

**STUDIES ON THE ARBUSCULAR MYCORRHIZAL FUNGI
ASSOCIATED WITH *MICHELIA CHAMPACA* L. AND THEIR
POTENTIAL ON ITS GROWTH**

(ABSTRACT)

BY

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Abstract

Arbuscular mycorrhizal fungi (AMF) are associated with majority of land plants in a symbiosis. The economic and ecological importance of these ancient biotrophic plant symbionts is therefore obvious. AMF transfer inorganic nutrients and water to the plant and receive carbohydrates in exchange. By driving this bidirectional nutrient transport between soil and plants, they are highly relevant for global phosphorus (P), nitrogen (N) and CO₂ cycles. Moreover, they affect directly and indirectly the diversity and productivity of land-plant communities by their central role at the soil–plant interface. They can also improve host plant pathogen resistance and drought stress tolerance (Krüger *et al.*, 2009).

Generally, several plant species have consistently high levels of mycorrhiza, some have intermediate, or variable levels of mycorrhiza or numerous are not mycorrhizal. The obligate AMF associations are typical of plants with coarse, slow growing, long-lived, and relatively thick roots (Brundrett, 2002). The primitive angiosperm and all the gymnosperms had short, thick fine roots, which had lower specific root length (Pregitzer *et al.*, 2002). Plants in the Magnoliaceae are considered part of the ancestral angiosperm complex. The family Magnoliaceae *sensu stricto* comprises approximately 240 species of evergreen and deciduous trees and shrubs which are distributed widely in the tropical, subtropical and temperate zones of southern and eastern Asia, southeastern North America, and northern South America (Shi *et al.*, 2000). The coarse structure of the root typified by the order Magnoliales are especially dependent on AMF for mineral uptake (Baylis, 1975).

There were few preliminary assessment of AMF and its morphology was examined in a few species from Magnoliaceae. However, there is lack of report on studies of species having coarse root structure from Magnoliaceae between non-mycorrhizal and mycorrhizal plants. Keeping in view the inadequacy, we have investigated the plants with the following objectives: (i) Assessment of arbuscular mycorrhizal colonization in *Michelia champaca* L. (ii) Diversity of arbuscular mycorrhizal fungi from the plantations of *M. champaca*. (ii) Isolation, characterization and identification of the arbuscular mycorrhizal fungi associated with *M. champaca*. (iii) Determination of the potential of the isolated arbuscular mycorrhizal fungi on growth of *M. champaca*. (iv) Root structure characteristics of *M. champaca* in association with arbuscular mycorrhizal fungi. (v) Quantification of root cortical cell and arbuscular mycorrhizal fungal colonization in *M. champaca*.

The findings of the study are summarized below:

- The assessment of mycorrhizal colonization conducted in the seedlings of *M. champaca* collected from nursery. The roots were stained with Trypan Blue, Acid Fuchsin and Stamp pad ink for the quantification of colonization. Among, all the stains used, the root was found to predominantly colonization by AMF. Nevertheless, stamp pad ink was found to be appropriate as other standard stains used normally for colonization assessment. The ink was found to be suitable and effective on this species as compared to standard methods. This is the first kind of report on the use of stamp pad ink as the staining reagent. The method with black stamp pad ink still further reduces health risks. The low cost availability of this dye is also reliable to carry out experimental research and teaching exercises in the developing country like

India. Moreover, this very simple technique often results in high quality images of visible competitive interactions at early stages of the plant growth by arbuscular mycorrhizal fungal colonization and symbiosis that may be used in a routine way for staining of other root colonizing fungi in different plant species.

- AMF and dark septate endophyte (DSE) colonization were investigated in three different plantation sites (Umdihar, Umsaw and Mawlein) of Meghalaya, northeast India. Isolation and identification of the AMF spore were conducted to evaluate the AMF diversity and host preference in terms of AMF species distribution and abundance in the plantation sites. Results showed that AMF colonization was significantly higher than dark septate endophyte colonization ($p < 0.05$). AMF and DSE colonization had a narrow range of colonization, varying from 50.91%–58.95% and 1.84%–4.11%, respectively. Spore density varied significantly in all the sites ($p < 0.05$). Out of 29 species identified from 7 genera, the species from *Glomus* was found to be highly abundant. Sorenson coefficient (Cs) ranged from 0.35–7.0. Species richness varied from 2.0–2.9 in the sites. Total species richness was significantly correlated with total relative abundance ($p = 0.001$). The distribution, abundance and principal component analysis plot suggest that *Glomus macrocarpum*, *G. multicaulis*, *G. constrictum* and *Acaulospora* sp 1 were the most host preferred species which possibly may favour the host with proper nutrient acquisition and growth.
- To evaluate the efficacy of native isolate of AMF, there was need to isolate from natural soil. Therefore, the trap plant culture was established with *Paspalum notatum* to isolate AMF. The single spore culture was found to possess successfully in case of

Glomus sp 1 in the first cycle. Nevertheless, in the first cycle of spores in *Glomus* sp 2 was found to be contaminated by a different morphotype of spore varying in 50-80 µm in size. However, the second cycle showed single morphotype of *Glomus* sp 2. The transfer of both the types of spore in the third cycle in large pots was found to contain pure monospecific spores and sporulation was in abundance. The density of spores in the third cycle was 752 spores of *Glomus* sp 1 per 25g of soil and 962 spores of *Glomus* sp 2 per 25g of soil. The trap culture technique used for isolation of AMF spore reveals two morphotypes: *Glomus* sp 1 and *Glomus* sp 2.

- In the present investigation, attempts were also undertaken to screen and select an efficient AMF for inoculating *M. champaca* for its propagation. For this reason, *Glomus* sp 1, *Glomus* sp 2 and *Glomus intraradices* were used to evaluate the efficacy of the isolate. There was significant influence of *G. intraradices* and *Glomus* sp 1 inoculation on the shoot dry weight, shoot length, and leaf area of *M. champaca* after 180 days of inoculation. Overall, there was no effect on AMF inoculation on the root dry weight, root/shoot dry weight ratio, roots length, stem girth, root to shoot ratio and number of leaves of the plants. There were significant differences between mycorrhizal (*G. intraradices* and *Glomus* sp1) and non-mycorrhizal plants in shoot dry weight, shoot length, and leaf area. *G. intraradices* exhibit more significant differences in growth parameters than *Glomus* sp1. *Glomus* sp 2 did not show significant variation in growth parameters than non-mycorrhizal ones.
- There was significant influence of *G. intraradices* inoculation on the shoot dry weight, shoot length, stem girth, leaf area and number of leaves after 270 days of inoculation. Moreover, there was significant influence of *Glomus* sp 1 inoculation on

the shoot dry weight, shoot length, stem girth, leaf area, root dry weight and root/shoot ratio of *M. champaca* after 270 days of inoculation. However, there was no effect on AMF inoculation on the roots length of the plants. There were significant differences between mycorrhizal (*G. intraradices* and *Glomus* sp1) and non-mycorrhizal plants on the shoot dry weight, shoot length, stem girth, leaf area root dry weight and root/shoot dry weight ratio. *G. intraradices* exhibit more significant differences in growth parameters than *Glomus* sp1. *Glomus* sp 2 showed significant variation in leaf area than non-mycorrhizal ones. *G. intraradices* exhibit significant higher number of leaves compared to others.

- The results suggest that of the native two species isolated, *Glomus* sp 1 influenced the growth of *M. champaca*. Moreover, the exotic species (*G. intraradices*) also improved the growth of host plant. However, *Glomus* sp 1 improved the belowground features after 270 days of inoculation whereas exotic species enhanced aboveground features.
- There were two root orders observed in both mycorrhizal and non-mycorrhizal plants observed after 180 days. There were significant differences in length and dry weight between orders of both types of plants. However, there were no significant differences in diameter of first and second order roots of non-mycorrhizal plants. Moreover, length and diameter of root orders of non-mycorrhizal plants were comparatively higher than mycorrhizal plants. In addition, there were no significant differences in the number between orders of mycorrhizal plants. However, the number of second order mycorrhizal roots was significantly higher than non-mycorrhizal roots. There were three root orders observed in both mycorrhizal and

non-mycorrhizal plants observed after 270 days. The mycorrhizal colonization in second and third orders was significantly higher than first order. However, height and diameter of first order was significantly higher than second and third orders. There was significant correlation noticed between hyphal and vesicles colonization and between height and diameter of root order.

- Roots collected from plantation sites exhibit *Paris* type of AMF morphology in all the roots examined. In addition, intermediate type of AMF morphology was also observed in few segments. Root hairs were not observed from the plantations sites. The colonization proceeds from epidermis and passes through 4th-6th layers to colonize the inner cortex. 10th-15th layers of cortex were observed where from 5th-7th layers onwards inner cortical layers colonization was observed. The colonization was found all around vascular bundle in the inner cortex.
- In the plants grown in greenhouse inoculated with AMF, root hairs were observed along with extraradical hyphae. *Paris* type AMF morphology was observed in the roots with intracellular hyphal coils; *Arum* type was detected in a few segments as well as intermediate type. 6th-8th cortical layers were found of which three layers onwards colonization was encountered. The presence of casparian strips in xylem, endodermis and exodermis is clearly shown. AMF colonization was also confined to the inner cortex surrounding the vascular tissue.
- The root order modification such as number of root order in mycorrhizal and non-mycorrhizal was conspicuous. Moreover, arbuscular mycorrhizal structure reveals three types of AMF morphology in this tree species.

- The sections revealed inner colonization of the cortex in longitudinal and transverse sections. All the three of diameter of root size reveal arbuscular mycorrhizal structure such as arbuscules, hyphal coils and hyphae. The colonization was observed to be in transverse and longitudinal section. However, outer cortical region of longitudinal section was not colonized. Highest number of cells was observed in 0.6 mm of diameter of root. Nevertheless, colonization was maximum in 0.4 mm of root segment. Total number of cell was maximum in 0.6 mm of diameter of root and total number of AMF structural colonization was maximum in 0.4 mm of root segment. Moreover, percentage of AMF structural colonization was found to be maximum in root diameter of 0.4 mm.

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**BY
PANNA DAS**

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**DEPARTMENT OF BOTANY
NORTH EASTERN HILL UNIVERSITY
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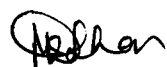
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17th FEBRUARY, 2011

I, Mr. Panna Das, hereby, declare that the subject matter of this thesis entitled “Studies on the arbuscular mycorrhizal fungi associated with *Michelia champaca* L. and their potential on its growth” is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the North Eastern Hill University, Shillong for the award of the degree of Doctor of Philosophy in Botany.


