pale brown, borne terminally on a septate hypha, 39-

Plate XII: Spores of *Scutellospora* species

- g) Broken spore of *S. biornata* (X 100).
- h) Germination shield with 'U' shaped configuration around the aperture (X 400).
- i) Broken spore of *S. gregaria* (X 100).
- j) Germination shield of *S. gregaria* (X 400).
- k) Intact spore of *S. pellucida* with bulbous suspensor (X 100).
- l) Broken spore of *S. calospora* (X 100).

Plate XII



80 μ m wide. Thick or thin walled hyphal projections arise from the suspensor cell towards the spore. Germination shield ovoid 116 μ m in diam. with many convolutions appearing as warts at the margins.

Glomus constrictum Trappe. *Mycotaxon* **6**: 359-366, 1977 (**Plate XV e**).

Spores 150-330 μ m, dark brown to black in colour. Spore walls 7-15 μ m thick, straight with a short funnel shaped projection. Attached hyphae straight or recurved at the point of attachment with dark brown walls 3-5 μ m thick. Just beyond the point of attachment the hypha constricted to 10-22 μ m diam. Just beyond the constriction the hypha inflated to 15-30 μ m diam. with yellow-brown walls of 2-3 μ m thick.

Glomus dimorphicum Boyetchko & Tiwari. *Can. J. Bot.* **64**: 90-95, 1986 (**Plate XV a**).

Spores yellow to reddish brown, 90-300 μ m in diam. Spore wall composed of three walls, outer wall hyaline laminated 2-8 μ m thick, middle layer light yellow 2-8 μ m thick, inner layer 1 μ m thick, light yellow in colour, sporogenous hyphae straight, 1.5-9 μ m thick, possessing a septum at the point of attachment. Sporogenous hyphae with clustered spores, light yellow in colour, 2-6 μ m thick, forming a loose network of septate hyphae, 7-12 μ m thick at the point attachment.

Glomus etunicatum Becker & Gerdemann. *Mycotaxon* **6**: 29-32, 1977 (**Plate XV f**).

Spores light brown in colour, globose 68-162 μ m in diam. Spore wall 4-13 μ m thick, composed of an outer wall 5 μ m thick, inner laminated wall 2-8 μ m thick, spore contents separated from attached hyphae by a thin curved septum.

Glomus fasciculatum (Thaxter) Walker & Koske. *Mycotaxon* **30**: 253-262, 1987 (**Plate XV b**).

Spores light brown to reddish brown in colour, 75-149 μ m in diam. Spore walls highly variable in thickness 3-17 μ m diam., perforated with thickened inward projections. Wall 1 smooth hyaline unit 0.2-1.0(-1.8) μ m thick. Wall 2 pale yellow to pale brown, laminated, 0.8-14.3 μ m thick. Wall 3 hyaline membranous wall, 0.1-0.9 μ m thick. Subtending hyphae often pale in colour than the spore, flared, straight or slightly constricted proximally, tapering to 1.5-2.0 μ m, thick distally.

Glomus geosporum Walker. *Mycotaxon* **15**: 49-61, 1982 (**Plate XV c**).

Spores 110-290 μ m in diam., ellipsoidal, light to dark brown in colour. Spore walls 4-8 μ m thick, 3 layered with thin tightly adherent outer wall (<1 μ m), yellow brown to red brown laminated middle wall (3-16 μ m), yellow-brown inner wall (<1 μ m) that appears membranous and forms a septum separating the spore contents from the subtending hypha. Spores with straight to recurved funnel shaped subtending hyphae of 10-24 μ m in diam. Spore contents of uniform droplets, granular in

appearance with age separated by a septum that protrudes slightly into subtending hypha.

Glomus macrocarpum Tulasne & Tulasne. *Can. J. Bot.* **61**: 2608-2617, 1983 (**Plate XV d**).

Spores reddish brown in colour, 80-180 μ m in diam. Spore wall composed of two distinct layers, outer thin hyaline layer (1-2 μ m), inner wall 6-12 μ m thick with laminations. Spores taper to the point attachment of single persistent hypha. The inner wall thickens to occlude the pore of attached hyphae. Pore is closed by a septum that is thinner than occluded wall thickening.

Glomus arboreense McGee. *Trans. Br. Mycol. Soc.* **87**: 123-129.1986.

Spores hyaline to pale yellow 26-55 μ m in diam. Spore wall 3 μ m thick, spore contents globular oil droplets, cut off infrequently by a septum 5-20 μ m down the subtending hypha. Subtending hyphae pale yellow, 1 μ m thick, cylindrical with slight flare at the spore wall.

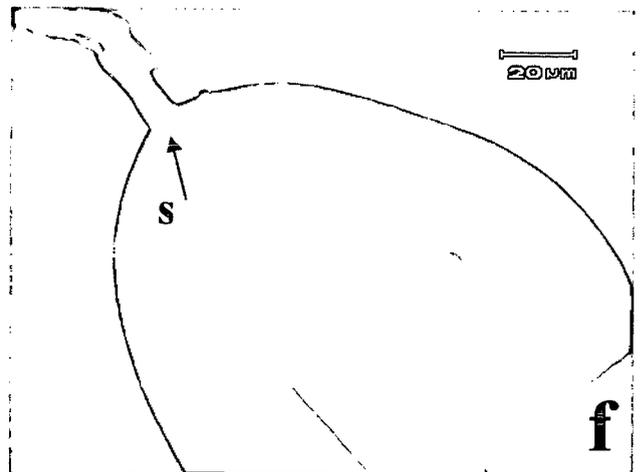
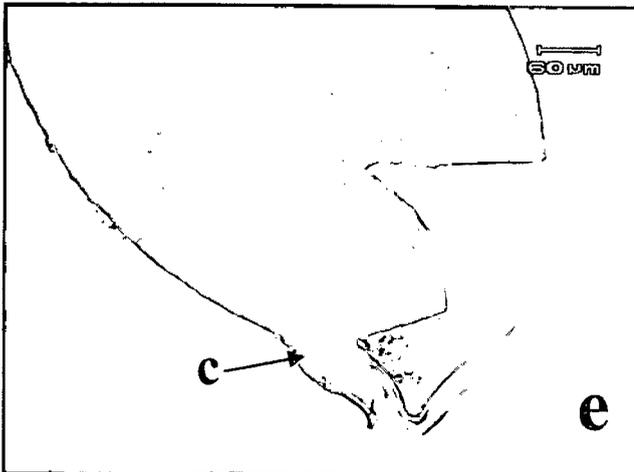
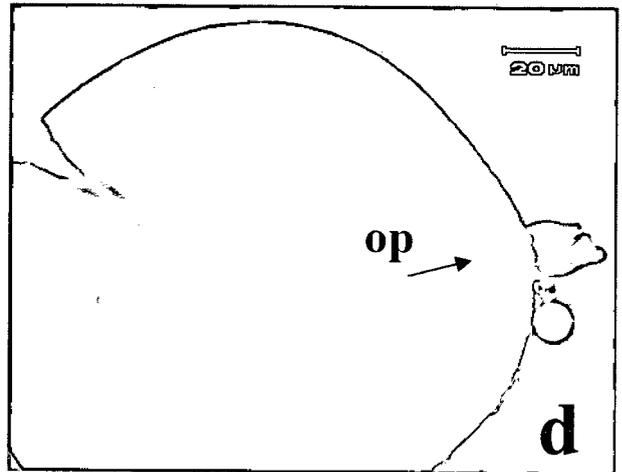
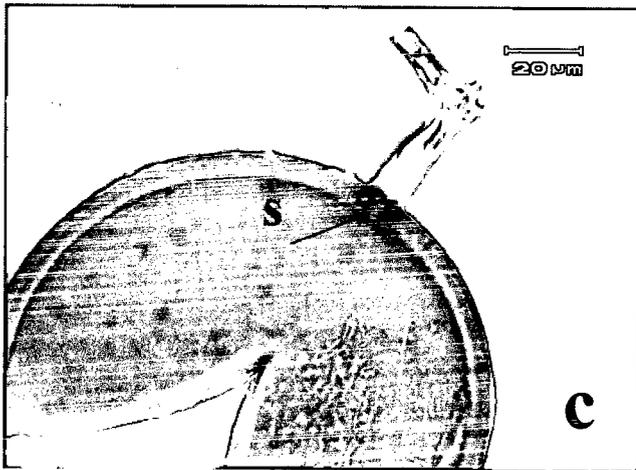
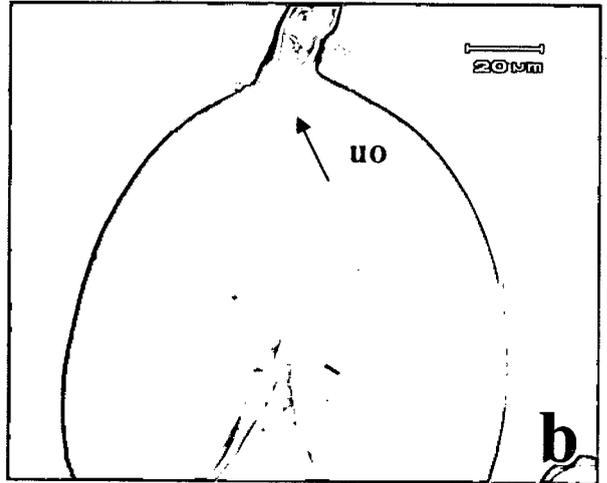
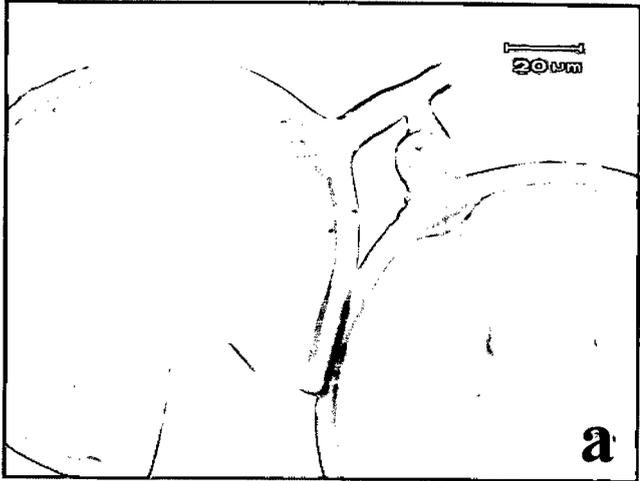
Glomus clarum Nicolson & Schenck *Mycologia* **71**: 178-198, 1979.

Spores hyaline 68-290 μ m in diam., composed of two wall layers. Spore contents hyaline consisting of globules of variable size, outer wall consists of 5-20 μ m thick, inner wall of 2-9 μ m thick. Subtending hyphae 15-80 μ m wide, becoming thinner with increasing distance from the spore.

Plate XV: Spores of *Glomus* species

- a) Broken spore of *G. dimorphicum* showing sporogenous hyphae with septa at the point of attachment (X 400).
- b) Crushed spore of *G. fasciculatum* unoccluded (uo) at the opening of the hyphae (X 400).
- c) Broken spore of *G. geosporum* with septum (s) separating spore contents from subtending hyphae (X 400).
- d) Spore of *G. macrocarpum* showing occluded pore (op) closed by a thinner septum (X 400).
- e) Crushed spore of *G. constrictum* with a constriction (c) just beyond the point of attachment (X 400).
- f) Broken spore of *G. etunicatum* showing thin curved septum(s) (X 400).

Plate XV



Glomus caledonium Gerdemann & Trappe. *Mycologia Memoir* No 5:76, 1974.

Spores dull yellow to brown, 130-272 μ m in diam. Spore wall 6-16 μ m thick, composed of a hyaline outer layer, 1-8 μ m thick and a yellow to brown inner layer, 4-10 μ m thick. Spore contents separated from attached hyphae by a thin curved wall formed at the hyphal attachment.

Glomus citricola Tang & Zang. *Acta Botanica Yunnanica* 6: 295-304, 1984.

Chlamydospores 35-90 μ m without ephemeral hyaline outer wall of spores. Spores are globose, exceeding 100- 120 μ m diam., the inner walls are often minutely perforated with thickened inward projections.

Glomus aggregatum Schenck & Smith emend. Koske. *Mycologia* 77: 619-630, 1985 (Plate XVI i).

Sporocarps 200-1400 μ m diam., lacking a peridium of loosely aggregate spores. Spores formed in sporocarps, 20-210 μ m diam., pale-yellow to yellow-brown. Spore wall laminated, 1-3(-5) μ m thick. Subtending hyphae straight, constricted, swollen or irregular upto 12 μ m wide at the spore base.

Glomus albidum Walker & Rhodes. *Mycotaxon* 12: 509-514, 1981 (Plate XVI I).

Spores 85-198 μ m in diam., yellowish in colour with subtending hyphae. Spore walls consisting outer hyaline wall 0.5-2 μ m thick, inner sub equal finely

lamine wall, yellow 0.5-2 μ m thick. Subtending hyphae straight, 2 walled, outer wall thickened at spore base, 3-15 μ m wide. Spore contents consist of crowded oil droplets.

Glomus flavisporum (Lange & Lund) Trappe & Gerdemann. *Freisa* 5: 90-95, 1955 (Plate XVI g).

Spores dark brown ovate constricted on the middle, 95-152 μ m in diam. Spore wall 6-13 μ m thick laminated with granular contents, subtending hyphae 6-12 μ m wide.

Glomus intraradices Schenck & Smith. *Mycologia* 74: 77-92, 1982 (Plate XVI k).

Spores 93-131 μ m diam., brown in colour. Spore walls 3-15 μ m thick, yellow to gray brown, with 4 laminated walls, inner walls 1-2 μ m thick, darker than outer walls. Spore contents globular, yellow to light brown. Wall of the spore extending into the hyphal attachment forming an apparent tubaeform flare at the juncture with the hyphal attachment. Hyphal attachment 9-33 μ m wide with wall thickness of 1.5-5.2 μ m at the base of the spore. Hyphal attachment constricted 2-3 μ m at the base of the spore.

Glomus maculosum Miller & Walker. *Mycotaxon* 25: 217-227. 1986 (Plate XVI j).

Spores 95-220 μ m, light brown to dark brown. Spore wall of three walls (1-3) in two groups. Group A, outer thin hyaline, unit wall (wall1), 0.3-1.0 μ m thick, tightly adherent to wall 2. Wall 2, pale straw-coloured, laminated, 4-13 μ m thick with 4-16

laminae. The innermost lamina appearing as separate unit wall often forming a septum at the spore base. Inner wall group (Group B, wall 3) thin ($<0.3\mu\text{m}$) and tightly adherent to wall 2. Wall 3 bearing dome shaped scalloped ingrowths, 6-15 μm diam., consisting of 2-8 concentric bulging discs increasing towards the inside of the spore. Subtending hyphae straight to sharply recurved parallel sided or funnel shaped constricted at the spore base, 5-25 μm wide proximally 5-7 μm .

Glomus microcarpum Tulasne & Tulasne. *Mycologia* **76**: 190-193, 1984 (**Plate XVI h**).

Sporocarps are irregularly ellipsoidal enclosed by a peridium. Peridium is 50-100 μm wide, composed of interwoven hyphae. Spores are globose, 30-40 μm in diam. Spore wall smooth, 4-6 μm thick, hyaline with laminations. Subtending hyphae is 4-8.5 μm thick at the point of attachment.

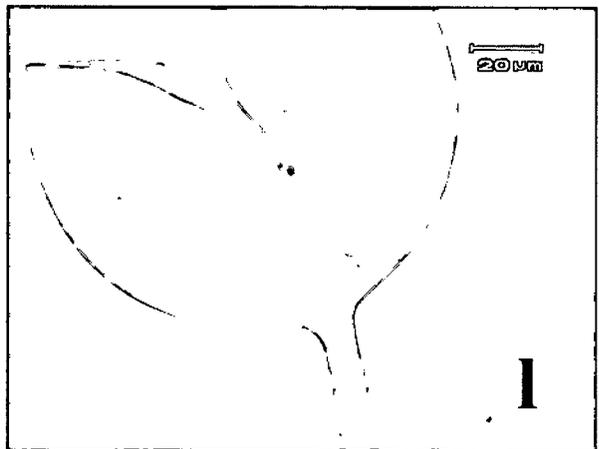
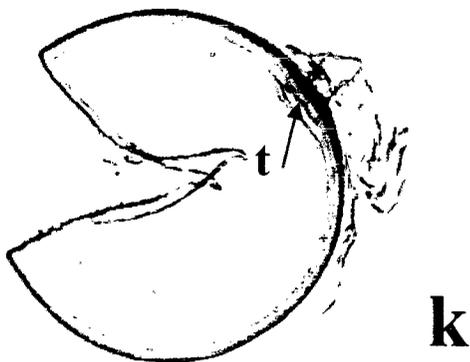
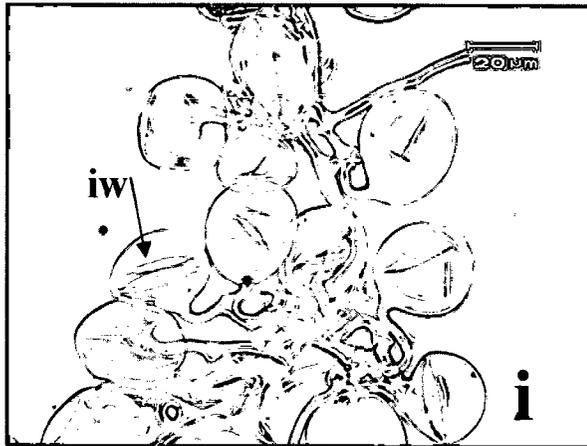
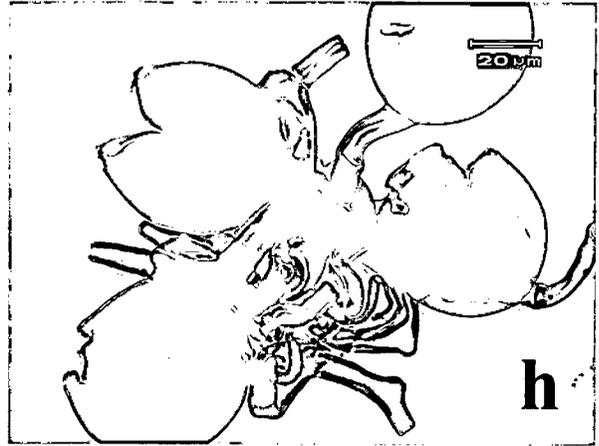
Glomus formosanum Wu & Chen. *Taiwania* **31**: 65-88, 1986 (**Plate XVII q**).