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General Introduction

The term mycorrhiza (meaning fungus-root) was coined by Frank (1885). Mycorrhizal fungi can be classified as ectomycorrhizal and endomycorrhizal depending on their morphological connection with the host. The former envelop the root with a hyphal sheathing structure and grow between root cortical cells to form the Hartig net, whereas the latter enter the root cell wall to form intracellular arbuscules (arbuscular mycorrhizal fungi) or coils (ericoid and orchid fungi). An intermediate behaviour is displayed by fungi that produce ectoendomycorrhizal colonization, i.e. a sheathing structure, a rudimentary Hartig net and intracellular structures such as pegs (monotropoid fungi) or coils (arbutoid fungi) (Smith and Read, 2008). Another type of mycorrhizal association is dark septate endophyte (DSE). It comprises of miscellaneous group of root inhabiting conidial sterile ascomycetous fungi that colonize living plant roots without causing noticeable harmful effects to the host (Jumpponen, 2001).

Arbuscular mycorrhizal fungi (AMF) are the most ancient, abundant, and ecologically important plant–fungal symbiosis on Earth (Parniske, 2008; Smith and Read, 2008). Despite their accepted keystone role in terrestrial ecosystems, ecological studies of AMF (Schüßler *et al.*, 2001; van der Heijden and Sanders, 2002) have generally been hampered by their obligate biotrophic lifestyle and complex genetics (Sanders, 2002; Smith and Read, 2008). Several studies have shown that the composition of AMF assemblages can strongly influence important ecosystem properties and processes, including plant diversity, nutrient acquisition, productivity, and soil structure (van der Heijden *et al.*, 1998; Vogelsang *et al.*, 2006; Maherali and Klironomos, 2007; Wilson *et al.*, 2009). However, there still exists a fundamental lack of knowledge concerning the functional capabilities of AMF assemblages in the field. AMF are functionally diverse at several levels of systematic integration and vary widely across a range of characters,

including nutrient uptake capabilities (Jakobsen *et al.*, 1992; Cavagnaro *et al.*, 2005), fungal growth patterns (Hart and Reader, 2002a, b; Koch *et al.*, 2004), carbohydrate requirements (Koch *et al.*, 2006), disease protection (Maherali & Klironomos, 2007), and drought stress tolerance (Michelson and Rosendahl, 1990; Aroca *et al.*, 2007).

The uptake of P by the extraradical mycelium (ERM) and its translocation to the mycorrhizal roots was stimulated and the metabolic and spatial distribution of P within the fungus was altered in response to increased carbohydrate availability. Sucrose supply resulted in a decrease of polyphosphates and an increased incorporation into phospholipids and other growth-related P pools and also caused elevated cytoplasmic P levels in the intraradical mycelium (IRM) within the root and higher cytoplasmic P levels in the root cortex (Bücking and Shachar-Hill, 2005).

AMF may transport considerable amounts of N to their host plants and they can acquire N from both mineral and organic sources (George *et al.*, 1995; Tu *et al.*, 2006; Leigh *et al.*, 2008). Root-organ studies demonstrate that extraradical mycelia of AMF are able to acquire inorganic ^{15}N (as either $\text{NO}_3\text{-N}$ or $\text{NH}_4\text{-N}$), convert it to arginine, and transport it to intraradical fungal structures where the amino acid is broken down, transported to the plant, and assimilated into plant proteins (Govindarajulu *et al.*, 2005; Jin *et al.*, 2005). The N, but not the C, from the arginine is transferred from the fungus to the host across the host–fungus interface. This mechanism transports N in a nontoxic and concentrated form (four N atoms per molecule). Govindarajulu *et al.*, (2005) showed that close to one-third of the N in root protein amino acids can be provided by symbiotic AMF. Furthermore, they suggest that N transport may be linked to polyphosphate transport, which is important because polyphosphate is the putative form of P translocated by the fungus (Smith and Read, 2008).

Mycorrhizal scavenge nutrients from soils and transfer a portion of these nutrients to their host plant in return for labile plant C. This exchange has important consequences for soil C balance, in that; mycorrhizal fungi can promote belowground storage of plant C. Indeed, in the conventional view of soil C cycling, mycorrhizal fungi are thought to primarily act as vectors for plant C input to soils. In contrast to this conventional view, there is accumulating evidence that mycorrhizal fungi may also contribute to the direct loss of soil C from ecosystems by acting as decomposers. Decomposition of soil organic matter involves two sequential processes: (i) the breakdown of polymeric organic substrates into monomers or oligomers by extracellular enzymes, and (ii) the metabolism of these small compounds and the release of CO₂ by soil microbes. However, recent experimental evidence indicates that arbuscular mycorrhizal fungi may play a larger role in organic matter decomposition than was previously assumed (Hodge *et al.*, 2001; Tu *et al.*, 2006).

In addition to their well-recognized roles in plant nutrition and communities, mycorrhizas can influence the key ecosystem process of soil aggregation. There are a suite of mechanisms by which mycorrhizal fungi can influence soil aggregation at each of these various scales. By extension of these mechanisms to the question of fungal diversity, it is recognized that different species or communities of fungi can promote soil aggregation to different degrees (Rillig and Mummey, 2006). Glomalin (Wright and Upadhyaya, 1996) is a fungal protein (or protein class) that is operationally quantified from soil as glomalin-related soil protein (GRSP). GRSP has received attention in the context of soil aggregation owing to the frequently observed correlation between GRSP and soil aggregate water stability (Wright and Upadhyaya, 1998). However, evidence linking GRSP to soil aggregation remains correlative, and the mechanisms involved are still unclear. Glomalin is hypothesized to act as 'glue' with hydrophobic properties, but

direct biochemical evidence for this is lacking. Contrary to original expectations, glomalin (in a sterile hyphal culture system) has recently been shown to be mostly tightly bound in the fungal mycelium, rather than being secreted into the medium (80% of glomalin was bound in the mycelium; Driver *et al.*, 2005). Given that it appears to not be secreted primarily, this implicates glomalin to have a role in the living fungus; functionality in the soil would then be only secondarily arising, perhaps by virtue of its relatively slow turnover rate in the environment (Steinberg and Rillig, 2003).

During development of AMF, the fungal symbionts grow out from the mycorrhizal root to develop a complex, ramifying network into the surrounding soil which can reach up to 30 m of fungal hyphae per gram of soil. This network can make up to 50% of fungal mycelium in soil thereby representing a major part of the soil microbial biomass. This mycelial network can have a binding action on the soil and improve soil structure. In addition, the secretion by AMF of hydrophobic, 'sticky' proteinaceous substances, referred to as glomalin, also contributes to soil stability and water retention. The combination of an extensive hyphal network and the secretion of glomalin is considered to be an important element in helping to stabilize soil aggregates, thereby leading to increased soil structural stability and quality (Gianinazzi *et al.*, 2010).

Abiotic stresses cause extensive losses to agricultural productivity. Mineral depletion, drought, salinity, heavy metals or heat are serious problems in many parts of the world, in particular in arid and semi arid areas. It is predicted that two thirds of cultivable land may disappear in Africa, a third in Asia and one fifth in South America by 2025 and that arable land area per inhabitant in the world will be reduced to 0.15 ha in 2050. In the USA and Spain, one third of the country is undergoing desertification. The potential of AMF to enhance plant tolerance in abiotic stress conditions has long been recognized and their manipulation in sustainable agricultural systems will be of

tremendous importance for soil quality and crop productivity under severe edapho-climatic conditions (Gianinazzi *et al.*, 2010).

To limit the spread of pests causing great yield losses in cultivated crops, conventional agriculture has been using large quantities of pesticides as well as plant breeding programmes in order to obtain disease-resistant plants. However, pesticides are often only partially effective against soil-borne diseases. Moreover, they are detrimental to human health and to the environment and as a consequence an ever-increasing number of pesticides is being taken off the market. In addition, disease resistance obtained by plant breeding programmes is often due to single plant genes, which can be rapidly overcome by evolutionary biodiversity in pathogenic agents. Complementary approaches have therefore to be developed to ensure durable tolerance of plants to pathogens. Numerous studies have demonstrated the beneficial effect of AMF in increasing plant tolerance to biotic stress caused by soil-borne pathogens interacting with many plant species. This has been consistently shown for a number of pathogenic fungi or Oomycetes such as *Fusarium*, *Rhizoctonia*, *Verticillium*, *Thielavopsis*, *Aphanomyces*, *Phytophthora* and *Pythium*, as well as nematodes from the genera *Heterodera*, *Meloidogyne*, *Pratylenchus* and *Radopholus*. Most of the research has been carried out under very controlled conditions at early stages of plant growth but a few studies conducted in the field or in the greenhouse under real production conditions confirm these results (Gianinazzi *et al.*, 2010).

Despite the enormous role of AMF in the entire terrestrial ecosystem, their biodiversity in relation to functional aspects is little understood. Most of the 214 currently described species (www.amf-phylogeny.com) are characterized only by spore morphology and the majorities have not yet been cultured. Moreover, from molecular ecological studies we know that the species described represent only a small fraction of

the existing AMF diversity (Kottke *et al.*, 2008; Öpik *et al.*, 2008). No evidence for recombination has been found in the fungi, suggesting that they reproduce clonally and have been asexual for the entire period of their association with plants (Kuhn *et al.*, 2001). Recently, AMF were placed into a new monophyletic group, the phylum *Glomeromycota*, which probably originated from the same ancestral group as the *Ascomycota* and *Basidiomycota* (Schussler *et al.*, 2001) 1,400–1,200 million years ago and is much older than the earliest land plants, which appeared ≥ 800 million years ago and whose primitive root systems were associated with ancestral AMF. AMF may, thus, have played a crucial role in facilitating the colonization of land by plants (Simon *et al.*, 1993; Remy *et al.*, 1994; Redecker *et al.*, 2000; Heckman *et al.*, 2001). It is also assumed that the ancient signaling pathways evolved in AMF symbiosis were recruited subsequently for the establishment of the evolutionary younger legume–Rhizobia nodulation symbiosis (Kistner and Parniske, 2002).

Symbiotic development results in the formation of tree-shaped subcellular structures within plant cells. These structures, which are known as arbuscules (from the Latin ‘arbusculum’, meaning bush or little tree) are thought to be the main site of nutrient exchange between the fungal and plant symbiotic partners. AMF intimately connects plants to the hyphal network of the fungi, which can be in excess of 100 metres of hyphae per cubic centimeter of soil (Miller, *et al.*, 1995). This hyphal network is specialized for nutrient (predominantly phosphate) and water uptake (Finlay, 2008). In return for supplying plants with nutrients and water, AMF obtain carbohydrates from plants (Solaiman and Saito, 1997; Bago, *et al.*, 2003). Up to 20% of the photosynthesis products of terrestrial plants (approximately 5 billion tonnes of carbon per year) are estimated to be consumed by AMF (Bago, 2000). Therefore, AMF symbiosis contributes significantly

to global phosphate and carbon cycling and influences primary productivity in terrestrial ecosystems (Fitter, 2005).

AMF development is accompanied by an exchange of signalling molecules between the symbionts. A novel class of plant hormones known as strigolactones is exuded by the plant roots. On the one hand, strigolactones stimulate fungal metabolism and branching. On the other hand, they also trigger seed germination of parasitic plants. Fungi release signalling molecules, in the form of 'Myc factors' that trigger symbiotic root responses. Plant genes required for AMF development have been characterized. During evolution, the genetic programme for AMF has been recruited for other plant root symbioses: functional adaptation of a plant receptor kinase that is essential for AMF symbiosis paved the way for nitrogen-fixing bacteria to form intracellular symbioses with plant cells (Parniske, 2008).

Several plant species generally have consistently high levels of mycorrhiza, some have intermediate, or variable levels of mycorrhiza or numerous are not mycorrhizal. The non-mycorrhizal plants normally have fine, active, extensive roots systems like those of facultative plants. Some non-mycorrhizal plants have evolved specialized root systems, such as cluster roots as well as dauciform and sand-binding roots, several different patterns of root hair production occur in eudicots and one type where hairs occur in linear files primarily in Caryophyllales and Brassicales. The principle attributes of the roots of non-mycorrhizal plants is its aptitude towards exclusion of glomalean fungi. Each type of mycorrhiza is associated with a characteristic type of root system. The obligate AMF associations are typical of plants with coarse, slow growing, long-lived, and relatively thick roots (Brundrett, 2002).

The primitive angiosperm and all the gymnosperms had short, thick fine roots, which had lower specific root length (Pregitzer *et al.*, 2002). Plants in the Magnoliaceae

are considered part of the ancestral angiosperm complex. The family Magnoliaceae *sensu stricto* comprises approximately 240 species of evergreen and deciduous trees and shrubs which are distributed widely in the tropical, subtropical and temperate zones of southern and eastern Asia, southeastern North America, and northern South America (Shi *et al.*, 2000). The coarse structure of the root typified by the order Magnoliales are especially dependent on AMF for mineral uptake (Baylis, 1975).

There were few preliminary assessment of AMF and its morphology was examined in a few species from Magnoliaceae (Muthukumar *et al.*, 2003; Kottke *et al.*, 2004; Ahulu *et al.*, 2005; Muthukumar *et al.*, 2006). The mycorrhizal and non mycorrhizal roots of *Liriodendron tulipifera* was compared for root morphology and mycorrhizal anatomy (Vozzo and Hacskeylo, 1964). The effect of a mycorrhizal isolate was evaluated on the growth and the mycorrhizal colonization in *Zea mays* and *L. tulipifera* (Gerdemann, 1965). However, there was an earlier report on *L. tulipifera* of having endotrophic and ectendotrophic mycorrhiza (Jackson and Driver, 1969). Fine root diameter, length, specific root length, and nitrogen concentration of nine North American tree species, including *L. tulipifera* was compared (Pregitzer *et al.*, 2002). The apocarotenoids in mycorrhizal roots of *Magnolia grandiflora* was analyzed along with 57 other species (Fester *et al.*, 2002). The influence of AMF inoculation on plant growth and phosphorus (P) uptake was studied in *Magnolia ovata* and other woody pioneer or late secondary species native to the Atlantic Rain Forest that are utilized to recover riparian forests (Pasqualini *et al.*, 2007).

Michelia champaca L. (Magnoliaceae) is known worldwide for its large, very aromatic yellow blossoms and the striking appearance of the tree with its smooth trunk and large ovate, glossy leaves. The species is a tropical evergreen native to India and Indonesia, extending into the Indonesia, Taiwan and also planted in Brazil (Candiani *et*

al., 2004). *M. champaca* is highly economical for perfume industries, furniture, etc. Flower buds of *M. champaca* in most of the herbal preparations for diabetes and kidney diseases. Traditionally, it is being used in fever, colic, leprosy, post partum protection and in eye disorders. It has been reported to possess antipyretic, anti-inflammatory, insecticidal, antimicrobial and leishmanicidal activities. The active constituents reported in this plant are alkaloids, saponins, tannins, sterols, flavonoids and triterpenoids (Jarald *et al.*, 2008).

However, there is lack of report on studies of species having coarse root structure from Magnoliaceae between non-mycorrhizal and mycorrhizal plants. Keeping in view the inadequacies, we have investigated *M. champaca*.

The objectives of the study are as follows:

- i) Assessment of mycorrhizal colonization in *M. champaca*.
- ii) Diversity of arbuscular mycorrhizal fungi from the plantations of *M. champaca*.
- iii) Isolation, characterization and identification of the arbuscular mycorrhizal fungi associated with *M. champaca*.
- iv) Determination of the potential of the isolated arbuscular mycorrhizal fungi on growth of *M. champaca*.
- v) Root structure characteristics of *M. champaca* in association with arbuscular mycorrhizal fungi.
- vi) Quantification of root cortical cell and arbuscular mycorrhizal fungal colonization in *M. champaca*.

Review of Literature

Bakshi (1974) was the first to publish an account of 14 spore types of arbuscular mycorrhizal fungi (AMF) in India: *Glomus macrocarpum*, *G. macrocarpum* var. *geosporum*, *G. mosseae*, *Glomus* sp., *Sclerocystis coremioides*, *Sclerocystis* sp., *Gigaspora calospora*, *Acaulospora* sp., *Endogone gigantean* *E. microcarpum*, *Endogone* 1, *Endogone* 2, *Endogone* 3. Gerdemann and Bakshi (1976) reported two new species, viz. *G. multicaule* and *Scl. sinuosa*. Inoculation of three crop plants, viz. cotton, cowpea and finger millet to inoculation with AMF, *G. fasciculatum*, in an unsterile Indian soil low in available phosphorus significantly increased their root and shoot weights (Bagyaraj and Manjunath, 1980). Bhattacharjee and Mukerji (1982) described the species *G. reticulatum* from soils of Bangalore. Bhattacharjee *et al.* (1982) reported the structure and hyperparasitism for *Gi. candida* while Bhattacharjee and Mukerji (1982) described the ultrastructure of *Scl. coremioides* sporocarp. Growth and phosphorus nutrition of finger millet on a sterile, phosphorus deficient soil was improved by inoculation with either *G. fasciculatum* or with *Streptomyces dnamomeous*. Dual inoculations stimulated plant growth less than individual inoculations (Krishna *et al.*, 1982).

Thirty-nine species and varieties of common Pteridophytes of southern Ontario were surveyed for the presence of AMF. In addition, various root-inhabiting fungi which could be confused with AMF were illustrated (Berch and Kendrick, 1982). Some 80 species of Australian Asteraceae were examined for their ability to form ectomycorrhizal associations. Twenty-seven species, all but one belonging to the tribe Inuleae, were found to form both ectomycorrhizas and vesicular-arbuscular mycorrhizas. Four species shown to form ectomycorrhizas gave significant growth responses to inoculation with either an ecto-or endomycorrhizal fungus in soil low in available phosphate (Warcup and McGee,

1983). Mukerji *et al.* (1983) reported two species of *Glomus*, viz. *G. multisubstensum* and *G. delhiense* both from soils of Delhi.

Effect of mycorrhizal colonization by *G. fasciculatum* on survival, penetration and development of the root knot nematode *Meloidogyne incognita* in tomato where root extract from the mycorrhizal plants brought about 50 % mortality of the nematode larvae in four days time (Suresh *et al.*, 1985).

The AMF status of plants in semiarid and arid regions of India where almost all plants were colonized and the fungi associated with most of the plants belonged to the genera *Glomus*, *Gigaspora* and *Acaulospora* (Mukerji and Kapoor, 1986). Spores of *Gigaspora* and *Scutellospora* species predominated in the root zones of three major plant species growing in the bamer dunes extending from northern New Jersey to Virginia. Twenty-three species of AMF were recovered and dominant species along the transect were *Gi. gigantea*, *Acaulospora scrobiculata*, and *Scutellospora dipapillosa* (Koske, 1987).

Three sterilized acid soils were inoculated with inocula of AMF. Soils were limed and/or P fertilized to produce different fertility levels. Red clover (*Trifolium pratense*) was seeded in pots and grown in a glasshouse for 4 months. *G. tenue* was the most efficient in improving plant growth and nutrient uptake (Arines *et al.*, 1988). Eighteen different AMF were screened for their symbiotic response with Troyer citrange in unsterilized soil. Of the 18 fungi, *G. macrocarpum*, *G. caledonicum*, *G. velum*, *G. monosporum*, *Gi. margarita*, gave the greatest improvements in growth and nutrition, resulting in larger leaf area, plant height, stem diameter, and plant biomass, with higher P, Zn and Cu contents (Vinayak and Bagyaraj, 1990).

AMF was reported for the first time in four species of Hawaiian Ericales, *Vacciniurn calycinurn*, *V. dentaturn*, and *V. reticulaturn* of the Ericaceae and *Styphelia*

tarneiarneiae of the Epacridaceae. The high frequency of AMF in Hawaiian populations of Ericales suggests that ancestral Ericales possessed the capacity to form both AMF and ericoid mycorrhizae. An evolutionary sequence of mycorrhizal dependency in the Ericales is presented (Koske *et al.*, 1990). AMF colonization of four species of pioneer salt marsh plants including two species of chenopodiaceae at the terminal Gangetic delta in India was reported. Five common species of AMF fungi were recorded from rhizosphere soils of the plant species (Sengupta and Chaudhuri, 1990). Out of 70 tropical hydrophytes screened for AMF, 33 harboured AMF. Spores of the AMF were *Entrophospora colombiana*, *G. aggregatum*, *G. claroideum*, *G. fasciculatum*, *G. microaggregatum* and *G. occultum* and *E. schenckii*, *G. aggregatum*, *G. ambisporum*, *G. fasciculatum*, *G. intraradices*, *G. occultum* and *G. pachycaulis* (Ragupathy *et al.*, 1990). Some members of the family Amaranthaceae, which has mostly been reported as non-mycorrhizal, were examined for a symbiotic association with mycorrhizae in the semiarid and arid zones. The spores isolated represented nine species belonging to four genera, *Glomus*, *Gigaspora*, *Sclerocystis*, and *Scutellospora* (Shanker *et al.*, 1991).

The effects of three soil temperatures on growth of spring barleys (*Hordeum vulgare*) and on their root colonization by AMF from agricultural soils in Montana (USA) or Syria at different inoculum concentrations were tested in soil incubators in the greenhouse (Grey, 1991).