Spores 65-117.5 μm in diam., 1-4 branched attached hyphae. Spore wall 5-6 μm thick, single layer, reddish brown, 5.5-12.5 μm thick. Frequently two nearby hyphae closely separated at the attachment. Hyphae 7-17.5 μm diam., with an opening at the attachment.

Plate XVI: Spores of *Glomus* species

- g) Broken spore of *G. flavisporum* showing constriction (c) (X 400).
- h) Sporocarp of *G. microcarpum* with interwoven hyphae (X 400).
- i) Sporocarp of *G. aggregatum* showing loose arrangement of spores on sporocarp with innerwall (iw) collapsed (X 400).
- j) Sporocarp of *G. maculosum* bearing scalloped ingrowths (si) on inner wall and 2-8 concentric disc (cd) on spore surface (X 400).
- k) Broken spore of *G. intraradices* showing tubaeform (t) flare at the juncture of hyphal attachment (X100).
- l) Crushed spore of *G. albidum* (X 400).

Plate XVI



Glomus fragilistratum Skou & Jakobsen. *Mycotaxon* **36**: 273-282, 1989 (**Plate XVII p**).

Spores yellow in colour, globose, 108-231 μ m diam. Spore wall consists of six walls in three groups. Wall group A consists of two walls upto 4.5 μ m thick. Wall 2 hyaline, 1.4-3 μ m thick. Wall group B consists of two walls (3 and 4). Wall 3 hyaline unit wall, 1 μ m thick. Wall 4 yellow laminated wall, 5.1 μ m thick. Wall group C consists of two hyaline walls of 1-2 μ m thick. The spore content consists of oil globules of different sizes. Walls 1-4 extends as subtending hyphae, 9-15 μ m widened at the spore base with 1-2 hyphal septa positioned close to the spore.

Glomus multicaule Gerdemann & Bakshi. *Trans. Br. Mycol. Soc.* **66**: 340-343, 1976 (**Plate XVII m & n**).

Spores dark brown, 149-162 μ m, ellipsoidal, occasionally triangular with 1-4 hyphal attachments generally occurring at opposite end of spores. Spore wall 8.6-34 μ m, thickest at the point of attachment with rounded projections of 1.2-3.7 μ m long distributed all over the spore surface.

Glomus magnicaule Miller & Walker. *Mycotaxon* **25**: 217- 227, 1986 (**Plate XVII o**).

Spores brown, 125-175 μ m diam. Spore wall with two wall layers, outer brown 9-20 μ m thick, inner upto 4 μ m thick, colourless to light brown. Subtending hyphae 35-58 μ m wide often slightly pinched in at the point of attachment.

Glomus halon Rose & Trappe *Mycotaxon*, **10**: 413-420, 1980.

Spores 200-280 μ m, reddish brown in colour. Spore wall consisted of two layers of 18-35 μ m thick. Outer wall 8-12 μ m thick, hyaline with obscure radial striations. Inner 10-15 μ m thick, brown ornamented with crowded spines 0.5 x 0.2 μ m that extends in to outer layer. Subtending hyphae straight, extending through the outer hyaline wall where it is constricted to 5-6 μ m thick.

Glomus hoi Berch. & Trappe. *Mycologia* **77**: 654-657, 1985.

Spores light brown in colour, ellipsoidal, 23-140 μ m in diam. Spore wall consists of two distinct separable layers. Outer layer orange-yellow, 2-8 μ m diam. Inner layer 0.5-1 μ m thick, hyaline to light yellow. Subtending hypha cylindrical or slightly flared toward the attachment of spore, 5-13 μ m wide, bearing thin walled septate lateral branches. Subtending hypha occluded by a fine curved septum below the attachment of spore.

Glomus glomerulatum Sieverding. *Mycotaxon* **29**: 73-79, 1987.

Sporocarps dark brown, globose to subglobose, 290-675 μ m diam. Spores yellow to brown, globose to subglobose, 40-70 μ m in diam. Composite spore wall is composed of two walls (walls 1 & 2) in Group A. Wall 1 is yellow to brown laminated, 4-9 μ m thick. Wall 2 hyaline, membranous, 0.5 μ m thick. Two to three hyphal attachments, straight to recurved, cylindrical to funnel shaped, yellow to

brown, 5-7 μ m diam. The pore of hyphal attachment is 1-2 μ m in diam., closed by the spore wall or by a septum. Spore contents are hyaline and oily.

Glomus mosseae Gerdemann & Trappe. *Mycologia Memoir* No, 5: 6, 1974

Spores yellow to brown, globose to subglobose, 105-305 μ m with one or two funnel shaped bases 20-50 μ m diam., divided from subtending hyphae by a curved septum, hyaline 2-7 μ m thick irregularly branched septate hyphae of 2-12 μ m wide.

Glomus pulvinatum (P. Hennings) Trappe & Gerdemann. *Aust. J. Bot.* 23: 849-866, 1975.

Spores 55-93 μ m in diam., yellow in colour, spherical, thin walled (2-4 μ m) densely granular. Spore wall two layers outer wall 1 μ m thick and inner wall 3 μ m thick. Subtending hyphae 12-17 μ m thick, a bulbous expansion below the spore and often constricted at the juncture with a septum of 1 μ m thick which separates spore from sporophore.

Glomus rubiforme Gerdemann and Trappe. *Mycologia Memoir* No, 5: 76, 1974.

Sporocarps dark brown, 180-675 μ m diam., surrounding a central plexus of hyphae. Peridium absent, individual spores partially enclosed in a thin network of tightly appressed hyphae. Spores dark brown, obovoid to ellipsoid or subglobose, 37-125 μ m with a small pore opening in to thick walled subtending hypha. Spore wall

lamine, 3-7.6µm thick upto 13.5µm thick at the spore base, perforated projections appears on the inner surface-walled subtending hyphae.

***Glomus* species (Unidentified)**

Spores brown in colour, 98.6µm in diam. Spore wall composed of 2 layers. Outer wall smooth hyaline 1µm thick, inner wall thin hyaline, 1µm thick. Spore contents hyaline, globular.

***Ambispora leptoticha* C. Walker, Vestberg & A. Schußler. *Mycol. Res.* 13: 111- 137, 2007 (Plate XVII r).**

Spores 104-262µm in diam., with single wall, 1.5-10.5µm thick, spore wall with an indistinct alveolate reticulum of shallow ridges (0.5-1µm wide). Spore contents globular, hyaline in young spores. Subtending hyphae 9-27µm diam., at spore attachment, hyphal wall continuous with the spore wall. Spore contents enclosed with a membrane, which occasionally bulges into subtending hyphae.

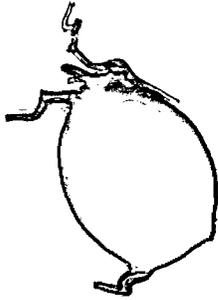
DISCUSSION

In the present study, seven out of 42 species of AM fungal species were multiplied using trap cultures. Brundrett *et al.* (1999) reported that AM fungal species frequently forming spores in the field soil were not detected in traps as conditions in the pots are less favourable for their sporulation. Bever *et al.* (1996) reported 23 AM fungal species from a 75m² region of a mown field with sorghum trap cultures. In an

Plate XVII: Spores of *Glomus* species

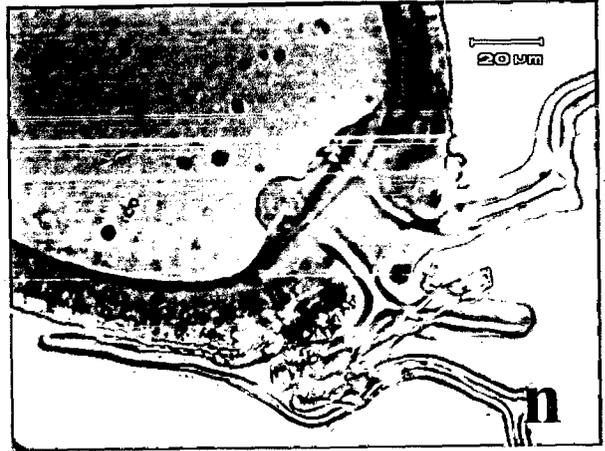
- m) Intact spore of *G. multicaule* showing 1-4 hyphal attachments(X 100).
- n) A portion of spore showing two hyphal attachments (X 400).
- o) Spore of *G. magnicaule* (X 100).
- p) Crushed spore of *G. fragilistratum* showing 1-2 hyphal septa (s) (X 400).
- q) A portion of spore of *G. formosanum* two hyphae (h) closely separated at attachment (X 400).
- r) Crushed spore of *Ambispora leptoticha* (X 400).

Plate XVII



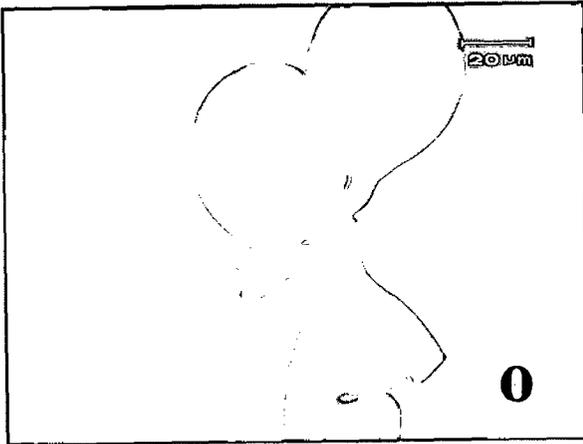
50 μm

m



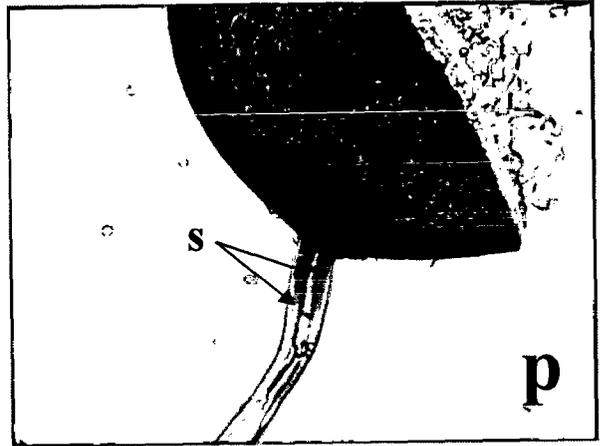
20 μm

n



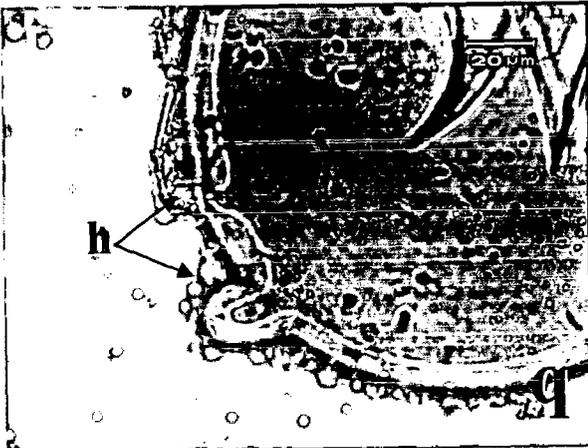
20 μm

o



s

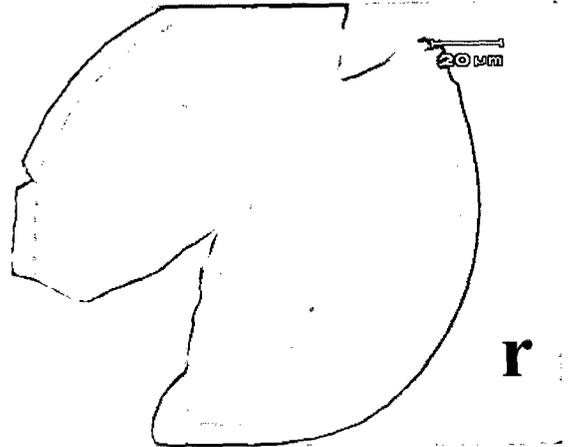
p



20 μm

h

q



20 μm

r

arid ecosystem, Stutz and Morton (1996) recovered 15 more AM fungal species than those detected in the field after three cycles of trap cultures.

Redecker *et al.* (2000) utilizing both morphological and molecular data transferred *Sclerocystis* to the genus *Glomus* and eliminated the genus *Sclerocystis* from the Kingdom Fungi. *Ambispora leptoticha* originally described as *Glomus leptotichum* (Schenck and Smith, 1982) and then renamed *Appendicispora leptoticha* (Walker *et al.*, 2007) is now included under new genera *Ambispora* (Walker, 2008).

In the present study, it was observed that a specific AM fungus is associated with different host plants. Moreira *et al.* (2007) reported that the effect of a single AM fungal species could differ in two different host plants, as each host plant would selectively produce a differentiated spore composition. However, depending on the growth conditions i.e. host plant and environmental conditions, there may be qualitative and quantitative change in the spore composition, which may not reflect the original composition in the field (Carrrenho *et al.*, 2002). The differences in spore number obtained from trap cultures may be due to the variations in host plant root type and morphology, carbon biomass, nutrient and endogenous hormonal levels. These factors may influence the richness of AM fungi isolated from soil in trap cultures (Brundrett *et al.*, 1999; Cuenca and Meneses, 1996; Stutz and Morton, 1996). Host plant and soil factors can influence both diversity and overall levels of P in soil

and plant are able to inhibit mycorrhiza formation (Douds and Schenck, 1990) and influence the diversity of AM fungi in field soil (Cuenca and Meneses, 1996).

CHAPTER 6

*EFFECT OF ARBUSCULAR
MYCORRHIZAL (AM) FUNGI ON GROWTH
OF ANDROGRAPHIS PANICULATA*

INTRODUCTION

Andrographis paniculata Nees (Acanthaceae), commonly known as 'king of bitters' and the Kalmegh of Ayurveda is extremely bitter in taste, has been used for centuries in Asia to treat GI tract and upper respiratory infections, fever, Herpes, sore throat and a variety of other chronic and infectious diseases. It is found in the *Indian Pharmacopoeia* and is a constituent in at least 26 Ayurvedic formulas. The genus *Andrographis* consists of 28 species of small annual herbs distributed mostly in tropical Asia.

Systematic position of *Andrographis paniculata* (Burm.f.) Nees

Division: Angiosperms

Class: Dicotyledonae

Subclass: Gamopetalae

Series: Bicarpellatae

Order: Personales

Tribe: Justiceae

Family: Acanthaceae

Genus: *Andrographis*

Species: *paniculata*

Andrographis paniculata is an annual herb, branched, erect, running 1/2 to 1 meter in height. The aerial parts of the plant (leaves and stems) are used to extract the active

phytochemicals. Since ancient times, *A. paniculata* been used as wonder drug in traditional siddha and ayurvedic systems of medicine as well as in tribal medicine in India and some other countries for multiple clinical applications. *Andrographis paniculata* has a surprisingly broad range of pharmacological effects which includes abortifacient, analgesic, antibacterial, antipyretic, antithrombotic, antiviral, cancerolytic, cardioprotective, choloretic, depurative, digestive, expectorant, hepatoprotective, hypoglycemic, immune enhancement, laxative, sedative, thrombolytic and vermicial (Jean Barilla, 1999).

The primary medicinal component of *A. paniculata* is andrographolide. It has a very bitter taste, is colourless crystalline in appearance and is a "diterpene lactone". Andrographolide, the major constituent of the extract is implicated with its pharmacological activity. The leaves contain the highest amount of andrographolide (2.39%), while the seeds contain the least amount (Sharma *et al.*, 1992). The medicinal properties include anti-inflammatory, hepatoprotective activities against galactosamine, paracetamol intoxication, cancer therapy and anti HIV activity (Puri *et al.*, 1993).