Of 147 species examined, 122 were mycorrhizal and 98% of the latter formed AMF. Ericoid, orchid, and ectomycorrhizae also were found. The high incidence of mycorrhizae in endemic species suggests that AMF and obligately mycotrophic plant species may have been present in the Hawaiian Islands from the earliest stages of the development of the angiosperm flora (Koske *et al.*, 1992). Forty-five percent of the Hawaiian pteridophytes floras were examined for mycorrhizae. A phylogram of

pteridophytes families is constructed incorporating the intensity of mycorrhizal colonization as one criterion for classification (Gemma *et al.*, 1992).

The interaction of *G. fasciculatum* with a wilt-causing soil borne pathogen, *Fusarium oxysporum*, was studied in cowpea (*Vigna unguiculata*). It was found that pre-establishment by AMF reduced the colonization of the pathogen and the severity of the disease, as determined by reduction in vascular discolouration index (Sundaresan *et al.*, 1993). The amount of phosphate fertilizer usually applied to rice may be decreased by 50%, without affecting yield, if *G. intraradices* is inoculated (Secilia and Bagyaraj, 1994). The present study reports AMF associations with 14 plant species colonizing a magnesite mine spoil in Salem, Tamil Nadu state, India. Thirteen AMF species were identified and spores of *G. fasciculatum* and *Gi. gigantea* were commonly found in the magnesite mine spoil (Raman *et al.*, 1993).

737 plant species from 121 families of angiosperms and four species of pteridophytes for mycorrhizal association. A total of 40 AMF species belonging to *Acaulospora*, *Entrophospora*, *Gigaspora*, *Glomus*, *Sclerocystis* and *Scutellospora* were isolated from the rhizosphere soils of different ecosystems (Ragupathy and Mahadevan, 1993). Twenty-four species of sedges from different vegetation types in Western Ghats, South India, were examined for AMF associations. Four *Glomus* spp., one *Acaulospora* sp., one *Sclerocystis* sp and one *Gigaspora* sp. were identified among the AMF spores (Muthukumar *et al.*, 1996).

A study was conducted to assess the dynamics of AMF associated with *Acacia farnesiana* and *A. planifrons* in moderately fertile alkaline soils. This was further confirmed from the presence of spores belonging to different AMF species in the rhizosphere soils. Spores of *A. foveata*, *Gi. albida*, *G. fasciculatum*, *G. geosporum* and *Scl. sinuosa* were isolated from the rhizosphere of *A. farnesiana* whereas *A. scrobiculata*,

G. pustulatum, *G. fasciculatum*, *G. geosporum* and *G. microcarpum* were isolated from that of *A. planifrons* (Udaiyan *et al.*, 1996).

Root colonization and spore density of AMF in the rhizosphere of 12 plant species growing on sand dunes in the west coast of India were studied during post-monsoon season. *Borreria articularis*, *Ipomoea pes-caprae* and *Launaea sarmentosa* were dominant plant species on the dunes. Out of a total of 16 species of AMF, nine species belonged to genus *Glomus*. Among the spore communities, *Gi. ramisporophora*, *G. albidum*, *G. clarum* and *Scu. gregaria* were dominant (Kulkarni *et al.*, 1997).

The fungal associations present in the roots and corms of four species of *Isoetes* found in Rajasthan, India, are described. Fungal fruiting bodies and chlamydospores are assigned to the mycorrhizal genus *Glomus*, and the septate branched intracellular mycelium is assigned to the genus *Rhizoctonia* of the Deuteromycotina (Sharma, 1998).

High concentrations of heavy metals have been shown to adversely affect the size, diversity, and activity of microbial populations in soil. The results show that size and diversity of AMF populations were modified in metal-polluted soils, even in those with metal concentrations that were below the upper limits accepted by the European Union for agricultural soils (del Val *et al.*, 1999).

A survey of 329 species for AMF status of plants growing in the Western Ghats region of Southern India was undertaken and AMF spores of 35 species belonging to *Acaulospora*, *Gigaspora*, *Glomus*, *Sclerocystis* and *Scutellospora* were recorded (Muthukumar and Udaiyan, 2000a).

The interaction of plant nutrients, root-soluble carbohydrate availability and AMF was examined in field grown cowpea (*Vigna unguiculata*). Organic amendments increased plant growth, AMF fungal colonization, soluble carbohydrate concentration in roots, and spore numbers. Increased AMF colonization due to organic amendment

significantly reduced nutrient imbalances (Muthukumar and Udaiyan, 2000b). Seed reserves in *Leucaena leucocephala* and *Zea mays* are important for mycorrhizal formation and seedling growth (Muthukumar and Udaiyan, 2000c).

Production of inoculum of *G. intraradices* was examined in locally available sand graded by particle size, planted with *Z. mays* and fertilized with a nutrient solution. Plants in sand with particle sizes of 0.50-0.78 mm had higher root fresh weights, spore production and percent mycorrhizal colonization than with other particle sizes (Gaur and Adholeya, 2000a). A field experiment was conducted to evaluate the benefit to growth of *Allium cepa* of inoculation with a mixed culture of indigenous AMF. Four locally adapted onions were grown at two phosphorus levels in a P deficient alfisol. All inoculated onion varieties showed higher values of bulb diameter, fresh weight, shoot dry matter, shoot P content and bulb yield than uninoculated plants (Mahaveer and Adholeya, 2000). Three vegetable crops, namely coriander, fenugreek, and carrot were inoculated with AMF and grown in nutrient-deficient sandy-loam soils amended with organic matter. The high levels of AMF colonization and large number of infectious propagules demonstrated the potential of these crops as substrates for inoculum production (Gaur *et al.*, 2000). *Eucalyptus tereticornis* was grown in a green house in a low phosphorus soil inoculated with mixed indigenous AMF. Taking dry matter yield into consideration, *Eucalyptus* plants were found to be highly dependent on 10 ppm of soil P for maximum dry matter production (Sharma and Adholeya, 2000). A mixed culture of indigenous endomycorrhizal fungi was multiplied in a nursery and tested for its ability to promote growth and yield of three agricultural crops i.e., onion, potato and garlic were successfully inoculated with AMF in nutrient deficient soil amended with composted leaves of albizzia, poplar and leucaena. Inoculation response in terms of yield increase



was maximum in onion whereas garlic and potato showed 30% and 48% increases, respectively (Gaur and Adholeya, 2000b).

A green house study was conducted under nursery conditions to study the efficacy of nine AMF on *Tectona grandis*. The extent of growth and nutritional status enhanced by AMF fungi varied with the species of AMF fungi inhabiting the roots of teak seedlings (Rajan *et al.*, 2000). Three seasonal ornamental plants, namely *Petunia hybrida*, *Callistephus chinensis* and *Impatiens balsamina*, were tested for their response to inoculation with mixed indigenous AMF culture when grown on a marginal wasteland amended with organic matter. In all the three plant species, mycorrhizal inoculation led to marked improvement in both reproductive (number of flowers) and vegetative (dry matter) phase of the plants (Gaur and Adholeya, 2000c).

AMF in soils from lowland evergreen forests and pastures in Nicaragua and Costa Rica was assessed. Of 28 distinct fungus morphospecies, 11 produced more spores in pasture, while only 1 produced more spores in forest (Picone, 2000).

An inventory of AMF status of 28 plant species belonging to 14 families established on the coastal sand dunes of southwest coast of India was performed where 23 plant species were colonized by AMF, whereas the rhizosphere of only 20 plant species possessed AMF spores. Among 30 AMF recovered, *Scu. erythropha* showed a wide host range (colonized 13 plant species), while *Scu. gregaria* had high spore abundance per plant species (Beena *et al.*, 2000).

Nem seedlings were inoculated with AMF, *G. intraradices* and *G. geosporum*, *Azospirillum brasilense*, and phosphate-solubilizing bacteria (PSB) individually or in various combinations in unsterile soil under nursery conditions. The results emphasize the importance of microbial inoculations for the production of robust, rapidly growing seedlings in nurseries and illustrate the advantage of inoculating soils of a low microbial

population with indigenous microbes (Muthukumar *et al.*, 2001). The influence of the levels of phosphorus on the symbiotic interaction between two subtropical tree species, *A. nilotica* and *Albizzia lebbek*, and a mixed inoculum of indigenous AMF was evaluated in a greenhouse study. *A. nilotica* colonized by AMF showed a significant increase in shoot P and dry matter at a soil P level of 10 ppm whereas in *A. lebbek*, such increase occurred at 20 ppm (Sharma *et al.*, 2001).

The mycorrhizal status of 112 plant species and the spore density of AMF in the rhizosphere soil of these plants in the tropical rain forest of Xishuangbanna, southwest China, were surveyed. Fungi belonging to the genera *Acaulospora* and *Glomus* are the dominant AMF in the soil of the tropical rain forest of Xishuangbanna (Zhao *et al.*, 2001). AMF spores in sand dune vegetation of India; Goa was obtained belonged to the five genera, viz. *Acaulospora*, *Gigaspora*, *Glomus*, *Sclerocystis* and *Scutellospora*. The identified species of AMF include *A. bireticulata*, *A. scrobiculata*, *A. spinosa*, *Gi. coralloidea*, *Gi. gregaria*, *Gi. margarita*, *Scl. sinuosa* and *Scu. verrucosa*. *Gi. coralloidea* was found to be the most frequently occurring fungus, followed by *S. verrucosa*, *Gi. margarita* and *S. sinuosa* (Varsha and Rodrigues, 2001). Commonly occurring pteridophytes from two sites namely Mollem and Chorlem located in Western Ghats region of Goa were selected for the present study. A total of 17 AMF species belonging to five genera namely *Acaulospora*, *Glomus*, *Gigaspora*, *Sclerocystis* and *Scutellospora* were recorded (Khade and Rodrigues, 2002).

Thirty-one species of mangrove and mangrove associates and 23 species of transported flora, belonging to 25 families at four physiographic stages of succession of the mangrove plant community at the terminal part of the Ganges river estuary in India were examined for AMF root association. Seven species of AMF in common with those of the upstream mesophytic plants were isolated from root-free rhizosphere soils of the

mangroves, three of which predominated in root association (Sengupta and Chaudhuri, 2002).

The effects of soil inoculation with AMF and a mycorrhiza helper bacterium (MHB) were investigated on mulberry and papaya plants already established in the field. Thus, mulberry and papaya already established in the field may respond to AMF inoculation and MHB may increase symbiosis development by efficient AMF (Mamatha *et al.*, 2002). Five fodder crops, *Z. mays*, *Medicago sativa*, *Trifolium alexandrinum*, *Avena sativa*, and *Sorghum vulgare* were inoculated with a consortium of indigenous AMF in non-sterile phosphate deficient sandy loam soil amended with organic matter under field conditions. This study clearly indicates the potential of using indigenous AMF inoculations in fodder crops grown in marginal soils along with in situ large-scale production of AMF inocula (Gaur and Adholeya, 2002).