The mycorrhizal symbiosis represents a series of complex feedbacks between host and fungus that is governed by their physiology and nutrition. The outcome of a mycorrhizal relationship depends on the balance between fungal demands for energy (in terms of carbon-based compounds) and the plant need for nutrients (Miller *et al.*,

2002). Despite the widespread distribution and ecological significance of AM symbiosis some characterization of its effects on secondary metabolites has been achieved, but relatively little is known about the effects of AM colonization on the accumulation of active phytochemicals in shoots of medicinal plants, which are often the harvest products and used for human consumption. Therefore, the present work was carried out to study the effect of different AM fungal inocula on growth and percent concentration of andrographolide in *A. paniculata*.

MATERIALS AND METHODS

Plant material: Seeds of *A. paniculata* Nees. collected from the wild were selected for the study. Seeds of uniform size were surface sterilized using hot water treatment (90⁰C) and were kept on moist filter papers in petri plates in a growth incubator at 26⁰C with 16hr photoperiod with Relative Humidity (RH) of 62%. Germination percentage was determined by counting the number of seeds germinated. Seeds were considered germinated only after the emergence of 2mm of radical.

Fungus material: Monospecific cultures of AM fungi prepared following trap cultures were selected for the study. Sand and soil based spore inocula of *Acaulospora scrobiculata* Trappe, *Gigaspora albida* Schenck & Smith, *Glomus fasciculatum* (Thaxter) Gerdemann & Trappe emend. Walker & Koske, *Scutellospora biornata* Spain, Sieverding & Toro and *Scutellospora calospora* (Nicolson &

Gerdemann) Walkers & Sanders were used. The inoculum of each monospecific culture consisted of approximately 80-120 spores 100g^{-1} of soil. Unsterilized control consisted of mixed AM fungal spores viz., *Acaulospora scrobiculata* Trappe, *A. laevis* Gerdemann & Trappe, *Glomus mosseae* (Nicolson & Gerdemann) Gerdemann & Trappe, *G. fasciculatum* (Thaxter) Gerdemann & Trappe emend. Walker & Koske, *G. maculosum* Miller & Walker, *G. magnicaule* Hall, *G. diaphnum* Morton & Walker, *Glomus multicaule* Gerdemann & Bakshi, *Glomus geosporum* (Nicolson & Gerdemann) Walker and *Glomus intraradices* Schenck & Smith. Sterilized sand soil (1:3) mix (control) used in the experiment was sterilized for 3hr at 15lbs pressure for three consecutive days to eliminate naturally occurring endophytes and other contaminants.

Growing conditions: The experiment was conducted for a period of five months (November 2007-March 2008) in the polyhouse. The temperature during the study period was maintained at 27°C and Relative Humidity (RH) was 65%. Throughout the experiment, the pots were watered on alternate days and Hoagland solution without phosphorus (Hoagland and Arnon, 1938) was added at an interval of 15 days.

Inoculation with mycorrhizal fungi: Uniform seedlings of germinated *A. paniculata* were planted in seven trays (22.5cm x 17.5cm) with five monospecific cultures of AM fungi, unsterilized (control) and sterilized (control).

Experimental design: Randomized block design experiment was conducted which comprised of following treatments. Each treatment consisted of four replicates.

Treatment 1: Control (C1)- Uninoculated sterilized soil

Treatment 2: Control (C2) - Unsterilized soil containing AM fungal spores of *A. scrobiculata*, *A. laevis*, *A. myriocarpa*, *G. albidum*, *G. mosseae*, *G. fasciculatum*, *G. maculosum*, *G. magnicaule*, *G. diaphnum*, *G. multicaule*, *G. geosporum* and *G. intraradices*.

Treatment 3: (M1) - *Scutellospora calospora*

Treatment 4: (M2) - *Acaulospora scrobiculata*

Treatment 5: (M3) - *Glomus fasciculatum*

Treatment 6: (M4) - *Gigaspora albida*

Treatment 7: (M5) - *Scutellospora biornata*

After 45 days, seedlings were transferred to pots (15cm diam.) filled with unsterilized sand soil (3:1) mix. The experiment was terminated after 150 days of growth and *A. paniculata* plants were harvested and subjected to analysis.

Plant growth measurements: Shoot and total plant dry weights and leaf number were recorded. There were three replicates for each treatment.

For measurement of fresh weight, plants were carefully removed from the polyethylene bags, both root and shoot portions were separated. Roots were thoroughly washed with tap water to remove the soil debris and then dried using

blotting paper. Number of leaves per plant was calculated by counting the total number of leaves in each plant. Dry weights of plant and shoot were determined after drying the tissue to constant weight in oven at 70°C.

Mycorrhizal dependency (MD): Using shoot dry weight data, degree of plant response to AM fungi (dependency) was calculated as the difference between the biomass of the shoot of inoculated and uninoculated plant and was expressed as percentage of the dry weight biomass of inoculated plants (Plenchette *et al.*, 1983).

$$\frac{\text{Shoot dry wt. of inoculated plant}}{\text{Shoot dry wt. of un-inoculated plant}} \times 100$$

Mycorrhizal Efficiency Index (MEI): Using plant dry weight, mycorrhizal efficacy in enhancing the growth was calculated by taking the average dry weight of the plant. The Mycorrhizal Efficiency Index (MEI) was estimated according to Bagyaraj (1994).

$$\text{MEI} = \frac{\text{Wt. of inoculated plant} - \text{Wt. of uninoculated plant}}{\text{Wt. of inoculated plant}} \times 100$$

Estimation of Phosphorus concentration:

Roots and shoot tissues of *A. paniculata* were subjected to for dry ash digestion procedure and were estimated for total P (ppm) using vanadomolybdate phosphoric yellow colour method (Chapman and Prat, 1961).

HPLC analysis for secondary metabolite concentration: Andrographolide concentration from the leaf extracts of *A. paniculata* were carried out using HPLC analysis (Pholphana *et al.*, 2004).

Sample preparation: For preparation of sample for HPLC analysis, *A. paniculata* leaves were oven dried at 70⁰C for 36 hours were then powdered, passed through 40 mesh sieve, placed in an airtight container and stored at 20⁰C till use.

Analysis of the andrographolide in *A. paniculata* leaf extract:

Methanol (HPLC grade) 4.0ml was added to accurately weighed amounts (300 mg each) of dried, powdered leaves of *A. paniculata*. Samples were agitated for 5 minutes and then centrifuged at 2000rpm for 10 minutes. The supernatant was transferred to a glass tube and marc re-extracted twice more with 3ml methanol. All extracts were combined and filtered through a 0.45 μ m nylon membrane prior to HPLC analysis.

Standard reference of andrographolide (98% purity) was procured from Sigma Aldrich Bangalore. Methanol (HPLC grade) was added to 1 μ l of reference standard solution before injection to the column.

Chromatographic conditions:

Crude leaf extracts were analysed by HPLC using a Spectra system UV, with thermostatically controlled column and a Photodiode Array Detector (PAD), RP18 reverse phase column (300×2.5mm) protected by a LiChrospher RP18 guard column (4 × 4 mm i.d.). The column was equilibrated with the mobile phase, consisting of 50.5% methanol in water, at a flow rate of 2 ml/min for 30 min before analysis. An aliquot (1µl) of standard and samples were injected onto the column and UV detection was set at 220nm. The temperature of the column was 25°C. The peak identification was based on retention time and comparison to the injected authentic reference standard. Using freshly prepared mobile phase, the baseline was monitored until stable before the samples were run.

Andrographolide content per plant was obtained by multiplying dry weight of the shoot by its andrographolide concentration.

Statistical analysis: Statistical analysis was carried out by Analysis of Variance (ANOVA) and correlation coefficient. Data on shoot and total plant dry weights and phosphorus analysis were tested for significance between the treatments using WASP 0.2. Correlation analysis was carried between shoot and total plant dry weights, mycorrhizal dependency, mycorrhizal efficiency index and phosphorus estimation in roots and shoots using Pearson correlation coefficient.

RESULTS:

Root colonization by Arbuscular mycorrhizal fungi:

Mycorrhizal colonization was observed in all the roots pieces of plants grown in inoculated and unsterilized soil while no colonization was observed in uninoculated sterilized soil. Arbuscular (**Plate XIX a, b, c, d, e & f**) and hyphal colonization was observed in all the treatments whereas hyphal, arbuscular and vesicular colonization was observed in unsterilized control. Young arbuscules were observed after 45 days of plant growth while mature and degenerating arbuscules were observed in roots after 150 days of growth. Arum type of arbuscules were observed in all the treatments and unsterilized control.

Growth parameters:

There was significant increase in growth of plants in all the treatments and in unsterilized soil compared to sterilized soil. After 150 days of growth, all the inoculated and control plants were harvested. At the time of harvesting, *S. calospora*, *S. biornata* and *G. fasciculatum* inoculated and sterilized control plants were in the vegetative stage, *Gi. albida* inoculated plants in flowering stage while *A. scrobiculata* and unsterilized control plants were in the flowering and fruiting stage. Thus, early flowering and fruiting was recorded in unsterilized control and *A. scrobiculata* inoculated plants (**Plate XVIII a & b**).

Plate XVIII: Effect of arbuscular mycorrhizal fungal inoculation on the growth of *Andrographis paniculata* plants

- a) *Andrographis paniculata* plants inoculated with mycorrhizal inoculum and control (after 150 days of growth).

- b) Flowering and fruiting stage of the plant.

Plate XVIII

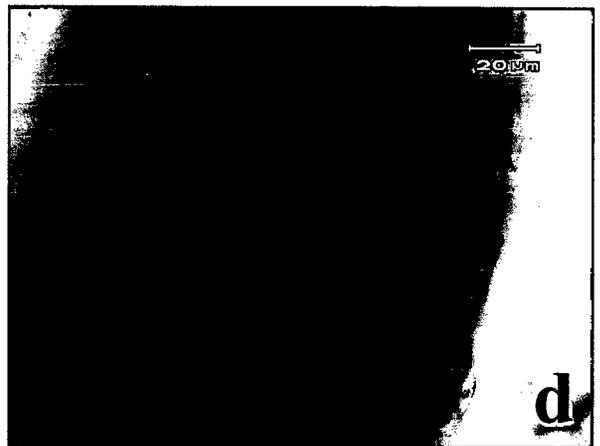


Plate XIX: Mycorrhizal colonization in *A. paniculata* plants inoculated with AM fungal species and control.

- a) Arbuscular colonization in roots of *A. paniculata* grown in *Acaulospora scrobiculata* (X 100).

- b) c) d) e) & f) Matured arbuscules in roots of *A. paniculata* inoculated with *Gi. albida*, *S. calospora*, *S. biornata* and unsterilized control (X 400).

Plate XIX



At the time of harvesting (150 days after transplantation), *A. paniculata* plants inoculated with all AM treatments and unsterilized control showed better growth and increased leaf number than sterilized control. However, growth responses varied among mycorrhizal treatments. Plants grown in unsterilized control and inoculated with *Gi. albida* and *A. scrobiculata* separately recorded a significant increase in leaf, shoot and total plant dry weights compared to other treatments and sterilized control ($F=0.028$, $CD=0.439$; $F=0.023$; $CD= 0.456$; $P\leq 0.05$) (Table 9; Fig. 26, 27 & 28). Observations revealed a significant increase in shoot and total plant dry weights in plants grown in unsterilized soil and *Gi. albida* compared to sterilized soil. The results in other AM fungal treatments did not differ significantly from the sterilized soil (Table 9). Maximum leaf number was recorded in plants inoculated with *Gi. albida* and minimum was recorded in sterilized control, while all the other treatments were significantly different from the control ($F= 0.000$, $CD= 5.309$, $P \leq 0.05$) (Table 9; Fig. 28).

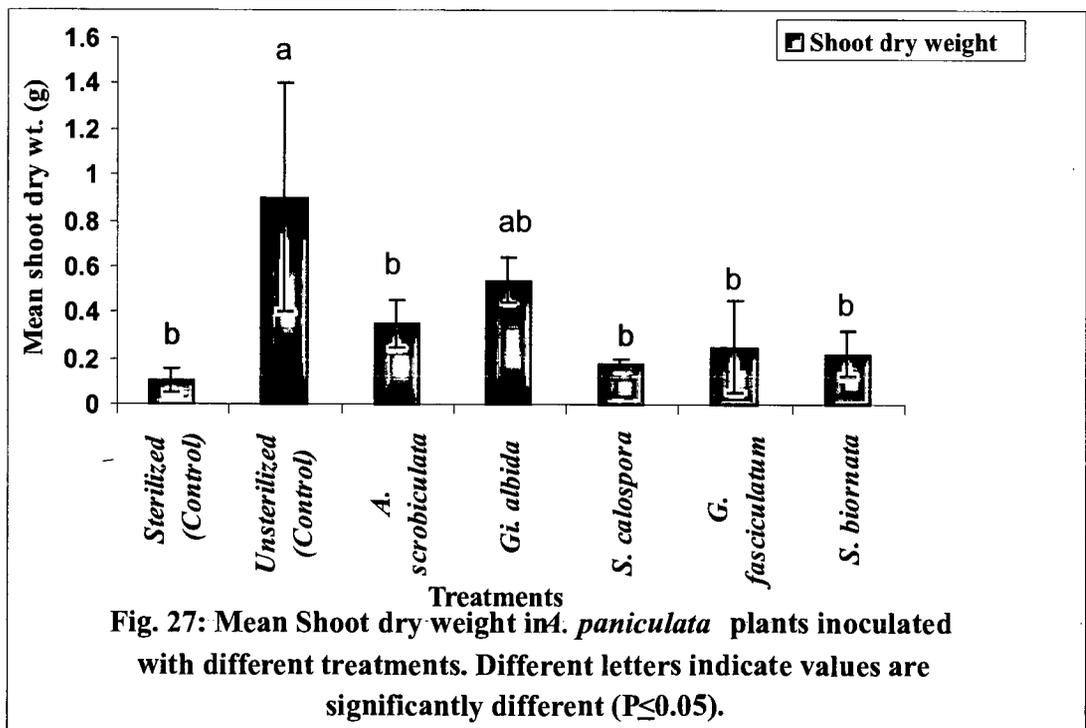
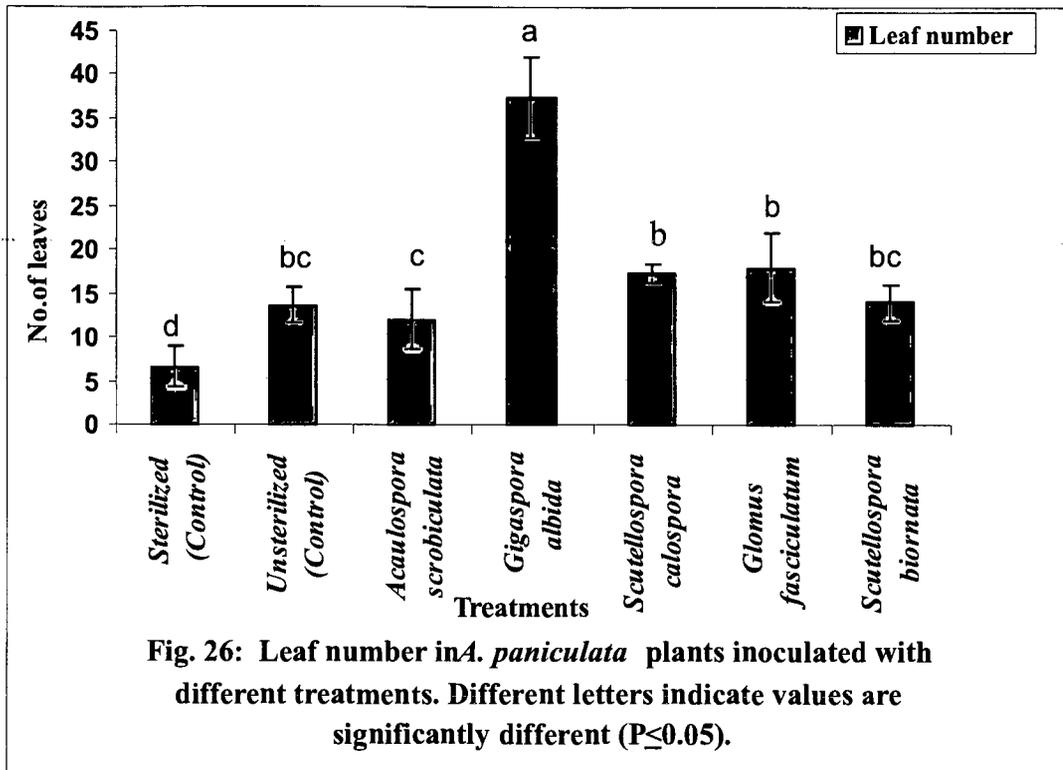
Mycorrhizal Dependency:

The Mycorrhizal dependency study revealed that plants inoculated with *Gi. albida* and those grown in unsterilized control showed high mycorrhizal dependency (81.4 & 88.8% respectively). Least mycorrhizal dependency was observed in plants inoculated with *S. calospora* (44.4%) (Fig. 29). Strong positive correlation was observed between shoot and plant dry weights and mycorrhizal dependency as the treatments were found significant at 5% ($r=1$; $r=0.78$, $P\leq 0.05$).

Table 9: Plant dry weight and shoot dry weight in *A. paniculata* inoculated with different treatments and control.

Sr. No.	Treatments	Shoot dry wt. (g)	Plant Dry wt. (g)	Number of leaves/plant
1	Sterilized (Control)	0.10 ^a ± 0.05	0.20 ^b ± 0.02	6.60 ^d ± 1.30
2	Unsterilized (Control)	0.90 ^a ± 0.05	1.00 ^a ± 0.52	13.66 ^{bc} ± 2.00
3	<i>Acaulospora scrobiculata</i>	0.35 ^b ± 0.01	0.36 ^b ± 0.06	12.00 ^c ± 3.40
4	<i>Gigaspora albida</i>	0.54 ^{ab} ± 0.10	0.59 ^{ab} ± 0.10	37.30 ^a ± 4.60
5	<i>Scutellospora calospora</i>	0.18 ^b ± 0.02	0.21 ^b ± 0.05	17.33 ^b ± 1.10
6	<i>Glomus fasciculatum</i>	0.25 ^b ± 0.06	0.28 ^b ± 0.09	18.00 ^b ± 3.00
7	<i>Scutellospora biornata</i>	0.22 ^b ± 0.02	0.26 ^b ± 0.07	14.00 ^{bc} ± 2.00

Legend: Values are means of 3 replicates. Columns with different letters indicate that treatments were significantly different at 5% level of significance ($P \leq 0.05$).



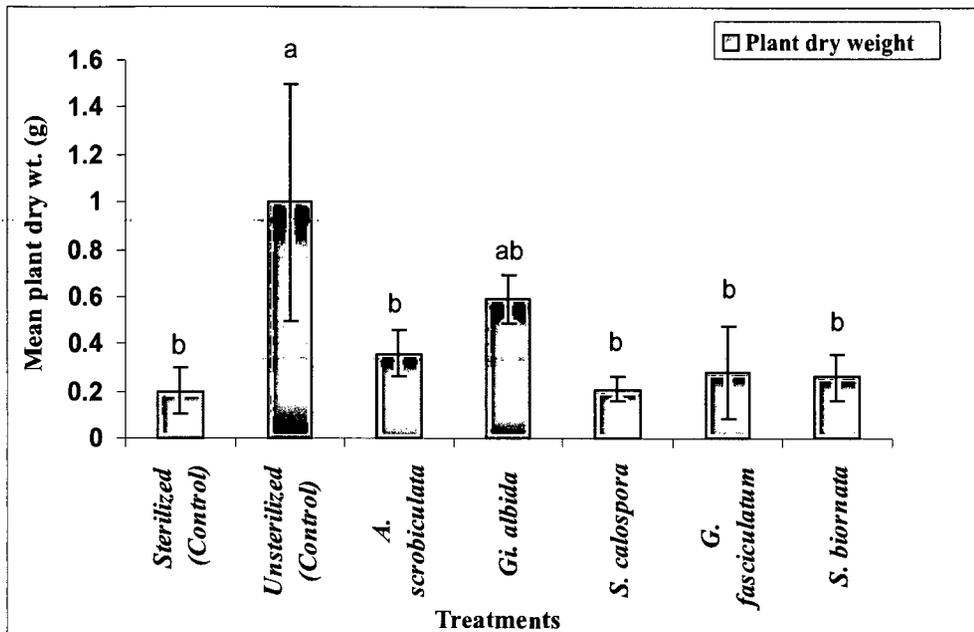


Fig. 28: Mean plant dry weight of *A. paniculata* plants inoculated with different treatments. Different letters indicate values are significantly different ($P \leq 0.05$).

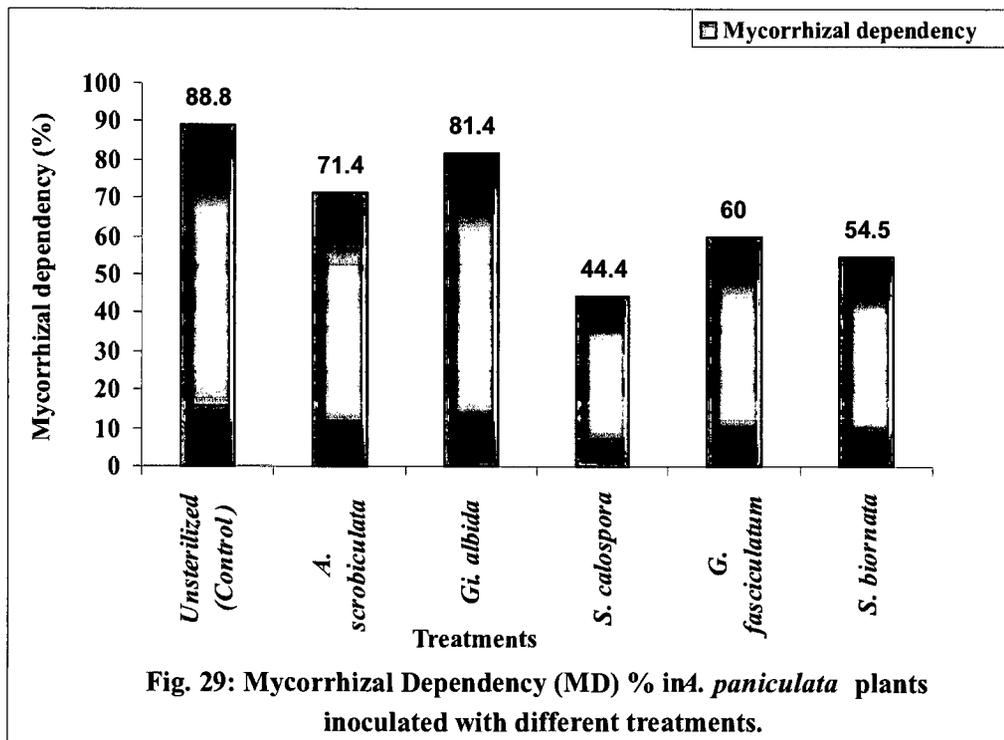


Fig. 29: Mycorrhizal Dependency (MD) % in *A. paniculata* plants inoculated with different treatments.

Mycorrhizal Efficiency Index:

Mycorrhizal efficiency study revealed that unsterilized control had high efficiency (80.00%) in enhancing the growth of *A. paniculata* followed by *Gi. albida* (66.10%) while *S. calospora* had least efficiency (4.76%). Mycorrhizal efficiency varied from 23.07% to 44.44% in other treatments. (Fig. 30). Significant weak positive correlation was observed between shoot and total plant dry weights and mycorrhizal efficiency index ($r=0.3$; $r=0.49$, $P\leq 0.05$).

Phosphorus Concentration:

Phosphorus concentration studies showed variation in P concentration among the treatments. Maximum P concentration was observed in both root (58.5ppm) and leaf (17.8ppm) tissues inoculated with *G. fasciculatum* followed by *S. biornata* and least in sterilized soil (Table 10; Fig. 31 & 32). A significant increase in P concentration was observed in the vegetative stage of plants inoculated with *G. fasciculatum* as compared to control (df= 6, CD (0.05) = 12.336; F=0.000; df=6; CD (0.05) = 4.375; F=0.000; $P\leq 0.05$). In the present study, a significant increase in P concentration was observed during the vegetative stage. It was observed that during the flowering and fruiting stage P concentration did not differ significantly in plants grown in sterilized soil (Table 10). Non-significant (at 5%) weak negative correlation was observed between mean total plant dry weight and P concentration in roots ($r= -0.46$, $P\leq 0.05$) and shoots ($r= -0.49$, $P\leq 0.05$).

Andrographolide concentration:

Table 10: Phosphorus estimation in roots and leaves of *A. paniculata* after 150 days of growth.

Treatments	Roots (ppm)	Shoots (ppm)
Sterilized (control) – Vegetative stage	20.90 ^c ± 3.50	7.00 ^c ± 0.60
Unsterilized (control) (Flowering & Fruiting stage)	23.80 ^c ± 2.70	10.90 ^{bc} ± 1.00
<i>A. scrobiculata</i> (Flowering & Fruiting stage)	26.80 ^c ± 2.50	7.30 ^c ± 0.80
<i>Gi. albida</i> (Flowering stage)	24.90 ^c ± 2.05	7.38 ^b ± 0.10
<i>S. calospora</i> (Vegetative stage)	44.92 ^{ab} ± 2.05	10.90 ^{bc} ± 1.90
<i>G. fasciculatum</i> (Vegetative stage)	58.50 ^a ± 6.00	17.80 ^a ± 1.10
<i>S. biornata</i> (Vegetative stage)	44.10 ^{ab} ± 4.60	17.60 ^a ± 1.40

Legend: Values are means of five replicates. Columns with different letters indicate that treatments were significantly different ($P \leq 0.05$).

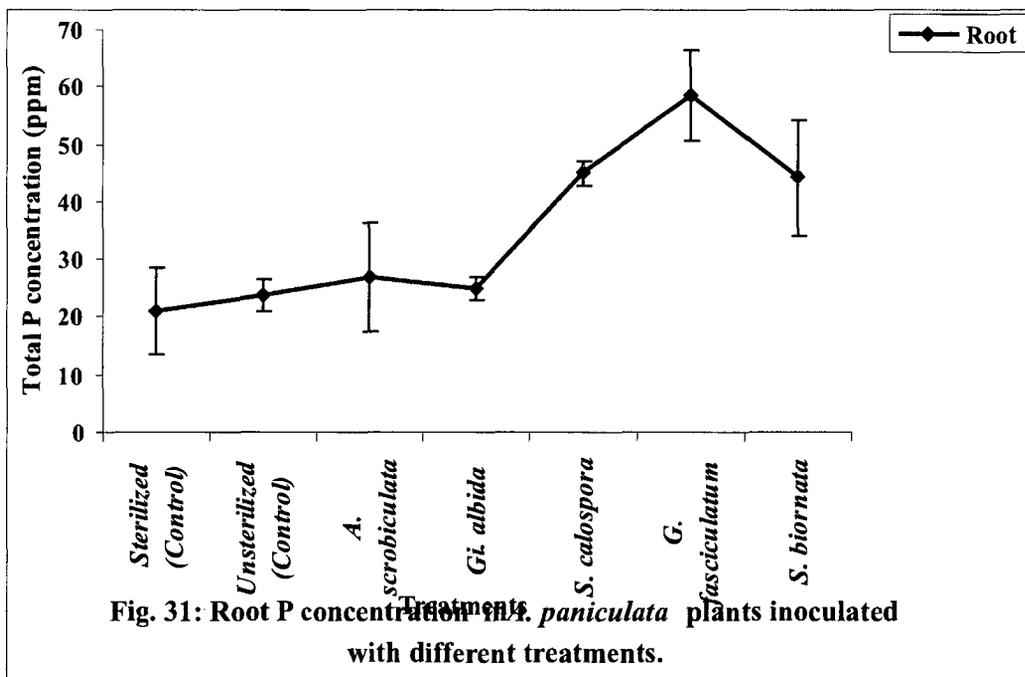
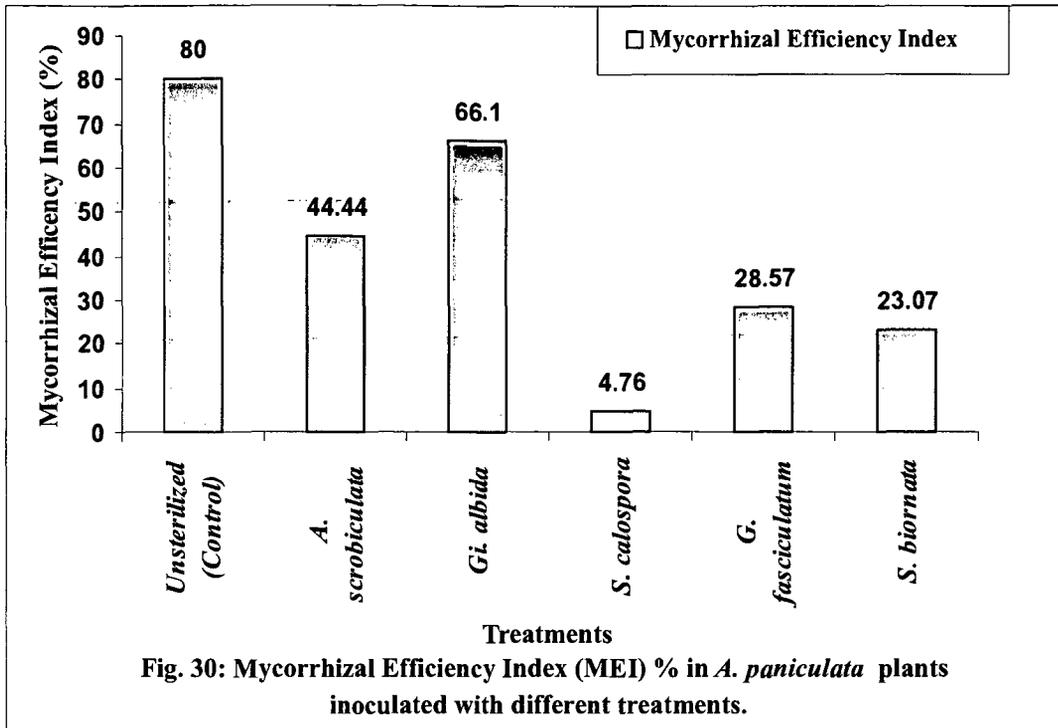
Concentration of andrographolide in leaf extracts varied among the treatments and the controls. Sharp symmetrical peaks were recorded in all the treatments and the controls. The standard reference andrographolide showed a percent recovery of 92% at a retention time of 15.2 (**Fig. 33**). The percent recovery of these compounds ranged from 1.98% to 58.53% at a retention time of 15.0 to 15.4 in all the treatments and control. The maximum percent recovery of andrographolide was observed in plants inoculated with *Gi. albida* (58.3%) whereas least was observed in plants grown in sterilized soil (1.98%) (**Table 11**) (**Fig. 34 - 40**). Maximum Andrographolide content per plant was observed in *Gi. albida* (31.49) and minimum in sterilized soil (0.19) (**Table 11**).

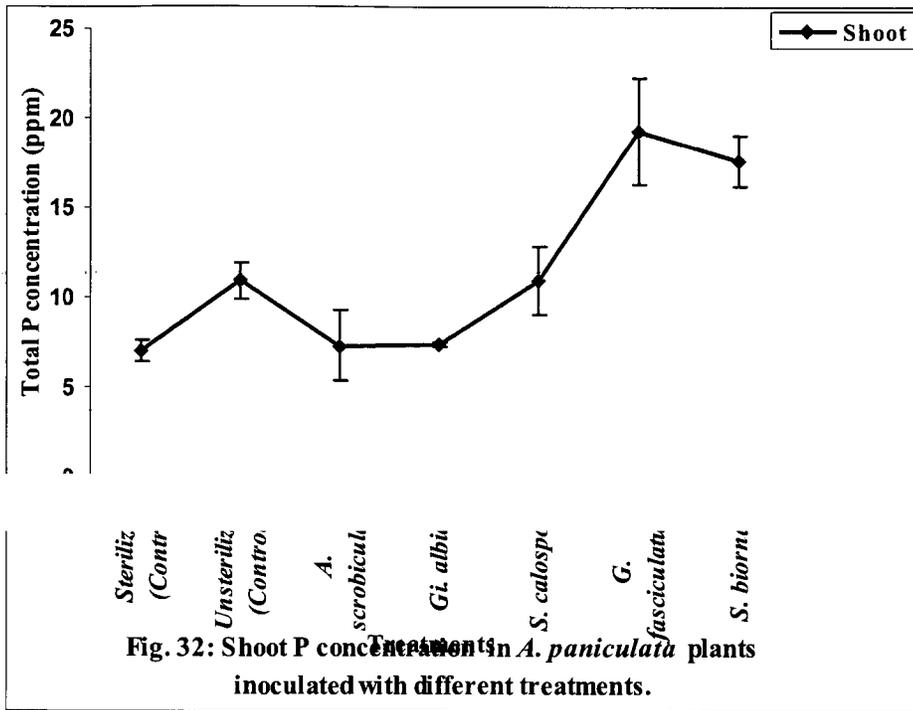
DISCUSSION

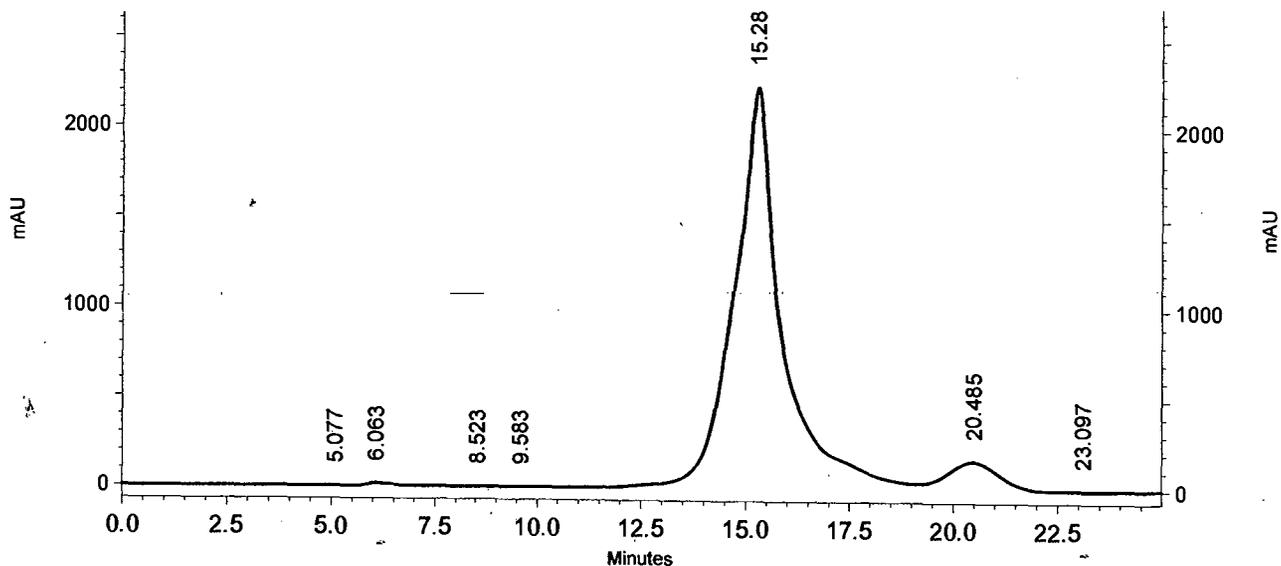
The present study confirms the benefit of mycorrhizae as biofertilizers on growth, yield, nutrient uptake and increase in andrographolide concentration in *A. paniculata*. Except for sterilized control, mycorrhizal colonization was observed in all the AM fungal treatments and unsterilized control. Similar observations were reported earlier (Chiramel *et al.*, 2006). Arum type of arbuscules was observed in roots of all the treatments. Arbuscule represents a dead end in the growth of AM fungi (Bonfante and Perotto, 1995) as they senesce and collapse after 4-10 days of symbiosis (Sanders *et al.*, 1977) and thus the plant cell recovers its original morphology (Jacquelinet-Jeanmougin *et al.*, 1987). In this way cortical cell is able to allow a second fungal penetration and arbuscule formation. The short life span of

Table 11: Percent andrographolide concentration and total andrographolide content per plant in treatments and control.