Cycas circinalis, *C. revoluta* and *Zamia* sp. surveyed for AMF colonization and spore populations of nine morphotypes belonging to three genera (*Acaulospora*, *Glomus*, *Scutellospora*) were extracted from soil (Muthukumar and Udaiyan, 2002).

Daucus carota are used extensively for culturing AMF under the root organ culture system (ROC) *in vitro*. The findings suggest the role of host on the differential expression of *G. intraradices* with respect to colonization, spore production, intraradical and extramatrical spread of fungus, nutritional kinetics of each host-fungus symbiosis, pattern of growth and differences in anatomical and morphological features of host roots (Tiwari and Adholeya, 2003). The benefit to the host plant through enhanced P uptake is expected to alter markedly according to the effectiveness of dominant AMF in roots. A positive effect of a mycorrhizal community in roots of *Phyllanthus calycinus* on P uptake has been observed (Solaiman and Abbott, 2003).

The impact of land use intensity on the diversity of AMF was investigated at eight sites in the “three-country corner” of France, Germany, and Switzerland. The increased land use intensity was correlated with a decrease in AMF species richness and with a preferential selection of species that colonized roots slowly but formed spores rapidly (Oehl *et al.*, 2003). The spatial and temporal distribution of the AMF community in a wet tropical rainforest in Costa Rica was examined. AMF communities are spatially distinguishable in the forest, even though all species are widespread. Sampling soils over seasons revealed that some AMF species sporulate profusely in the dry season compared to the rainy season (Lovelock *et al.*, 2003).

A field study of traditional agroforestry systems in six districts of the arid and semiarid zones of Rajasthan was undertaken where field investigation showed that *Glomus*, *Gigaspora* and *Sclerocystis* were the genera of AMF present in the neem-based agroforestry system. Among the three genera, *Glomus* occurred most frequently, with 15 species of *Glomus*, 3 of *Gigaspora* and 2 of *Sclerocystis* observed (Pande and Tarafdar, 2004).

The importance of spatial scale and forest size on changes in AMF spore communities by sampling AMF spores in soils of forested mainland and island sites in the vicinity of Gatun Lake, Republic of Panama was assessed. A total of 27 AMF species with dominance of *Glomus*, *Acaulospora*, *Sclerosystis* and *Scutellospora* was encountered (Mangan *et al.*, 2004).

Strawberries grown in alfisols of semiarid areas in India results in suboptimal yields. AMF response as measured by yield varied with P concentration. Inoculated plants had a significantly greater fruit yield when yield as comparable with uninoculated plants (Sharma and Adholeya, 2004). Four organic amendments: leaf compost (LC), vegetable compost (VC), poultry manure (PM) and sewage sludge (SSL) applied at four

doses were evaluated for their effect on the herbage yield, essential oil content and inoculum potential (IP) of native AMF on three varieties of Java citronella, Bio-13 performed better and produced the highest herbage, essential oil and dry matter yield. Highest numbers of AMF propagules were recorded in the LC amended plots in all the three varieties. Amongst the varieties, highest native mycorrhizal inoculum was recorded in the Bio-13 (Kaur *et al.*, 2004).

Seven isolates along with two more AMF namely *A. laevis* and *G. mosseae*, which were found to be better symbionts for cashew during the earlier study, were used to study their effectiveness on the growth and nutrition of cashew rootstock Ullal-1. Grafts with rootstock treated with *G. etunicatum* and *A. laevis* survived and performed better when planted in the field compared to the uninoculated and other AMF treatments (Lakshmiathy *et al.*, 2004). Based on the plant biomass, P uptake and forskolin content per plant, *G. bagyarajii* was found to be the best AMF symbiont for inoculating *Coleus forskohlii*, the next being *Scu. calospora* (Sailo and Bagyaraj, 2005).

The colonization by AMF, spore abundance and community were investigated in a valley-type semi-savanna vegetation of Yuan River in southwest China. These results revealed that AMF are a common and important component in this semi-savanna vegetation; the high spore density and colonization were presumably a selective adaptation toward the hot and arid ecosystem (Tao and Zhao, 2005). The diversity of AMF in agricultural fields was reported on *L. leucocephala* from Bangalore, ornamentals and cultivated plants at Allahabad and adjoining areas, crop fields of Konkan and Solapur, tea plantation at Nilgiris, Tamil Nadu, pearl millet, maize, wheat, pigeon pea and chick pea in Gwalior and different agroclimatic regions of India (Manoharachary *et al.*, 2005).

The vertical distribution of spores of AMF was investigated in soil profiles of extensively and intensively managed agroecosystems, including two permanent grasslands, a vineyard and two continuously mono-cropped maize fields. The results indicate that the AMF communities in deep soil layers are surprisingly diverse and different from the topsoil. Thus, deep soil layers should be included in studies to get a complete picture of AMF diversity (Oehl *et al.*, 2005). AMF status of five species of rhododendrons distributed in Kumaun region of the Indian Central Himalaya. Spore populations were found to belong to five genera: *Acaulospora*, *Glomus*, *Gigaspora*, *Sclerocystis* and *Scutellospora*; genus *Glomus* was found to be dominant in the rhizosphere soil samples of all the rhododendron species (Chaurasia *et al.*, 2005).

The temporal and spatial dynamics of AMF were investigated in Indian Thar Desert. A total of fifteen AMF species were associated with *Mitragyna parvifolia*. Percent spore density and species richness suggest that the genus *Glomus* was the predominant AMF under Thar Desert (Panwar and Tarafdar, 2006). Mangroves form an important ecosystem of Great Nicobar, a continental island in the Bay of Bengal with luxuriant tropical rainforests. The rhizosphere of the mangrove plants of Great Nicobar was investigated for the presence of AMF and phosphate solubilising bacteria (PSB) (Kothamasi *et al.*, 2006).

AMF status of native plant species and AMF diversity in the grasslands of southern Tibet was reported. After trap culture for 5 months, 25 AMF taxa were identified in the soil samples collected, of which nine belonged to *Glomus*, ten to *Acaulospora*, one to *Entrophospora* and five to *Scutellospora*. The frequency of occurrence of different genera and species varied greatly. *Glomus* was the dominant genus, and the most frequent and abundant species was *G. mosseae* (Gai *et al.*, 2006). The occurrence of AMF and the mycotrophism of the plants of Madhupur forest area and

the applicability of AMF technology in the forest management of Madhupur forest (Dhar and Mridha, 2006).

A nursery experiment was conducted to assess the effect of bioinoculants (*G. aggregatum*, *Bacillus polymixa*, *A. brasilense*) on seedling growth promotion of bamboo (*Dendrocalamus strictus* in two soil types (alfisol, vertisol) with or without fertilizer application. Though soil type did not affect microbial inoculation response, fertilizer application significantly affected plant response to microbial inoculation (Muthukumar and Udaiyan, 2006). A pot experiment was conducted to examine the effect of AMF, *G. fasciculatum*, and salinity on the growth of *Acacia nilotica*. Our results indicate that mycorrhizal fungus alleviates deleterious effects of saline soils on plant growth that could be primarily related to improved P nutrition. The improved K/Na ratios in root and shoot tissues of mycorrhizal plants may help in protecting disruption of K-mediated enzymatic processes under salt stress conditions (Giri *et al.*, 2007).

Colonization and diversity of AMF associated with five common ephemerals were investigated in four typical desert plant communities in Junggar Basin, northwest China. *Glomus* was the dominant AMF genus. (Shi *et al.*, 2007). The diversity and community structure of AMF in two sites (Pudu and Xiao Rivers) with different vegetation in the hot-dry valley of Jinsha River, southwest China, were investigated. The most common and frequent genus was *Glomus*, and several species of *Glomus* and *Gigaspora* were the most common and frequent among the 43 species present in this hot-dry ecosystem (Zhao and Zhao, 2007).

Biodiversity of AMF colonization and spore population was investigated in different agroforestry trees and crop species collected from different locations of Dinajpur district of Bangladesh (Mridha and Dhar, 2007). Of the 20 plant species of hydrophytes screened for AMF root colonization, 10 aquatic plants and five marshy

plants were found to be mycorrhizal, while the remaining species were non mycorrhizal. In all, two genera viz., *Glomus* and *Scutellospora*, the former being dominant, were recorded (Radhika and Rodrigues, 2007). Twelve plant species having underground storage organs (tubers, rhizomes, corms and bulbs) belonging to five families (Liliaceae, Dioscoreaceae, Amaryllidaceae, Zingiberaceae and Araceae). Nineteen species of AMF belonging to four genera (*Acaulospora*, *Glomus*, *Gigaspora* and *Scutellospora*) were recovered (Khade and Rodrigues, 2007).

The AMF status of fifteen mangroves and one mangrove associate was investigated from 27 sites of three inundation types namely, diurnal, usual springtide and summer springtide. *G. mosseae* was the predominant species in terms of frequency in the soils of the Sundarbans (Kumar and Ghose, 2008). The colonization and diversity of AMF associated with the rhizosphere of tea growing under 'natural' as well as 'cultivated' conditions in the Kumaun region of Uttaranchal Himalaya (India), during the periods of active growth and dormancy were investigated. The rhizosphere of cultivated tea bushes was found to be dominated by *Glomus* morphotypes along with three morphotypes of *Acaulospora*; occurrence of 35 morphotypes belonging to four genera viz. *Acaulospora*, *Gigaspora*, *Glomus* and *Scutellospora* was recorded in the rhizosphere of tea plants from the natural ecosite. A total of 51 AMF morphotypes were detected (Singh *et al.*, 2008).

A glass house experiment was conducted to study the interaction between the mycorrhizal fungus, *G. mosseae* and six soil yeasts (*Rhodotorula mucilaginosa*, *Metschnikowia pulcherrima*, *Trichosporon cutaneum* var. *cutaneum*, *Saccharomyces cerevisiae*, *Cryptococcus laurentii*, *Debaryomyces occidentalis* var. *occidentalis*), and their effect on growth and nutrition of cowpea. All the yeasts had a synergistic interaction

with the mycorrhizal fungus and dual inoculation improved plant growth compared to single inoculation with *G. mosseae* alone (Boby *et al.*, 2008).