Sr. No	Treatments	Retention time	Andrographolide concentration (%)	Total Andrographolide content per plant (mg/plant)
1	Sterilized (Control)	15.0	1.98	0.198
2	Unsterilized (Control)	15.1	5.78	5.2
3	<i>A. scrobiculata</i>	15.3	14.6	3.21
4	<i>Gi. albida</i>	15.4	58.53	31.6
5	<i>S. calospora</i>	15.3	8.06	1.44
6	<i>G. fasciculatum</i>	15.4	8.06	2.01
7	<i>S. biornata</i>	15.3	13.8	4.83





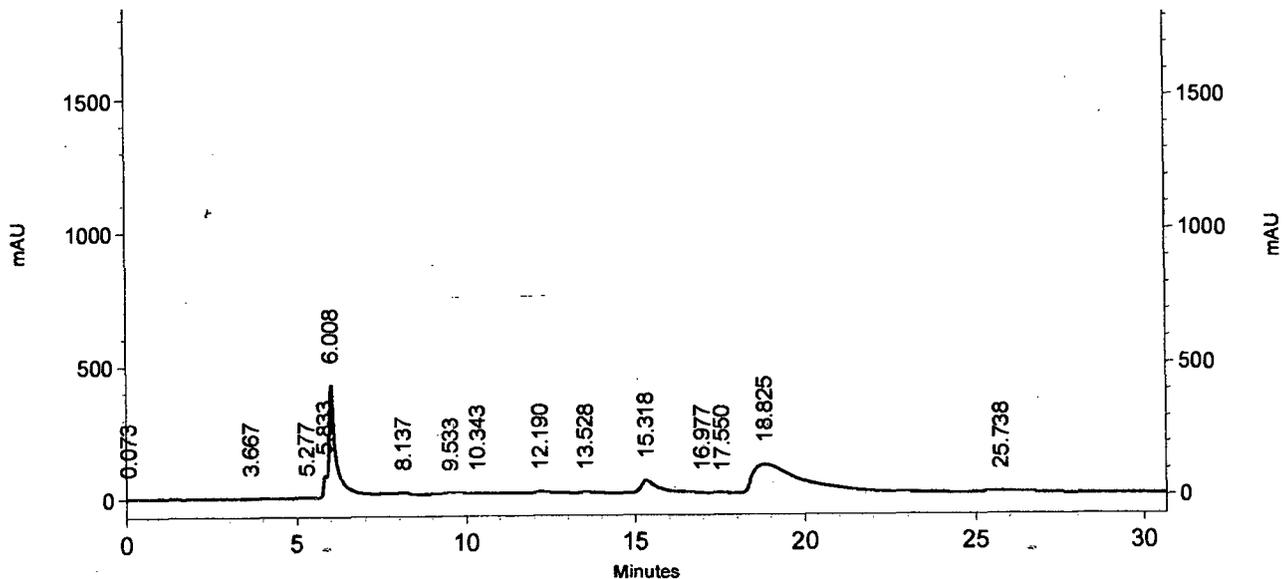


Detector
1-220nm Results
(System
(29/04/08
04:58:22)
(Reprocessed)
(Aborted Run))

Name	Retention Time	Area	Area Percent	Integration Codes
	5.077	35996	0.023	BV
	6.063	714088	0.464	VV
	8.523	27156	0.018	VB
	9.583	21092	0.014	BB
	15.280	142020384	92.370	MM
	20.485	10929696	7.109	VV
	23.097	2965	0.002	VV

Totals		153751377	100.000	
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Fig. 33: Percent andrographolide concentration in reference standard.

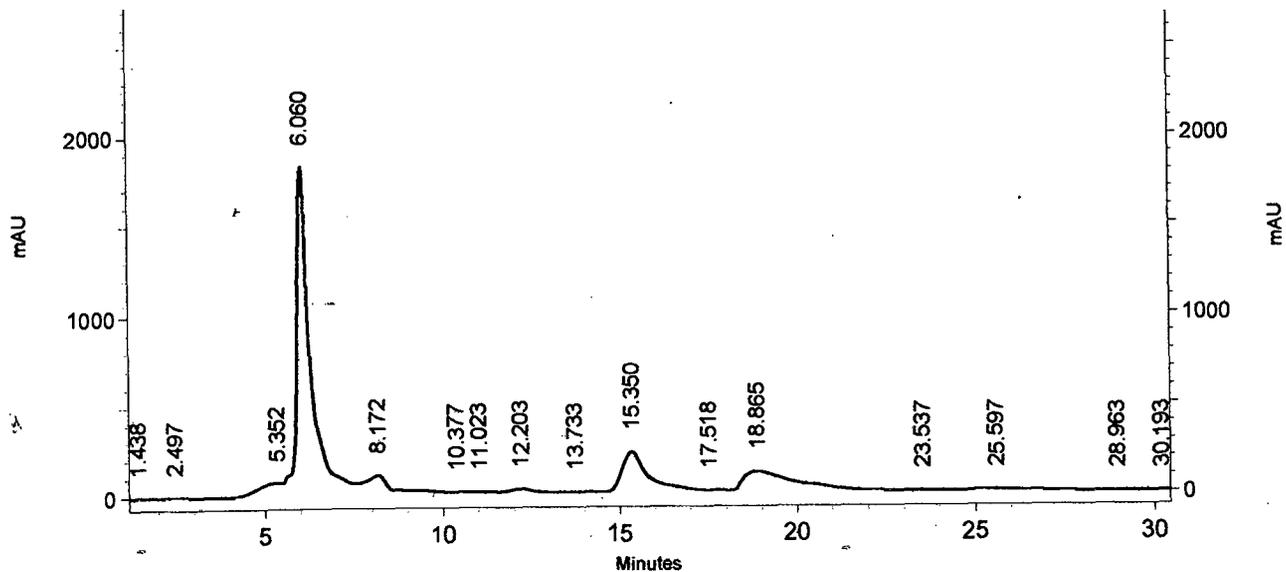


Detector
1-220nm Results
 (System
 (29/04/08
 05:25:09)
 (Original)
 (Aborted Run))

Name	Retention Time	Area	Area Percent	Integration Codes
	0.073	13013	0.060	BB
	3.667	19999	0.093	BV
	5.277	6768	0.031	BV
	5.833	548696	2.540	VV
	6.008	6192586	28.666	VV
	8.137	655278	3.033	VV
	9.533	319172	1.477	VV
	10.343	30864	0.143	VV
	12.190	151905	0.703	BV
	13.528	138700	0.642	VV
	15.318	1749536	8.099	VV
	16.977	55961	0.259	VV
	17.550	35877	0.166	VV
	18.825	11167739	51.697	VB
	25.738	516184	2.389	BV

Totals		21602278	100.000	
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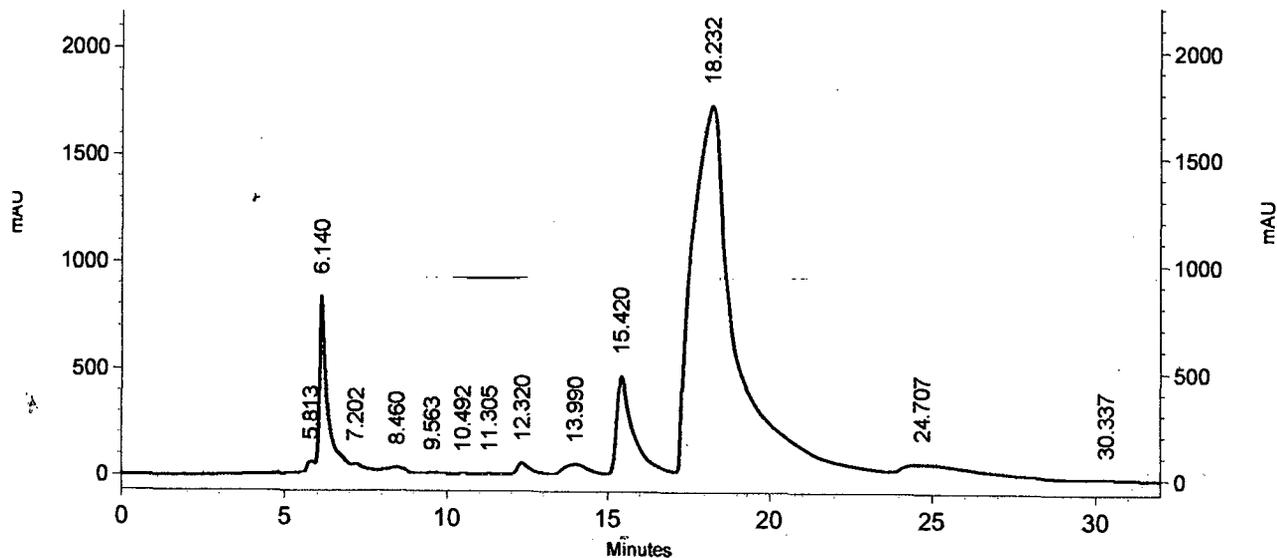
Fig. 34: Percent andrographolide concentration in *A. paniculata* inoculated with *S. calospora*.



Detector
1-220nm Results
(System
(01/01/98
05:31:25)
(Reprocessed)
(Aborted Run))

Name	Retention Time	Area	Area Percent	Integration Codes
	0.147	99904	0.109	BV
	0.598	74416	0.082	VV
	1.438	202696	0.222	VV
	2.497	293864	0.322	VV
	5.352	3655126	4.005	VV
	6.060	50244590	55.061	VV
	8.172	7253602	7.949	VV
	10.377	508587	0.557	VV
	11.023	752308	0.824	VV
	12.203	1672025	1.832	VV
	13.733	673855	0.738	VV
	15.350	12652175	13.865	VV
	17.518	359892	0.394	VV
	18.865	11899137	13.040	VV
	23.537	6210	0.007	BB
	25.597	814172	0.892	BV
	28.963	32708	0.036	VV
	30.193	57500	0.063	VE

Fig. 35: Percent andrographolide concentration in *A. paniculata* inoculated with *S. biornata*.

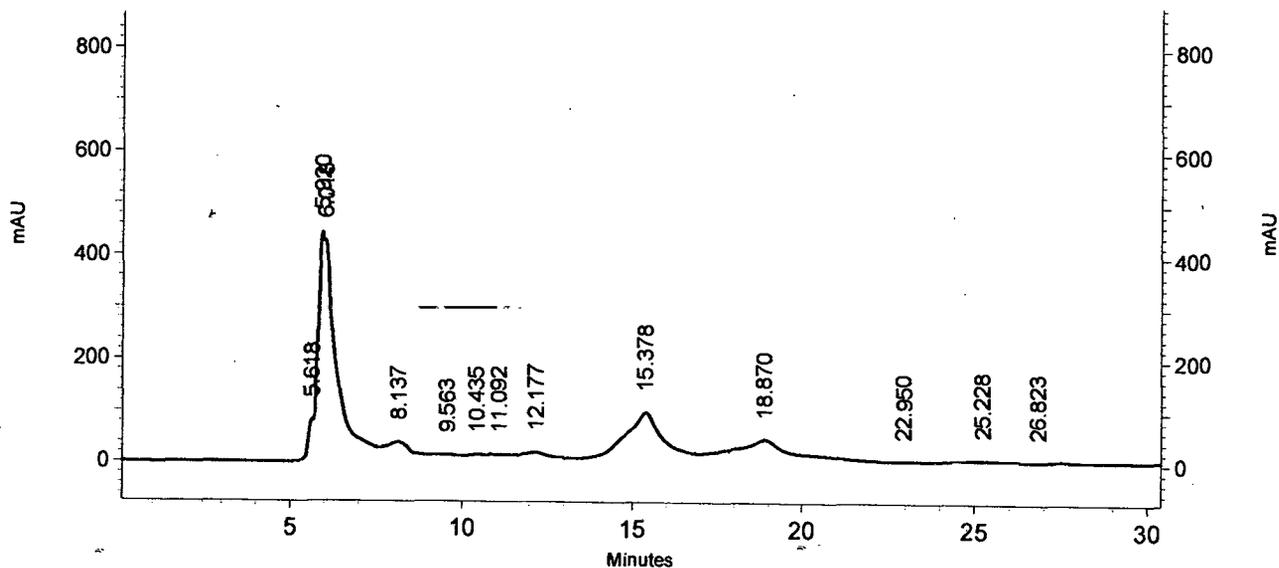


Detector
1-220nm Results
 (System
 (29/04/08
 05:36:49)
 (Original)
 (Aborted Run))

Name	Retention Time	Area	Area Percent	Integration Codes
	5.813	864341	0.395	BV
	6.140	14762112	6.739	VV
	7.202	1303504	0.595	VV
	8.460	1379115	0.630	VV
	9.563	176208	0.080	VV
	10.492	47825	0.022	VV
	11.305	71962	0.033	VV
	12.320	1658461	0.757	VV
	13.990	2900392	1.324	VV
	15.420	17672936	8.068	VV
	18.232	166133102	75.841	VV
	24.707	11912773	5.438	VV
	30.337	170330	0.078	VB

Totals		219053061	100.000	
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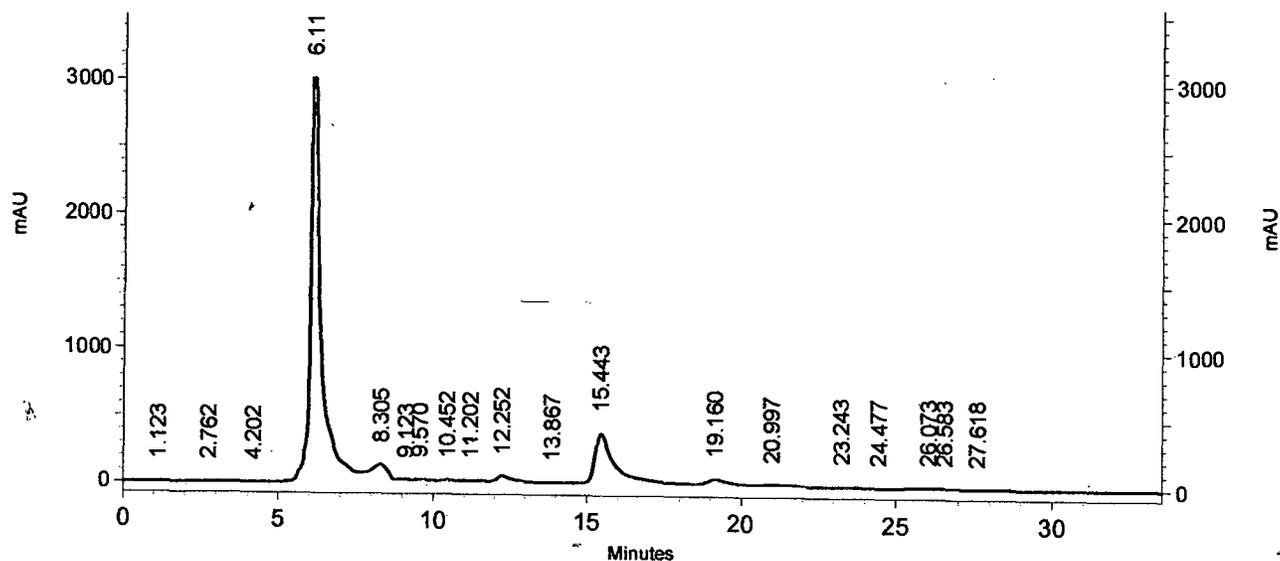
Fig. 36: Percent andrographolide concentration in *A. paniculata* inoculated with *G. fasciculatum*.