In glasshouse experiments, dominants and subordinates from subtropical grasslands were colonized by native AMF or introduced AMF, *G. versiforme*. Results show that plant community structures are partly determined by MD on native AMF, and could be modified by introducing exogenous AMF species (Yao *et al.*, 2008). The effects of *G. clarum* and *G. aggregatum*, on the early growth of two slow-growing peat-swamp forest tree species, *Ploiarium alternifolium* and *Calophyllum hosei*, under greenhouse conditions was determined. The results suggest that inoculation with AMF improves early growth of *P. alternifolium* and *C. hosei* in a tropical peat-swamp forest and can therefore contribute to rehabilitation of peat-swamps (Turjaman *et al.*, 2008)

The feasibility of using mycorrhizal roots of seedlings of *Inga acreana*, *Tabebuia chrysantha*, *Cedrela montana* and *Heliocarpus americanus* was studied. Assessment of plant growth and mycorrhizal status of 6 months old *Cedrela montana* and *Heliocarpus americanus* revealed an improvement in growth and diverse associated fungi through mycorrhizal root inoculation in comparison with moderate fertilization (Urgiles *et al.*, 2009). The influence of *G. intraradices* (AMF), earthworm inoculation (*Pheretima* sp.) and rhizobium (*Rhizobium melilotus*) separately, and in combination, on growth of annual medic (*Medicago scutellata*) were studied in potted culture at different planting density with light stress at seed formation. At low population plant, EW inoculants caused annual medic to retain fresh green leaf and leaf chlorophyll content after 96 h light stress at seed formation (Zarea *et al.*, 2009).

The effects of inoculating *Panax ginseng* seedlings with AMF by examining the root colonization, plant nutrition uptake, growth characteristics, and soil aggregation of *P. ginseng* seedlings inoculated at the time of transplantation. Results indicate that AMF

inoculation may enhance the growth of ginseng seedlings by improving the uptake of mineral nutrients and the soil structure in mycorrhizosphere (Cho *et al.*, 2009). The symbiosis between AMF and mangrove plant species was investigated in two mangrove swamps in south China. Six AMF species belonging to the genera *Glomus* or *Acaulospora* were identified. The inoculated AMF significantly improved growth in *Sonneratia apetala*, resulting in greater plant height, diameter at ground level and plant biomass, as well as increased absorption of N, P and K (Wang *et al.*, 2010).

The populations of the general microflora (bacteria, actinomycetes and fungi) in the rhizosphere and their corresponding non-rhizosphere soil samples of *Ginkgo biloba* L. of two age groups growing under a temperate location of Indian Himalayan Region (IHR) have been determined. All the spores detected, belonged to the genus *Glomus* with five different types (Kumar *et al.*, 2009). Commonly occurring plant species on metal-contaminated soils and non-contaminated soils adjoining Kanpur Tanneries, Uttar Pradesh, India were surveyed for AMF. A total of six species of AMF belonging to two genera viz., *Glomus* and *Scutellospora* were recovered during the study (Khade and Adholeya, 2009).

The diversity of AMF species in the rhizosphere of an endangered anticancerous herb—*Curculigo orchioides* in its natural habitat was reported. A total of 18 species of AMF, belonging to three genera (*Acaulospora*, *Glomus* and *Gigaspora*) were recorded, with *G. microcarpum* being the most abundant species type. *A. laevis* spores were restricted to sites where the soil pH was acidic (Sharma *et al.*, 2009). Shah *et al.* (2009) examined 63 alien plant species representing 26 families, collected from diverse habitat types in the Kashmir Himalaya, India, for the extent and type of their AMF association. The AMF colonization in 33 plant species was of *Arum*-type, 18 species was of *Paris*-type, and eight species harbored an intermediate type.

Morphological types of AMF in crops and associated weeds were examined in agro-ecosystems. In total, 48 plant species (8 crops and 40 weeds) belonging to 43 genera in 18 families were examined. The number of plant species with *Arum*-type AMF was higher than those with *Paris*-type AMF in the examined plants (Muthukumar and Prakash, 2009). Mycorrhizal fungal associations in a shola vegetation of Western Ghats region, southern India. Plants belonging to 29 species of 19 families were assessed for mycorrhizal type and dark septate endophyte (DSE) fungal association. AMF association was the most predominant mycorrhizal type (Bagyalakshmi *et. al.*, 2009). Comparison of AMF and DSE associations in 2 crops and 31 weeds commonly occurring in pulp and paper mill effluent irrigated and well-water irrigated soils. Twelve morphotypes of AMF spore belonging to *Acaulospora*, *Dentiscutata*, *Glomus*, *Racocetra* and *Scutellospora* were isolated from the well-water irrigated soils, whereas spores of six morphotypes were isolated from effluent irrigated soils (Muthukumar and VEDIYAPPAN, 2009).

Sporocarpic species of AMF were extracted and described from the rhizosphere of *Alnus nepalensis*, *M. champaca* and from the cultivation field of *Solanum tuberosum*. The identified morphotypes were *G. aureum*, *G. clavispurum*, *G. fuegianum*, *G. glomerulatum*, *G. macrocarpum*, *G. microaggregatum*, *G. rubiforme*, *G. sinuosum* and *G. taiwanense* (Das and Kayang, 2009a). The occurrence and inoculum density of AMF associated with *Blechnum orientale* were studied in a subtropical pine forest, a coalmine, an abandoned coalmine and the roadside of Meghalaya, northeast India. *Glomus*, *Acaulospora*, *Gigaspora* and *Entrophospora* were identified comprising of 11 morphotypes (Das and Kayang, 2009b). Associations of AMF and DSE with 38 ginger species (Zingiberaceae) and two spiral gingers (Costaceae) were investigated. Spores of 16 morphotypes belonging to 4 genera (*Glomus*, *Acaulospora*, *Scutellospora*, *Racocetra*) were identified from the rhizosphere soil (Uma *et al.*, 2010).

AMF and DSE association in 50 south Indian grasses from four different sites. Forty-four of the 50 grasses examined had AMF association and dual association with DSE fungi occurred in 25 grasses. AMF morphology is reported for the first time in 35 grasses. Spores of 11 AMF taxa were isolated from the soil samples of grasses of which nine belonged to *Glomus*, one to *Acaulospora* and one to *Scutellospora*. *G. aggregatum*, *G. viscosum* and *G. mosseae* were most frequent species at different sites (Sathiyadash *et al.*, 2010).

A survey was conducted to determine the AMF spore population in rhizosphere soils and its root colonization of ten plants belonging to seven families having tubers in different seasons of Anaimalai Hills, Western Ghats. *Glomus* species was recorded as dominant root symbionts (Nisha *et al.*, 2010). *Dentiscutata nigerita* Khade (family *Dentiscutataceae*), a new species is reported and described from the rhizosphere of *Carica papaya* plants from Kodar, Goa, India (Khade, 2010). Thirty-six medicinal plant species belonging to 25 families were surveyed to study AMF diversity from different localities of North and South Goa of Western Ghats, Goa region, India. *Glomus* was found to be the most dominant genera in the study sites, and *G. fasciculatum* the most dominant AMF species (Radhika and Rodrigues, 2010).

A study of diversity of AMF in *Camellia sinensis* was conducted in four plantation territories of Uttarakhand. *Glomus* was represented by three species; that is, *G. mossae*, *G. fasciculatum*, *Glomus* sp. 1 (an unidentified species) (Gupta and Sharma, 2010). AMF distribution and DSE colonization on four species of bamboo from Northeast India. Of 17 species isolated from *Acaulospora*, *Ambispora* and *Glomus*, 12 were isolated from *Phyllostachys manii*. *A. tuberculata*, *A. rehmii*, *Glomus intraradices* and *G. tortuosum* were the most frequently distributed species (Das and Kayang, 2010a). AMF and DSE colonization were investigated in three different plantation sites

(Umdihar, Umsaw and Mawlein) of Meghalaya, northeast India. The distribution, abundance and principal component analysis plot suggest that *G. macrocarpum*, *G. multicaulis*, *G. constrictum* and *Acaulospora* sp 1 were the most host preferred species which possibly may favour the host with proper nutrient acquisition and growth (Das and Kayang, 2010b). Sixteen AMF morphotypes were identified from the potato field in Meghalaya belonging to genera *Acaulospora*, *Gigaspora*, *Glomus*, *Pacispora* and *Scutellospora*; a few remain unidentified with the dominance of *Glomus tortuosum*, *Pacispora boliviana* and *Gi. margarita* from Meghalaya, northeast India (Das and Kayang, 2010c). The distribution of AMF in the marshy and shoreline vegetation of Deepar Beel Ramsar site of Assam, India was studied. All total 18 AMF morphotypes were recorded which comprises four genera viz. *Glomus*, *Acaulospora*, *Gigaspora* and *Scutellospora* (Choudhury *et al.*, 2010).

The efficacy of different *Glomus* species, AMF (*G. aggregatum*, *G. fasciculatum*, *G. mosseae*, *G. intraradices*) on various growth parameters such as biomass, macro and micronutrients, chlorophyll, protein, cytokinin and alkaloid content and phosphatase activity of pink flowered *Catharanthus roseus* plants showed that all *Glomus* species except *G. intraradices* enhanced the chlorophyll, protein, crude alkaloid, phosphorus, sulphur, manganese and copper contents of *C. roseus* plants along with phosphatase activity significantly over uninoculated plants. (Ratti *et al.*, 2010).

Assessment of mycorrhizal colonization in *Michelia champaca*

Introduction

The process of staining plant roots for observing mycorrhizal structure and quantification of the extent of arbuscular mycorrhiza fungi (AMF) colonization is probably the most frequently performed procedure in AMF research. All the modifications to standard clearing and staining procedures have been proposed for poor contrast images and safety reasons. Acid Fuchsin destains rapidly and produces relatively low-contrast images as observed by Brundrett *et al.* (1984). Grace and Stribley (1990) have shown that Chlorzool Black E or Trypan Blue can be replaced by Methyl Blue or Aniline Blue as less harmful dyes. However, there is not much evidence to confirm that these other dyes are non-toxic, so they must also be handled carefully. Again, Brundrett *et al.* (1994) suggested that Trypan Blue does not provide good contrast. Trypan Blue and Acid Fuchsin are also expensive and suspected carcinogenic as referred to Veirhilig *et al.* (2005). For environmental reasons it is preferable, wherever possible, to find substitutes for harmful chemicals. In an attempt to eliminate some of the hazardous effect and cost of these chemicals, a procedure by Veirhilig *et al.* (1998) for staining of AMF in roots has already been made to stain AMF with ink and vinegar.

The interactions between stain and plant species depended on the plant species as examined by Morton (1985). Most model plants (e.g. leek, onion, maize, clover, etc.) used in greenhouse studies with AMF have always been employed for their ease in staining with Trypan blue or the other stains. Plants whose roots have several layers of cortical cells or contain high levels of phenolic compounds and tannins are much more difficult to stain and study. These species also need alterations to the staining protocols, which are only achieved by trial and error as discussed by Dodd *et al.* (2000).