Detector
1-220nm Results
(System
28/04/08
05:22:59)
(Reprocessed)
(Aborted Run))

Name	Retention Time	Area	Area Percent	Integration Codes
	5.618	777743	2.155	BV
	5.920	5926678	16.422	VV
	6.018	10534711	29.190	VV
	8.137	2097326	5.811	VV
	9.563	332126	0.920	VV
	10.435	366915	1.017	VV
	11.092	398422	1.104	VV
	12.177	683767	1.895	VV
	15.378	5303012	14.694	MM
	18.870	301444	0.835	BV
	22.950	215	0.001	VV
	25.228	268974	0.745	BV
	26.823	5414	0.015	VB
	33.337	7053	0.020	BV
	35.550	779	0.002	BB
	39.297	8986196	24.899	MM
	46.710	22960	0.064	VV
	48.478	8063	0.022	VV

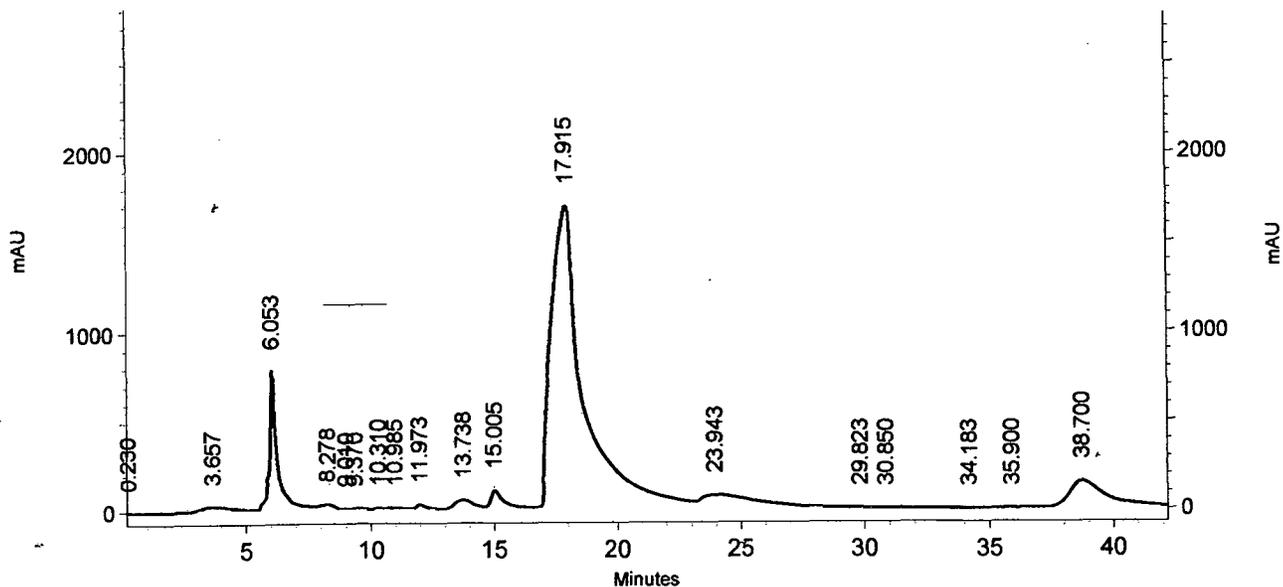
Fig. 37: Percent andrographolide concentration in *A. paniculata* inoculated with *A. scrobiculata*.



Detector
1-220nm Results
 (System
 (29/04/08
 05:39:10)
 (Original)
 (Aborted Run))

Name	Retention Time	Area	Area Percent	Integration Codes
	1.123	83963	0.286	BV
	2.762	87339	0.298	VV
	4.202	1155	0.004	VB
	6.110	3027618	10.321	BV
	8.305	2737679	9.333	VV
	9.123	8389	0.029	VV
	9.570	134401	0.458	VV
	10.452	128381	0.438	VV
	11.202	301783	1.029	VV
	12.252	1422865	4.850	VV
	13.867	14114	0.048	VV
	15.443	17169540	58.530	VV
	19.160	2397674	8.173	VV
	20.997	1005394	3.427	VV
	23.243	124878	0.426	VV
	24.477	5443	0.019	VV
	26.073	388275	1.324	VV
	26.583	186964	0.637	VV

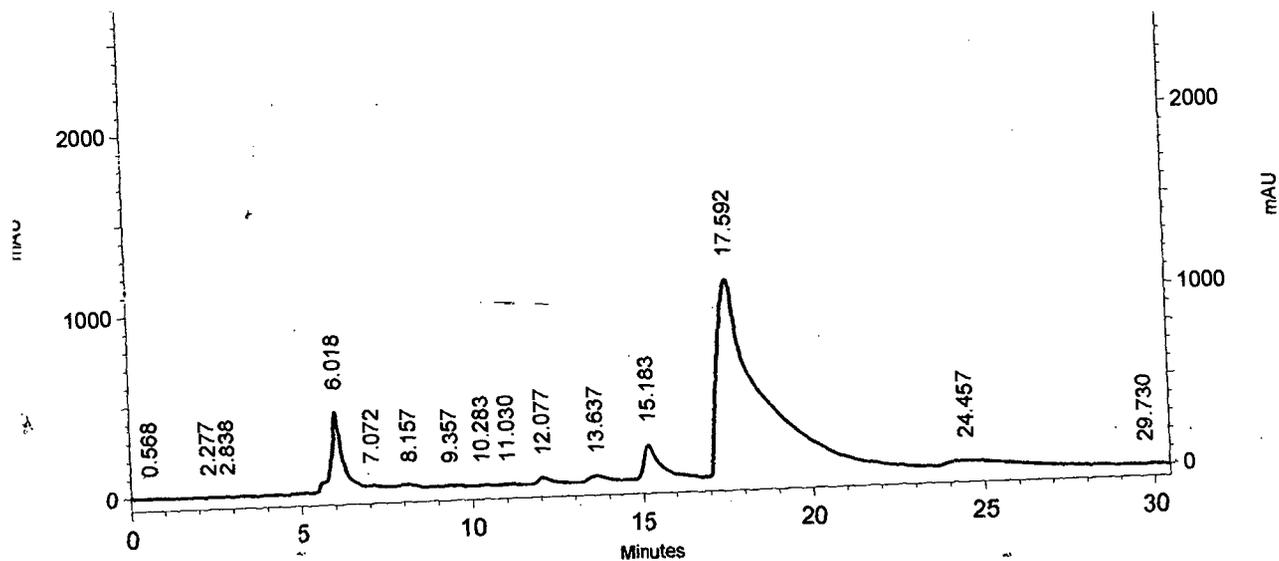
Fig. 38: Percent andrographolide concentration in *A. paniculata* inoculated with *Gi. albida*.



Detector
1-220nm Results
 (System
 (29/04/08
 05:12:48)
 (Original)
 (Aborted Run))

Name	Retention Time	Area	Area Percent	Integration Codes
	0.230	1159	0.001	BV
	3.657	2268710	1.077	BV
	6.053	17128638	8.133	VV
	8.278	1486866	0.706	VV
	9.010	115935	0.055	VV
	9.370	307904	0.146	VV
	10.310	233883	0.111	VV
	10.985	552297	0.262	VV
	11.973	887902	0.422	VV
	13.738	2782358	1.321	VV
	15.005	4178496	1.984	VV
	17.915	156524226	74.317	VV
	23.943	11905089	5.652	VV
	29.823	34987	0.017	BV
	30.850	55464	0.026	VB
	34.183	6797	0.003	BV
	35.900	5557	0.003	BV
	38.700	12140160	5.764	BE

Fig. 39: Percent andrographolide concentration in *A. paniculata* grown in sterilized soil.



Detector
1-220nm Results
(System
(29/04/08
04:27:47)
(Reprocessed)
(Aborted Run))

Name	Retention Time	Area	Area Percent	Integration Codes
	0.568	41869	0.030	BV
	2.277	4371	0.003	BV
	2.838	2212	0.002	VV
	6.018	11038973	7.789	BV
	7.072	1023074	0.722	VV
	8.157	1648256	1.163	VV
	9.357	860708	0.607	VV
	10.283	315198	0.222	VV
	11.030	535083	0.378	VV
	12.077	1487189	1.049	VV
	13.637	2061527	1.455	VV
	15.183	8194289	5.782	VV
	17.592	105480577	74.431	VV
	24.457	8909631	6.287	VV
	29.730	113598	0.080	VE

Totals		141716555	100.000	
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Fig. 40: Percent andrographolide concentration in *A. paniculata* grown in unsterilized soil.

arbuscules is perhaps they are digested by the host cell presumably when no longer needed for transfer (Toth and Miller, 1984).

Mycorrhizal and non-mycorrhizal plants differed significantly in shoot and total plant dry weights. A significant increase in shoot and total plant dry weights was observed in unsterilized control indicating that native AM fungi were more efficient in stimulating plant growth than the inoculated strains. Similar observations have been reported earlier (Bagyaraj and Manjunath, 1980). *Gigaspora albida* showed a significant increase in plant and shoot dry weights compared to other treatments but there was no significant difference between plants grown in other AM fungal treatments and sterilized control. The study also confirms the earlier findings that plant growth varies with plant–fungus interaction, certain combinations of host and fungus are more or less compatible than others (Ravnskov and Jakobsen, 1995; Parke and Kaeppler, 2000). Growth and mineral nutrition of plants are known to be enhanced by inoculation with AM fungi (Clark and Zeto, 2000). The reasons for species-specific responses to AM fungal colonization and plant benefit are probably mediated by a complex combination of plant and fungal signals based on genetically controlled substances (Gianinazzi-Pearson *et al.*, 1991).

The Mycorrhizal Dependency (MD) describes the degree of plant responsiveness to AM colonization by producing maximum growth or yield at a given level of soil fertility. Mycorrhizal dependency varied with AM fungal treatments in

A. paniculata and this could be due to differential growth response to specific AM fungi. Similar observations were made earlier (Van der Heijden *et al.*, 1998a). The high mycorrhizal dependency observed in *Gi. albida* compared to other AM fungal treatments indicate *Gi. albida* as the efficient inoculum for growth of *A. paniculata*. High mycorrhizal dependency observed in plants grown in unsterilized control could be due to AM fungal species richness. Negative correlation was observed between mycorrhizal dependency and P concentration in plant tissues, as P concentrations in plant tissues do not necessarily govern mycorrhizal dependency. Mycorrhizal dependency of a given plant species can be altered mainly by mycorrhizal species (Schenck *et al.*, 1974). Factors such as root hairs (Baylis, 1974), root geometry (Mosse *et al.*, 1973), plant growth rates (Hall, 1975), phosphate transport and utilization (Nassery, 1970) could influence mycorrhizal dependency. In the present study *A. paniculata* showed high mycorrhizal dependency in unsterilized soil which had low P concentration in roots and shoots. This is in conformity with an earlier study (Soares and Martins, 2000).

Mycorrhizal efficiency for *A. paniculata* varied among the treatments and between the two controls. Plants grown in unsterilized soil recorded high mycorrhizal efficiency compared to all other treatments. The efficiency of *Gi. albida* in enhancing the growth of *A. paniculata* was found maximum compared to other treatments. The study also contradicting to the effect of mycorrhizal inoculation on plant growth being attributed to improved P absorption by the inoculated plants. Negative

correlation was observed between mycorrhizal efficiency index and P concentration in *A. paniculata*.

In *A. paniculata* high P concentration was observed during the vegetative stage of the plant and least during the fruiting stage. This could be due to the fact that higher tissue P concentrations are required at early growth stages. As the plant matures, an increasing proportion of its dry weight is composed of low-P structural and storage tissues (Grant *et al.*, 2001). Phosphorus concentration in plants is very important as an indicator of mycorrhizal activity. Smith and Read (1997) reported that AM fungi increased plant biomass through an increased P uptake in the plant and the amount of P uptake is related to the amount of root colonization by AM fungi (Sanders *et al.*, 1977; Graham *et al.*, 1982). However, in this study the relationship was found to be plant-AM fungal species dependent. High P concentration observed in plants inoculated with *G. fasciculatum* could be attributable to P absorption being dependent on specific plant-AM fungal combination which supports earlier work (Smith *et al.*, 2004). A negative correlation observed between plant biomass and P concentration indicates that P uptake cannot be used as the only mechanism to explain AM fungal effects on plants as reported earlier (Van der Heijden *et al.*, 1998).

In the present study, high percent concentration of andrographolide was observed in the leaf extracts of *A. paniculata* inoculated with *Gi. albida* which confirm that AM symbiosis enhances the production of secondary metabolites.

Minimum recovery of secondary metabolite concentration was observed in plants grown in sterilized soil. The highest concentration of andrographolide was observed during the flowering stage of the plant. The synthesis of secondary metabolites is also dependent on plant age and developmental stage (Maffei *et al.*, 1989; Zheng, 1982). Andrographolide content per plant was maximum in plants inoculated with *Gi. albida* which confirms host preference by AM fungi. Host preference has been reported in many forest tree species (Rajan *et al.*, 2000; Lakshmipathy *et al.*, 2003) but few medicinal plant species (Earanna, 2001; Gracy and Bagyaraj, 2005). In the present study the concentration of andrographolide varied with treatments and was independent of P level, which suggests that colonization rather than nutritional status is responsible for increase in secondary metabolites of the plant which confirm earlier reports (Copetta *et al.*, 2006; Khaosaad *et al.*, 2006). Also colonization of plant roots by AM fungi has been shown to induce the accumulation of secondary metabolites arising from phenolic and terpenoid metabolism (Tang *et al.*, 2000; Krishna *et al.*, 2005). Other studies showed AM fungus-induced accumulation of abscisic acid (Danneberg *et al.*, 1993) and of a yellow "C14 carotenoid" ("mycorradicin") in maize (Klingner *et al.*, 1995) and other graminaceous plants (Klingner *et al.*, 1995b). A recent study showed that AM symbiosis enhances the biosynthesis of several economically useful secondary metabolites like carotenoids and terpenes in annual and perennial plants (Zhi-Lin *et al.*, 2007). Freitas *et al.* (2004) also observed that inoculation with AM fungi increased essential oil and menthol content in mint.

In the present study *Gi. albida* was found the most efficient AM fungal inoculum for *A. paniculata* cultivation. Arbuscular Mycorrhizal symbiosis induces changes in the accumulation of secondary compounds, some of them acting as signal molecules (Akiyama and Hayashi, 2002; Smith *et al.*, 2006). Enhancement of secondary product accumulation in medicinal plant is of great importance in the medicinal plant cultivation industry. Secondary metabolite accumulation in plants in the course of plant-symbiotic fungal interaction definitely impels the development of attractive strategies to bring medicinal plants cultivation into a new era for pharmaceutical purpose.

SUMMARY

Medicinal plants are known as sources of phytochemicals and active compounds, which are widely used for their natural properties. A few attempts have demonstrated the association of AM fungi with medicinal plants but the effect of AM fungi on the medicinal values received little attention. Relatively little is known about the effects of AM fungal colonization on the accumulation of active phytochemicals in shoots of medicinal plants, which are often the harvest products. The present study was undertaken to evaluate AM fungal diversity in medicinal plants, phenology and effects on growth and secondary metabolite concentration.

The work carried out in the present study can be summarized as follows:

A survey of AM fungal status in medicinal plant species of Western Ghats, Goa region was undertaken. Studies on soil characteristics at the study sites revealed differences in soil properties in macro- and micro-nutrients. Soil pH was acidic and was also deficient in available P at both the study sites. The micronutrients also exhibited variations at both the sites.

Mycorrhizal colonization was recorded in 30 out of 36 medicinal plant species selected for the study. The AM colonization was characterized by arbuscules and/or vesicles and intraradical hyphae. Hyphal and vesicular colonization was observed in 16 plant species whereas four plant species exhibited arbuscular colonization. Variation in percent colonization was observed throughout the season. Higher root colonization levels were recorded during pre monsoon and least in monsoon season.

Studies on AM fungal diversity and abundance revealed a rich diversity of AM fungal species associated with medicinal plant species. No host specificity was recorded in the present study. Forty-two AM fungal species belonging to five genera viz., *Glomus*, *Acaulospora*, *Scutellospora*, *Gigaspora* and *Ambispora* were recovered from the rhizosphere soil. Wild medicinal plant species showed higher spore diversity than cultivated medicinal plants. *Glomus* was the most dominant genera and *G. fasciculatum* the most dominant species. Negative correlation was observed between percent colonization and spore density. Studies on seasonal variations in spore density recorded higher mean spore density during monsoon and least during post monsoon period. Maximum species richness was recorded in *P. nigrum* where eight AM fungal species belonging to three genera were recovered. Diversity studies showed less variation, indicating a stable and a diverse AM fungal community.

Seven out of 42 AM fungal species was successfully multiplied using trap cultures with *Coleus* sp. as host plant includes *Gi. albida*, *A. laevis*, *A. scrobiculata*, *Glomus aggregatum*, *G. fasciculatum*, *G. geosporum* and *S. calospora* which were further used for preparation of monospecific cultures.

Studies on histochemical staining of polyP and lipid bodies revealed the localization of polyphosphate granules in *Gi. albida* stained pinkish purple and lipid bodies stained blue black in *G. clarum* in roots of *Coleus* sp. Vesicles developing into intraradical spores were also observed in the root cortex of *Coleus* plant inoculated

with *G. clarum*. Formation of arbuscules was observed in roots of *Coleus* plant inoculated with *Gi. albida*.

Studies on variation in P concentration in different developmental stages of three medicinal plant species viz., *Andrographis paniculata*, *Catharanthus roseus* and *Rauwolfia serpentina* growing in wild revealed an increase in the P concentration during flowering stage of *A. paniculata* and *C. roseus* and is directly related to the presence of arbuscules as flower initiation required extra uptake of P. High P concentration was observed in plant tissues of *C. roseus*, but recorded less number of spores in the rhizosphere soil. In *R. serpentina*, no arbuscules were observed in all the growth stages and maximum P concentration was observed during vegetative stage indicating that plants require P during the early growth stage for optimum yield. Hyphal and vesicular colonization was observed in all the three medicinal plant species at different growth stages where as arbuscular colonization was observed only during the flowering stage in *C. roseus* and *A. paniculata*. Twenty AM fungal species belonging to five genera viz., *Glomus*, *Acaulospora*, *Scutellospora*, *Gigaspora* and *Ambispora* were recovered from the rhizosphere soil. *Glomus* was the most dominant genus and *G. fasciculatum*, the most dominant species and was recorded in all stages of development in the three plant species.

Studies on the effect of different AM fungal inocula on growth and percent concentration of Andrographolide in *A. paniculata* revealed a significant increase in

growth in all the treatments and unsterilized control compared to sterilized control. Plants grown in unsterilized control and inoculated with *Gi. albida* enhanced leaf number, shoot and total plant dry weights significantly compared to other treatments and sterilized control. *Andrographis paniculata* revealed high mycorrhizal dependency and efficiency when grown in unsterilized control and *Gi. albida*. Phosphorus concentration in plant tissues was observed more during the vegetative stage in *A. paniculata* on inoculation with *G. fasciculatum*. Increase in andrographolide concentration was observed on inoculation with *Gi. albida* confirms host preference in AM symbiosis and also identifies *Gi. albida* as an efficient AM fungal inocula for commercial cultivation of *A. paniculata*.

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SYNOPSIS

INTRODUCTION

India is endowed with a rich wealth of medicinal plants. Although a good proportion of the medicinal plant species do occur throughout the country, peninsular Indian forests and the Western Ghats are highly significant with respect to varietal richness (Parrota, 2001). Medicinal plants are important for pharmacological research and drug development, not only as plant constituents used directly as therapeutic agents, but also as starting materials for the synthesis of drugs or as models for pharmacologically active compounds (Mukherjee, 2003). It is reported that in India, 4,365 ethnic communities, including over one million folk healers, use around 8,000 species of medicinal plants. They are also increasingly becoming economically important due to the growing demand for herbal products in the domestic and global market.

Across the country, the forests are estimated to harbour 90% of the country's medicinal plant diversity and only about 10% of the known medicinal plants of the country are restricted to non-forest habitats. Demand for medicinal plants is increasing in both developing and developed countries due to growing recognition of natural products, being non-toxic, having no side effects and are easily available at affordable prices. Due to an increasing demand for medicinal plants and a loss and fragmentation of natural habitats, close to 300 species of Indian medicinal plants have been so far assessed as under threat in the wild (based on International Union for Conservation for Nature (IUCN) (Red List Criteria). Around 1,000 species are

estimated to be facing various degrees of threat across different biogeographic regions in the country (Seth and Sharma, 2004).

Arbuscular mycorrhizal (AM) fungi are a major component of rhizosphere microflora in natural ecosystems and play significant role in the re-establishment of nutrient cycling (Peterson *et al.*, 1985). They can modify the structure and function of plant communities (Douds and Miller, 1999) and may be useful indicators of ecosystem change (McGonigle and Miller, 1996).

Although AM fungi have not been used specifically to increase the production of medicinal compounds in plants, their ability to enhance the plant growth and root health has been demonstrated earlier in many crop species (Maier *et al.*, 1995; Van Loon *et al.*, 1998). The use of microbial association for medicinal crops provides a sustainable approach to improve crop quality and yield. It provides the potential to increase production, value and export of human health enhancing crops and products.

The Western Ghats, a valuable repository for biodiversity after the Himalayas, is one of the 34-mega diversity hot spots of the world. It contains 4000 (27%) of the country's plant species, of which 38% (1500 species) are endemic and also a treasure house of over 8,000 ethnic and endemic varieties belonging to 386 botanical families which accounts for one-fourth of the world's medicinal plants. The high biodiversity of the Western Ghats can be attributed to its varied habitat types ranging from semi-arid grasslands to tropical rainforests. In the recent years, mycorrhizal association in

several plant species from different habitat types of the Western Ghats region in Southern India and Goa have been reported (Appasamay and Ganapathi 1995; Muthukumar *et al.*, 1996; Muthukumar and Udaiyan, 2001; Khade *et al.*, 2002 and Bukhari *et al.*, 2003). However, the species diversity and composition of AM fungal communities from medicinal plants of the Western Ghats, Goa region is largely unknown. Therefore, the present work was undertaken to study the AM fungal diversity in medicinal plant species of Western Ghats of Goa region.

AIMS AND OBJECTIVES:

1. To study root colonization of AM fungi associated with the medicinal plants selected for the study.
2. To isolate, identify and study the diversity of AM fungal spores from the rhizosphere soil of medicinal plants.
3. To identify dominant indigenous AM fungi and their multiplication in the roots of compatible host in pot cultures.
4. To study the histochemical localization of polyphosphate granules and lipid bodies in intraradical mycelium of selected AM fungal species.
5. To investigate mycorrhizal status of medicinal plants selected for the study as influenced by phenology.
6. To study the response of selected AM fungal species on growth of selected medicinal plant species.

METHODOLOGY

1. Roots and rhizosphere soil samples of selected medicinal plant species were collected from different localities in north and south Goa of Western Ghats region.
2. Assessment of AM fungal colonization was carried out in roots of selected plant species by trypan blue staining method (Koske and Gemma, 1989).
3. Quantification of AM fungal colonization in roots was carried out using slide method (Giovannetti and Mosse, 1980).
4. Arbuscular Mycorrhizal fungal spores were isolated by wet sieving and decanting technique (Gerdemann and Nicolson, 1963; Muthukumar *et al.*, 1996) and quantification of spore density was carried out as described by Gaur and Adholeya, (1994).
5. Trap cultures of the above isolated AM fungal spores were carried out by open pot cultures (Gilmore, 1968) using *Coleus* sp. as host plant.
6. Taxonomic identification of intact and unparasitized AM fungal spores was carried out by using various bibliographies (Almeida and Schenck, 1990; Bentivenga and Morton, 1995; Walker and Vestberg, 1998; Redecker *et al.*, 2000; Morton and Redecker, 2001) and INVAM (International Culture collection of Vesicular Arbuscular Mycorrhizal Fungi) (<http://invam.caf.wvu.edu>).
7. Diversity studies were carried out using Simpsons Diversity Index, (Simpson, 1951), and Shannon Wiener Index (Weaver and Shannon, 1949).

8. Histochemical analysis were carried out for the localization of polyphosphate granules and lipid bodies using histochemical stains *viz.*, Toluidine blue O (TBO) (Kumble and Kornberg, 1996), and Sudan Black (McGee-Russell and Smale, 1963).
9. Estimation of phosphorus (P) concentration (ppm) in selected medicinal plant species was carried out using Vanadomolybdate phosphoric yellow colour method (Chapman and Prat, 1961) following dry ash digestion procedure.
10. Mycorrhizal dependency (RMD) (Plenchette *et al.*, 1983) and Mycorrhizal Efficiency Index (MEI) (Bagyaraj, 1994) were calculated for growth response studies in selected medicinal plant species.
11. Estimation of andrographolide, the main secondary metabolite in *Andrographis paniculata* was carried out using HPLC analysis (Pholphana *et al.*, 2004)

OBSERVATIONS:

The first chapter deals with study of AM fungal association in selected wild and cultivated medicinal plants growing in the Western Ghat area of Goa State. The objective of this study was to survey the medicinal plants for AM fungal association. In all, a total of 36 plant species from north and south Goa areas of Western Ghats were selected for the study. A total of 30 plant species were found to be mycorrhizal

and characterized by the presence of hyphae, vesicles and arbuscules. Absence of colonization was observed in *Commelina benghalensis*, *Physalis minima*, *Adathoda vasica*, *Murraya koenigii*, *Piper nigrum* and *Euphorbia pulcherrima*. Maximum root colonization was recorded in *Azadirachta indica* and *Cajanus* sp. (100%) while it was minimum in *Alpinia galanga* (8.33%).

The second chapter deals with diversity of AM fungal species in selected medicinal plants growing in the Western Ghat areas of Goa State. The objective of this study was to record the spore density and AM fungal diversity in medicinal plants. Forty-two AM fungi belonging to five genera viz., *Glomus*, *Gigaspora*, *Acaulospora*, *Scutellospora* and *Ambispora* were recovered from the rhizosphere soil samples. No significant positive correlation was found between percent colonization and spore density. Spore density varied from 1197 spores (*Hemidesmus indicus*) to 14 spores (*Eclipta alba*). Simpson's and Shannon Weiner Diversity Index studies carried out in north and south Goa exhibited less variations in both the sites indicating a stable and a diverse plant community.

The third chapter deals with taxonomy of AM fungi associated with medicinal plants. The objective of this study was to identify the dominant AM fungal species. The study revealed that *Glomus* was the most dominant genera with *Glomus fasciculatum*, as the most dominant species followed by *A. scrobiculata*. Trap cultures of AM fungal spores were prepared using pot cultures with *Coleus* sp. as host plant.

In all, a total of seven AM fungal species viz., *A. laevis*, *A. scrobiculata*, *Glomus aggregatum*, *G. fasciculatum*, *G. geosporum*, *Gigaspora albida* and *Scutellospora gregaria* were recovered from trap culture which were later used to prepare monospecific cultures.

The fourth chapter deals with histochemical localization of polyphosphate granules and lipid bodies in the intraradical mycelia of two selected AM species. The objective of this study was to locate the accumulation of polyP granules and lipid bodies in the intercellular hyphae in roots of *Coleus* sp. inoculated with monospecific cultures of *Gigaspora albida* Schenck & Smith and *Glomus clarum* Nicolson & Schenck. Accumulation of polyP granules was located in the intercellular hyphae in roots inoculated with *Gi. albida* which stained pinkish purple in Toluidine blue O at pH 1. The study revealed accumulation of lipid bodies in the form of droplets of varying sizes in the intraradical hyphae and spores of *G. clarum*, which stained bluish black in Sudan Black.

The fifth chapter deals with AM fungal status of medicinal plants as influenced by its phenology. The objective of this study was to record the variations in sporulation of AM fungi, and to study P concentrations in three herbaceous medicinal plants viz., *Rauwolfia serpentina* Benth, *Catharanthus roseus* L. and *Andrographis paniculata* Nees growing in wild. Results revealed that all the plant species were colonized by AM fungi but varied in the extent of colonization and

sporulation during different growth stages. Twenty AM fungal species were identified from the rhizosphere soil samples. *Glomus fasciculatum* followed by *G. geosporum*, *G. maculosum* and *A. scrobiculata* were found to be the dominant AM fungal species in terms of relative abundance and frequency of occurrence at different growth stages. The results of the present study revealed that increase in the P concentration during flowering stages of *A. paniculata* and *C. roseus* is directly related to the presence of arbuscules as flower initiation required extra uptake of P.

The sixth chapter deals with the response of AM fungal inoculation on the growth of *Andrographis paniculata*, an important medicinal plant. The experiment consisted of seven treatments viz., Un-inoculated control (sterilized soil), Un-inoculated control (unsterilized soil) viz., *Scutellospora biornata* Spain, Sieverding & Toro, *Acaulospora scrobiculata* Trappe, *Gigaspora albida* Schenck & Smith, *Scutellospora calospora* (Nicolson & Gerdemann) Walkers & Sanders and *Glomus fasciculatum* (Thaxter) Gerdemann & Trappe emend. Walker & Koske. The study revealed that plants grown in unsterilized soil showed high Mycorrhizal Dependency (MD), and Mycorrhizal Efficiency Index (MEI) followed by *Gi. albida*, whereas the maximum concentration of andrographolide (secondary metabolite) was recorded in plants inoculated with *Gi. albida* compared to other treatments and control.

CONCLUSION:

The present study revealed a rich and stable diversity of AM fungi associated with medicinal plant species of Western Ghat areas of Goa. The study also showed an increase in the P concentration due the presence of arbuscules which corresponds to active P accumulation in wild medicinal plant species. The growth response studies in *A. paniculata* on inoculation with AM fungi enhanced plant growth and increased the concentration of secondary metabolite. The concentration of andrographolide varied among the treatments and was independent of P level and is mainly due to colonization and plant defense response induced by the presence of AM fungi and not by the nutritional status of the plant.

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Signature of the Guide

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APPENDIX

- RESEARCH PAPERS PUBLISHED
- RESEARCH PAPERS IN PRESS
- RESEARCH PAPERS PRESENTED

RESEARCH PAPERS PUBLISHED

K.P. Radhika & B.F. Rodrigues (2007). Arbuscular Mycorrhizae in association with aquatic and marshy plant species in Goa, India. *Aquatic Botany* 86: 291-294.

K.P. Radhika & B.F. Rodrigues (2007). Orchid mycorrhizal colonization in *Rhyncostylis retusa* (L.) Blume. *Mycorrhizal Newsletter* 19(3); 22-23.

RESEARCH PAPERS IN PRESS

K.P. Radhika & B.F. Rodrigues (2008). Histochemical localization of polyphosphate (Poly P) granules and lipid bodies in intraradical mycelium of two Arbuscular Mycorrhizal (AM) fungi. *Kavaka*

RESEARCH PAPERS PRESENTED

- “Studies of Arbuscular mycorrhizal fungi in Aquatic plants of Goa” in National Seminar on “Recent Advances in Mycology” in Mangalore University, Mangalore (December 2-3, 2004).
- “Studies of Arbuscular mycorrhizal association in some commonly occurring medicinal plants of Goa” in National Seminar on “Recent trends in Plant Sciences” organized in Smt. Parvatibai Chowgule Cultural Foundation’s College of Arts and Science, Margao, Goa (February 23-24, 2005).

- “Arbuscular mycorrhizal (AM) fungal studies in commonly occurring medicinal plants of Goa” for Global Conference II entitled “Plant Wealth-Global Wealth” organized by Indian Society of Mycology and Plant Pathology, (ISMPP) & Maharana Pratap University of Agriculture & Technology, Udaipur (November 25 - 29, 2005).
- “Growth and biomass studies in ectomycorrhizal fungus -*Pisolithus albus* (Cook & Masee) Priest” for 2nd Asian Conference of Mycology and Plant Pathology organized by Indian Society of Mycology and Plant Pathology, (ISMPP) & Osmania University, Hyderabad (December 19-22, 2007).
- “Arbuscular Mycorrhizal (AM) fungi associated with wild medicinal plants exhibit variation in phosphorus concentration during growth developmental stages” for Asian Conference of Mycology and Plant Pathology organized by Indian Society of Mycology and Plant Pathology (ISMPP) and Mysore University, Mysore (November 19-22, 2008).



Short communication

Arbuscular Mycorrhizae in association with aquatic and marshy plant species in Goa, India

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Abstract

Of the 20 plant species of hydrophytes screened for Arbuscular Mycorrhizal (AM) fungal root colonization, 10 aquatic plants (out of 14 species) and five marshy plants (out of six species) were found to be mycorrhizal, while the remaining species were non mycorrhizal. Vesicular colonization occurred in 12 plant species while arbuscular colonization was restricted to only three plant species. A rooted submerged pteridophyte viz., *Isoetes macrospora* L. was found to be mycorrhizal exhibiting vesicular colonization. In all, two genera viz., *Glomus* and *Scutellospora*, the former being dominant, were recorded. The most common AM fungal species *Glomus claroideum* was recovered from 14 plant species.