The objective of our investigation was to determine mycorrhizal colonization types and whether stamp pad ink technique could be adapted for staining arbuscular mycorrhizal structure in the root of *Michelia champaca* L.

Materials and Methods

Root sampling and processing

Ten seedlings of healthy root system of *M. champaca* (Family: Magnoliaceae) of 40-50 cm heights were collected from nursery of Social Forestry Division, Upper Shillong, Meghalaya. The seedlings were six months old growing on soil collected from the nearby areas of the nursery in the polythene containers size of 12x20cm. The plant is characterized by having coarse root system diameter varies from 0.5-5.0mm. All root samples washed free of soil particles prior processing of roots. The diameter of the root sample was measured with the vernier caliper.

The washed roots were normally cut into small segments of 1cm and stored in cold water for 30 minutes before clearing. The roots were normally cleared in 10% (w/v) KOH kept overnight at 25°C. Cleared roots were captured on a fine sieve and rinsed with water before transferring them into the bleaching solution. The roots were then bleached in alkaline hydrogen peroxide as followed by Bevege (1968) in the water bath at 70°C for 15 minutes. The roots were rinsed with copious amount of water as soon as the samples were bleached white or become transparent, at least with five changes of tap water.

Root staining

The roots were acidified with 1% of hydrochloric acid and roots were directly stained from this solution with Faber Castell black stamp pad ink. Adding 30 ml of ink in 70 ml of distilled water does the preparation of the suitable concentration of the stamp pad ink. The advantage of large diameter roots (2.0-3.0 mm) of *M. champaca* renders to cut into equal halves longitudinally before staining.

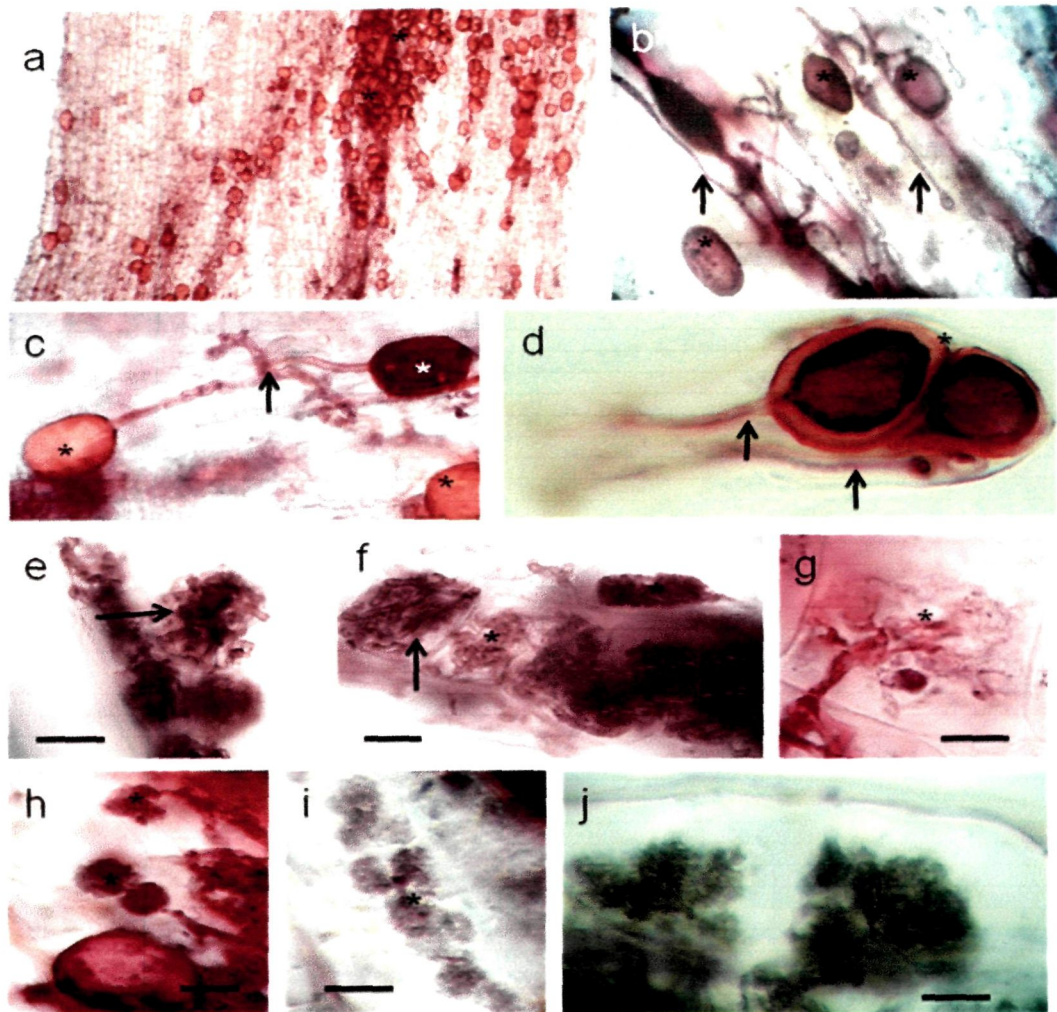


Figure 3.1: Light photomicrographs of arbuscular mycorrhizal colonization of roots of *Michelia champaca* stained with stamp pad ink. (a) Portion of root showing vesicles. Bar=60 μ m. (b) Darkly stained vesicles and hyphae. Bar=40 μ m. (c) Vesicles are connected by hyphae. Bar=40 μ m. (d) Two intracellular vesicles and hyphae. Bar=20 μ m. (e) The intracellular hyphae. Bar=25 μ m. (f) Arbuscules and intracellular hyphae colonizing the segment of the root. Bar=50 μ m. (g). Tree-like structure of arbuscule. Bar=20 μ m. (h) Clearly visible arbuscules and vesicles. Bar=60 μ m (i) Arbuscule with lobe. Bar=25 μ m. (j) The arbuscules stained lightly. Bar=20 μ m. (Asterisk showing vesicles and arrow pointing hyphae).

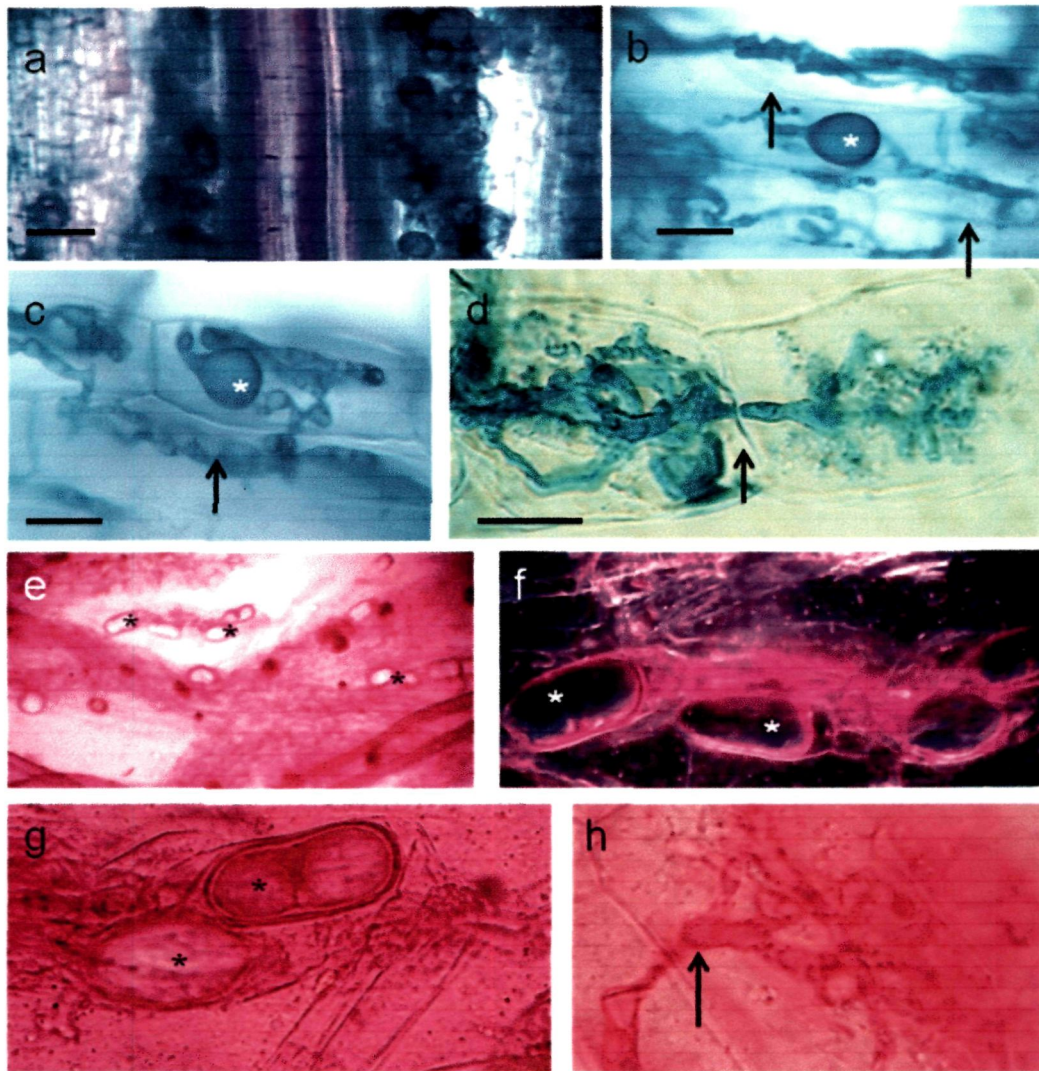


Figure 3.2: Light photomicrographs (a-d) of arbuscular mycorrhizal colonization of roots of *Michelia champaca* stained with Trypan blue (a) Roots segment showing colonization in inner cortex. Bar=300 μ m. (b) Segment of root showing vesicles. Bar=50 μ m. (c) Vesicles and intracellular hyphae. Bar=40 μ m. (d) Arbuscules and intracellular hyphae. Bar=30 μ m. Light photomicrographs (e-h) of arbuscular mycorrhizal colonization of roots of *M. champaca* stained with Acid Fuchsin (e-g) Presence of vesicles in the root segment. Bar=100, 50 μ m, 50 μ m. (h) Presence of arbuscules. Bar=20 μ m. (Asterisk showing vesicles and arrow pointing hyphae).

The root material was kept in staining solution at 90°C for 1 hour and rinsed the samples with two changes of tap water followed by keeping immersed in tap water for 05-20 minutes. The stained roots were squashed gently under cover slips. The stained roots were mounted in lacto glycerol as found suitable by Harinikumar and Bagyaraj (1988).