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Keywords: Aquatic; Marshy; Hydrophytes; Plant species; AM fungi; Colonization

1. Introduction

Plant root-fungal interactions, called mycorrhizae, are found in approximately 90% of all vascular plants (Allen, 1991). Arbuscular mycorrhizae clearly play important roles in terrestrial ecosystems, such as grasslands, where they influence plant community structure and nutrient cycling (Jackson and Mason, 1984). Since AM fungi require oxygen to thrive and since many wetland plants have been described as non-mycorrhizal (Mosse et al., 1981; Anderson et al., 1984; Mejsstrik, 1984), it has been assumed that AM fungi have little significance in wetland ecosystems. However, recent field studies show that AM fungi exist in wetlands and colonize many hydrophytic plants (Brown and Bledsoe, 1996; Cooke and Lefor, 1998; Turner and Friese, 1998; Cantelmo and Ehrenfeld, 1999; Thormann et al., 1999; Turner et al., 2000). Arbuscular mycorrhizal (AM) fungi have been found in roots of submerged macrophytes (Clayton and Bagyaraj, 1984; Tanner and Clayton, 1985), salt marsh plants (Rozema et al., 1986; Van Duin et al., 1990), plants in oligotrophic wetlands (Sondogaard and Laegaard, 1977), wetland woody species (Keeley, 1980; Lodge, 1989), plants in prairie potholes (Wetzel and van der Valk, 1996) wetland plants in Everglades (Azi et al., 1995) and

plants in recently rehabilitated wetlands (Turner and Friese, 1998). Nine of 49 species examined in aquatic habitats of Denmark were found to be mycorrhizal (Beck-Nielsen and Vindaek, 2001).

The association between AM fungi and aquatic plants may mediate co-existence of aquatic plant species and keep balance of the hydrophytes community as in terrestrial ecosystems (Hart et al., 2003). The primary abiotic factors known to influence the abundance and distribution of AM fungi in aquatic ecosystems are water, nutrient and oxygen availability (Read, 1991). One of the main functions and ecological roles of AM fungi is providing enhanced phosphorus nutrition to plants; thus the effect of soil available phosphorus is often assessed in wetland ecosystems (Smith and Read, 1997). The AM fungal colonization of wetland plants may be particularly dependent on the interaction between plant phenology and soil wetness (Miller, 2000). One of the recently accepted theories of arbuscular mycorrhizae suggest that these fungi were influential in colonizing land by aquatic plants, based on fossil evidence from Rhynie chert (Smith and Read, 1997). This paper presents a study of the AM fungal diversity in plants growing in aquatic and marshy habitats of Goa.

2. Materials and methods

Fourteen aquatic plant species and six marshy plant species were collected from three localities viz., Santa Cruz, Taleigao

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d Vasco of Goa State during the period July 2004–September 2004. Roots were gently washed and fixed in FAA (Formalin–acetic acid–Alcohol) and transported to the laboratory for processing. Fixed roots were washed free of FAA, and cut into 2-cm root bits, cleared in 10% (w/v) KOH at 90 °C for 30-min, thoroughly rinsed in water, acidified with 5 N HCl and cleared in acetic glycerol containing 0.5% (w/v) trypan blue and observed under light microscope for the presence or absence of AM colonization (Phillip and Hayman, 1970).

Trap cultures were established by using roots and rhizosphere soils except for free floating plants where only roots were used. The soil samples were mixed with autoclaved sand in a ratio of 2 (v/v) and filled in 15-cm diameter pots. Pots were planted with cuttings of *Coleus* sp. and watered regularly. After three months, the plants were cut at the soil surface and replanted with the same host. The plants were allowed to grow for an additional three months, at which time they were sampled for determination of AM fungal species. The spores isolated from the trap cultures using the wet sieving and decanting procedure (Gerdemann and Nicolson, 1963) were used to identify the AM fungal species. Intact and crushed spores in PVLG (Poly vinyl alcohol–lactic acid–glycerol) solution (Koske and Tessier, 1983) with or without Melzers reagent were examined under Leica compound microscope and identified based on spore morphology and subcellular characters and compared with original descriptions (Schubert and Perez, 1990; Almeida and Schenck, 1990; Redecker et al., 2000; Morton and Redecker, 2001; Schüßler

et al., 2001). Spore morphology was also compared with the culture data established by International Collection of Vesicular Arbuscular Mycorrhizal Fungi (<http://invam.cag.wvu.edu>). Voucher specimens of AM fungi have been retained in the Botany Department, Goa University, Goa, India.

3. Results

In the present study, a total of 20 species of aquatic and marshy plants were surveyed for AM fungal root colonization. Out of the total 20 plant species, root colonization of AM fungi was recorded in 16 species. Vesicular colonization was the most prevalent type and was recorded in 12 species (Table 1). In rooted plants with floating leaves, *Marsilea quadrifolia* showed the presence of vesicular colonization whereas no AM fungal root colonization was observed in *Nymphaea stellata*. The free-floating plant species viz., *Pistia stratiotes* and *Salvinia natans* exhibited vesicular colonization. The two submerged floating plants viz., *Najas minor* and *Ceratopteris thalictroides* did not show any AM fungal root colonization. Rooted submerged plants viz., *Blyxa echinosperma*, *Isoetes coromandelina* (a peridophyte), *Eriacaulon cinereum*, *Rotala malampuzhensis* and *Rotala densiflora* recorded the presence of AM fungal colonization; whereas *Scripus lateriflorus* (Cyperaceae) recorded the absence of fungal root colonization. The rooted emergent plants viz., *Lymnophila indica* and *Monocoria vaginalis* showed vesicular colonization in the roots. Out of

Table 1
Arbuscular Mycorrhizal fungal colonization and AM fungal species identified in aquatic and marshy plants

no.	Hydrophytes	Family	Locality	AM fungal colonization	AM fungi identified
Rooted submerged plants					
1	<i>Blyxa echinosperma</i> (Clarke) Hook.f.	Hydrocharitaceae	Taleigao	H V	<i>Glomus claroideum</i> , <i>Scutellospora</i> sp.
2	<i>Eriacaulon cinereum</i> R. Br.	Eriocaulaceae	Taleigao	H V	<i>Glomus claroideum</i>
3	<i>Isoetes coromandelina</i> L.	Isoetaceae	Taleigao	H V	<i>Glomus claroideum</i>
4	<i>Rotala malampuzhensis</i> Nair ex Cooke.	Lythraceae	Taleigao	H A	<i>Glomus claroideum</i>
5	<i>Rotala densiflora</i> (Roth) Koehne	Lythraceae	Taleigao	H A	<i>Glomus claroideum</i>
6	<i>Scripus lateriflorus</i> Gmel.	Cyperaceae	Taleigao	-	-
Rooted emergent plants					
7	<i>Lymnophila indica</i> (L.) Druce	Scrophulariaceae	Santacruz	H V	<i>Glomus claroideum</i>
8	<i>Monocoria vaginalis</i> (Burm. f.) Persl. ex Kunth	Pontederiaceae	Taleigao	H V	<i>Glomus claroideum</i>
Rooted plants with floating leaves					
9	<i>Marsilea quadrifolia</i> L.	Marsiliaceae	Santacruz	H V	<i>Glomus</i> sp.
10	<i>Nymphaea stellata</i> Willd.	Nymphaeaceae	Taleigao	-	-
Submerged floating plant					
11	<i>Najas minor</i> (Pers.) All Fl.	Najadaceae	Santacruz	-	-
12	<i>Ceratopteris thalictroides</i> L.	Ceratophyllaceae	Taleigao	-	-
Free-floating plants					
13	<i>Pistia stratiotes</i> L.	Araceae	Vasco	H V	<i>Glomus claroideum</i> , <i>Scutellospora verrucosa</i>
14	<i>Salvinia natans</i> Allioni.	Lamiaceae	Santacruz	H V	<i>Glomus claroideum</i>
I. Marshy plants					
5	<i>Drosera indica</i> L.	Droseraceae	Taleigao	H V	<i>Glomus claroideum</i>
16	<i>Lindernia ciliata</i> Cols (Penn).	Veronicaceae	Taleigao	H V	<i>Glomus claroideum</i>
17	<i>Ludwigia parviflora</i> Roxb.	Onagraceae	Taleigao	H V	<i>Glomus claroideum</i>
18	<i>Murdannia semeteres</i> Dalz.	Commelinaceae	Taleigao	H V	<i>Glomus claroideum</i>
19	<i>Utricularia reticulata</i> Smith.	Lentibulariaceae	Taleigao	-	-
20	<i>Centella asiatica</i> (L.) Urb.	Apiaceae	Santacruz	H A	<i>Glomus claroideum</i>

Hyp: A: Arbuscular and V: vesicular, (-): absent.

a total six marshy plant species, five species viz., *Drosera indica*, *Ludwigia parviflora*, *Murdania semeteris*, *Centella asiatica* and *Lindernia ciliata* showed the presence of AM fungal colonization. Except for *C. asiatica*, the remaining four marshy species exhibited vesicular colonization.

Four species of AM fungi belonging to two genera viz., *Glomus* and *Scutellospora* were recovered in the pot cultures prepared from 10 hydrophytes and five marshy plant species (Table 1). *Glomus* was the dominant genus and was recovered from pot cultures prepared from all the 15 plant species. The genus *Scutellospora* was recovered from two hydrophytes viz., *B. echinosperma* and *P. stratiotes*. *Glomus claroideum* was the most dominant species colonizing a total of 14 plant species.

4. Discussion

The present study confirms the AM association in hydrophytes. Colonization of AM fungi was indicated by the presence of darkly stained vesicles and arbuscules in the roots. In this study, 71.43% of aquatic plants and 83.33% of marshy plants were found to be arbuscular mycorrhizal. The present study confirms the earlier reports that arbuscules are rare in hydrophytes (Cantelmo and Ehrenfeld, 1999). The study also confirms the earlier findings of Bagyaraj et al. (1979) who reported the absence of mycorrhizal root colonization in *Nymphaea stellata*. However, Firdaus-e-Bareen (1990) reported AM fungal root colonization in *N. stellata* and also in free floating plant *P. stratiotes*. Free floating plant species viz., *P. stratiotes* and *S. natans* and rooted emergent species viz., *Lymnophila indica* and *M. vaginalis* accounted for 100% AM fungal colonization. An explanation for the increased colonization of emergent and free floating plants might be that in the present study these four species were collected from shallow water where there was a relatively high redox potential in the sediments compared the deeper part of water and a potentially greater photosynthetic efficiency of the host. Higher photosynthetic efficiency of the host and redox potential were considered respectively to facilitate the colonization of AM fungi indirectly and directly (Khan, 1993; Wigand et al., 1998). Arbuscular mycorrhizal colonization in *M. quadrifolia* was also reported by Iqbal et al. (1988) and Firdaus-e-Bareen (1990). The observations recorded in the present study are contradictory to the findings of Dharmarajan et al. (1993) who reported the absence of AM fungal colonization in *L. parviflora*. Many common wetland plant families, most notably the Cyperaceae had been previously categorized as non-mycorrhizal because in few species roots were observed to harbor the fungus (Powell, 1975; Newman and Reddell, 1987; Brundrett, 1991; Peat and Fitter, 1993; Smith and Read, 1997), but there have been numerous reports of mycorrhizal colonization in certain Cyperaceae species (Koske et al., 1992; Wetzel and van der Valk, 1996; Lovera and Cuenca, 1996). Variation in the mycorrhizal condition in Cyperaceae may be due to environmental and edaphic factors rather than a phylogenetic constraint per se (Read, 1984), because AM colonization seems to be negatively correlated with soil moisture (Anderson et al., 1984). It has been speculated that

non-mycorrhizal state of some Cyperaceae members might result from their presence in marshy, anaerobic soils rather than their taxonomic position (Tester et al., 1987).

A relatively few species of AM fungi were found to be associated with the plant species selected for the study. *Glomus* species were predominant among two genera viz., *Glomus* and *Scutellospora* identified from rhizosphere soil, of which *Glomus claroideum* was the most dominant accounting for 70% of total AM fungal species. This could be due to the fact that the number and type of AM fungal spores will depend on both the fungal species (Tommerup and Abbott, 1981) and the extent of root colonization (Douds and Schenck, 1990).

This study also confirms the fact that AM fungi are an important part of aquatic community but they are less frequently than the terrestrial plants. This may be attributed to the poorly developed root system and also due to the fact that nutrients can directly be absorbed from water by root as well as shoot system of the plant. Although the roles of AM fungi are still not fully understood in aquatic and marshy environments, the results from this study imply that AM associations are functional in and are a significant component of aquatic plant communities mainly for plant competition, succession and diversity in fens and marshes (Newman and Reddell, 1988; van der Heijden et al., 1998; Carvalho et al., 2001; Facelli and Facelli, 2002). Because of these ecological implications, AM associations and the seasonal dynamics of AM colonization should be considered in plans for the ecological restoration of functional wetlands and should be a significant component of studies assessing aquatic ecosystem dynamics.

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Orchid mycorrhizal colonization in *Rhyncostylis retusa* (L.) blume

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Introduction

The family Orchidaceae is the most species-rich plant family in the world, with an estimated 17 500 to 35 000 species, and a circum global distribution (Dressler 1993). It is also one of the most advanced plant families with many adaptations that enable long-term survival. All orchids have an obligate relationship with mycorrhizal symbionts during seed germination, with most of the symbionts being *Rhizoctonia*-like fungi (Arditti 1992). Reliance on mycorrhizal interactions is an adaptive mechanism that has allowed orchids to persist in relatively less ideal habitats, and has led to their occurrence worldwide.

Understanding mycorrhizal symbiosis is of great importance, as the fungal symbionts may play a key role in determining orchid distribution and diversity. The relationship of orchids with fungi is relatively unique in the plant kingdom. Orchid species vary in their fungal specificity, both among genera and over the course of their life cycle. Orchid mycorrhizal fungi are found intracellularly in cells of the cortex, and are

confined to the roots (Hadley 1982). Within the cells, the mycorrhizae form dense coils of mycelium called pelotons, which are considered as adaptations to the host cell (Hadley 1982). Ecologically, orchid mycorrhizae are important in that they allow the plant to have two possible sources of nutrition, and the plant may use fungal carbohydrates to supplement, replace or alternate with its own photosynthetic activity. Since the fungal partner is generally able to break down complex organic materials, the orchids that grow with them are able to tap unusual substrate for nutrients, including bog peat, highly calcareous soils, and dust debris or tree branches.

Rhyncostylis retusa (L.) Blume, commonly known as foxtail orchid, is a medium-sized species, found in deciduous and dry lowland forests, and savanna-like woodlands with stout, repent, short stem, curved, fleshy, ligulate, deeply channelled, and apically retuse leaves. It blooms on a 60-cm long axillary pendant called racemose, and is densely flowered (100-140), with small, magenta and white flowers (2 cm in diameter) with spots, in cylindrical inflorescence. In

Methodology

Epiphytic orchid *R. retusa* (L.) Blume collected from Mollem area (North Goa) was brought to the laboratory, and velamen roots were processed for mycorrhizal colonization. The roots of the orchid plant species were washed thoroughly in water, cut into 1-cm fragments, and analysed for mycorrhizal association, using the method described by Phillips and Hayman (1970). The presence of mycorrhizal fungi in the cortical cells was examined under a light compound microscope.

Observations and discussion

The roots of the epiphytic orchid *R. retusa* are consistently and heavily colonized by pelotons (Figure 1), representing a potentially substantial source of carbon and other nutrients. Within the cells, hyphae form coils called pelotons, which greatly increase the interfacial surface area between orchid and fungi. A membrane and an interfacial matrix material surround these pelotons (Peterson, Uetake, and Zelmer 1998). Each intracellular peloton has a short life span, lasting only a few days, before it degenerates and is digested by the orchid cell. In fact, hyphae have short life span; the older hyphae develop large vacuoles and thick cell walls, and the cytoplasm degenerates. The hyphal cells eventually collapse, and are consumed by the orchid cell. During this process, the plant cell remains functional, and can be recolonized by any surviving hyphae, or by fungi invading from adjacent cells.

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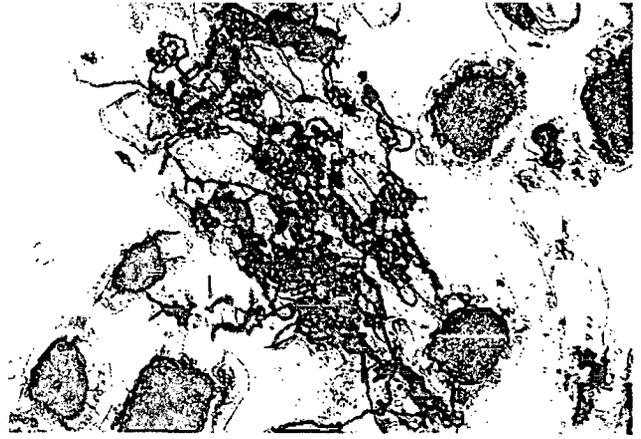


Figure 1 Orchid mycorrhizal fungi in *Rhyncostylis retusa* with pelotons in the cortical cells of the roots (400×)

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