The standard methods were then compared with Faber Castell black stamp pad ink method. The standard methods by Phillips and Hayman (1970) and Kormanik and McGraw (1982) were applied to stain the roots. Therefore, Trypan Blue (Colour Index: 23850) and Acid Fuchsin (CI: 42685) were prepared for comparison.

From each replicate, 50 root samples of 1cm were stained with the different stains and quantified for colonization in root tissues under Olympus 41209 by magnified intersection method (McGonigle *et al.*, 1990). Stained roots by stamp pad ink that showed arbuscular mycorrhizal structures (arbuscules and vesicles) were selected for light microscope studies. The light microscopy of the samples was observed under Leitz Wetzlar Germany 513467.

Results

Coarse branching and lack of root hairs characterize the roots. The root was observed to be colonized by AMF. The cortex is several cell layers; AMF colonizes the root diameter of 0.5-3.0 mm. The staining of AMF with the stamp pad ink shows excellent results (Figure 3.1). The Faber Castell stamp pad ink stains epidermal cells in roots, in particular, xylem, as well as fungal hyphal structures. The fungus penetrates the cortical cells of the roots which are characterized by the formation of unique structures. The intraradical hyphae of AMF exhibit modifications dependent on their locations within the cortex. The hypertrophic hyphae, called vesicles (Figure 3.1a-d), intracellular hyphae (Figure

3.1e&f); and intracellular hyphae with many ramifications, known as arbuscules (Figure 3.1g-j) are visible.

The stained segment of root without heating gives light brown to the vesicles (Figure 3.1a&c) with heating results black (Figure 3.1b). Vesicles are hyphal swellings in the root cortex that may be intercellular or intracellular. They can be regular or irregular in shape and several times wider than the hyphae on which they form. The vesicles are connected with hyphal connection, which are easily discernible (Figure 3.1c). Therefore, vesicles are usually very distinctive and can resemble spores (Figure 3.1 c&d).

The staining of arbuscule on heating gives fast and effective results (Figure 3.1g-j). Arbuscules are usually found in the inner cortex, are formed from penetrating hyphae (Figure 3.1j) which invaginate the host plasmalemma and repeatedly bifurcate to form a bush-like structure (Figure 3.1i&j) with progressively thinner branches. Because of the extensive ramifications and thinness of the ultimate branches, these arbuscules present a challenging task to their study with light microscopy. The arbuscules exhibiting tree-like structures are also clearly seen (Figure 3.1g). Therefore, the staining of arbuscules is visible (Figure 3.1g-j) which are the preferential sites of exchange for phosphorus, carbon, water and other nutrients. Observing the prepared slide after two months assessed the reliability of stamp pad ink. The colour of the arbuscule changed slightly after two months without any other change in the artifacts.

The stain was compared with the standard stain such as Trypan Blue and Acid Fuchsin. The comparison was based on the ability to visualize the structure distinctly under the microscope as well as the colonization percentage was determined. The vesicles are clearly visible stained with stamp pad ink, which can be easily comparable to the Trypan blue (Figure 3.2a-d) that is still one of the highly utilized standard stains. The arbuscules stained by stamp pad ink are distinct and clear as compared to the Trypan

blue. Acid Fuchsin stains vesicles (Figure 3.2e-g) very well as compared to stamp pad ink. The arbuscule stained does not provide good contrast (Figure 3.2h) as compared to the stamp pad ink.

Table 3.1. Staining of roots samples with standard method and Faber Castell black stamp pad ink

Stain	Root colonization*	Percentage of roots colonization
Trypan Blue	26.3±2.37	52.6
Acid Fuchsin	21.6±2.03	43.2
Stamp pad ink	27.9±2.6	55.8

*Mean ±SE

The arbuscular mycorrhizal colonization was almost similar, with mean colonization percentage levels of 55.8% and 52.6% stained with Faber Castell stamp pad ink and Trypan Blue, respectively (Table.3.1). The colonization was found to be 43.2% stained with Acid Fuchsin, comparatively lower than the other two methods.

Discussion

The arbuscular mycorrhizal fungal (AMF) colonization was reported earlier (Muthukumar *et al.*, 2006), however, the same author found zero colonization previously (Muthukumar *et al.*, 2005). Nevertheless, AMF colonization was found to be comparatively less in the seedlings than reported by Muthukumar *et al.* (2006).

Prolonged times of clearing with KOH often removes more phenolic pigments from roots, but it may not be appropriate if root samples tend to disintegrate or contain wall-bound secondary metabolites which are resistant to clearing. Roots that are insufficiently cleared will still have cell contents, which obscure mycorrhizal structures. Clearing the roots in 10% KOH kept overnight sufficiently remove the cell contents and does not interfere the staining process. The clearing of root of *M. champaca* takes longer

time due to their woody nature and high amount of phenolics. Post-clearing bleaching with alkaline hydrogen peroxide and sodium hypochlorite as effectively removes any phenolic compounds left in cleared roots. Decolourization with hydrogen peroxide is slower than sodium hypochlorite, but there is less danger of complete destruction of fungal and cortical tissue. However, H₂O₂ is fast and effective bleaching agent at high temperature as referred to Hurst *et al.* (2002).

Stains such as Trypan Blue and Chlorzool Black E are believed to bind most strongly to phenolic-like materials (melanins) in hyphal walls but will also stain plant cell walls with encrusted phenolics (suberin and lignin) and any residual cell contents as discussed by Brundrett *et al.* (1984). Staining of plant cell walls results from the nature of cell wall components, which cannot be eliminated completely either by clearing or bleaching steps. Staining of these structures can conceal mycorrhizal structural details in some roots unless they are squashed under a cover slip. The slight modification in sectioning longitudinally the large diameter roots reveals the fungal structure brilliantly. The sectioning is appropriate for studying morphological criteria of AMF but it is not recommended for staining and scoring colonization large samples.

Staining and microscopic methods not only provide reliable data on the degree of root colonization but also permit to visualize the presence of key features such as arbuscules, which are the morphological criteria that define AMF associations as described by Brundrett (2004). Stamp pad ink also gives reliable colonization data as compared to Trypan blue. Acid Fuchsin fails to reveal fungal structure especially arbuscules at higher magnification as also demonstrated by Gange *et al.* (1999). Trypan Blue gives excellent contrast at higher magnification when materials were squashed properly.

An interesting colour change observed when the roots were stained either with or without heating. The vesicles and hyphae are stained exhibiting differences in colour. The unique structure of AMF i.e., arbuscules stains without heating unless kept for longer period immersed in the staining reagent. The vesicles rapidly stain than arbuscules. The arbuscules after staining for suitable time gives black colour and depending upon the time the colour may vary from black to brown. The change in colour of the arbuscule after two months is may be due to the fact that it was mounted in lactoglycerol, which destain the fungal structure.

The penetration of stain depends upon several factors of which thickness of the root is important as found by Hayat (1993). The staining period and destaining time, also varies with the diameter of the roots. For root samples of size 0.5-1.2 mm destaining may vary time between 1-5 minutes and root size of 1.4-2.0, time varies from 6-10 minutes. The destaining of all the roots sizes together may result to complete loss of stain from the small diameter roots.

The method with black stamp pad ink still further reduces health risks. The method of Vierhilig *et al.* (1998) reporting several pen inks are not available in the South Asian countries and also the ink used in this method is used for rubber stamp. The low cost availability of this dye is also reliable to carry out experimental research and teaching exercises in the developing country like India. Moreover, this very simple technique often results in high quality images of visible competitive interactions at early stages of the plant growth by arbuscular mycorrhizal fungal colonization and symbiosis that may be used in a routine way for staining of other root colonizing fungi in different plant species.

Diversity of arbuscular mycorrhizal fungi from the plantations of *Michelia champaca*

Introduction

Mycorrhiza is widespread in natural ecosystems, and plays a crucial role in the uptake of mineral nutrition of forest trees, which is one of important nutrient acquiring mechanisms (Pate, 1994). Arbuscular mycorrhizal fungi (AMF) form associations with the majority of terrestrial plant species (Smith and Read, 1997). AMF stimulate plant uptake of nutrients such as P, Zn, Cu, and Fe in deficient soils and mycorrhizal hyphae can significantly improve ¹⁵N, P, and K uptake (Chen and Zhao, 2009). It plays an important role in ecological system protection, restoration, and reconstruction (Wu *et al.*, 2009). Moreover, AMF are now well practiced in the forestry management (Mridha and Dhar, 2007). They belong to four orders: Glomerales, Archaeosporales, Paraglomales and Diversisporales in the division Glomeromycota (Schubler *et al.*, 2001).

The coarse structure of the root typified by the order Magnoliales are especially dependent on AMF for mineral uptake (Baylis, 1975). Moreover, occurrence of DSE and *Paris* type of AMF colonization in *Michelia champaca* was reported earlier (Muthukumar *et al.*, 2006). However, there is no detailed study of AMF diversity of this extremely important multipurpose tree. Composition of indigenous AMF spores in the plantation sites may be helpful in indicating the preference of AMF spores in inoculation program for seedling production in nursery. Therefore, the study was undertaken to evaluate (1) the status of AMF and DSE colonization in the three plantation sites, (2) the biodiversity of AMF in the plantation sites, and (3) whether some level of host preference in terms of frequency and abundance exist among AMF species in the plantation sites.

Materials and methods

Study sites and sampling

Three plantation sites were selected in Ribhoi District of Meghalaya, Northeast India. They were located in Umdihar (N25°51.156'; E91°52.699'; with an elevation of 544.0 m.a.s.l.), Umsaw (N25°49.051'; E91°52.642'; 553.5 m.a.s.l.), and Mawlein (N25°42.642'; E91°53.55'; 828.5 m.a.s.l.), respectively (Figure 4.1). The plantations in Umdihar and Mawlein are privately managed sites, while the plantation in Umsaw is under State Forestry Department, Meghalaya.

The plant individuals with circumference breast height (CBH) <5 cm were considered for sampling. Tree height (H) was measured with the help of a Clinometer (Suunto pm-5/1520). From each sampling site, five trees were randomly selected with sampling points approximately 5 m apart. *M. champaca* from the order Magnoliales is distinguishable by its coarse root structure (Baylis, 1975) and characteristic interesting aroma. The rhizospheric soil and roots at depths of 0–20 cm around each tree, at four different points for each plant were collected. Combined samples of approximately 500 g of soil per plant were placed in 3-kg polythene bags, labeled and transported for further analysis in the laboratory.

Root processing

To determine percent root colonization, the root samples were washed in tap water, processed and stained with black Faber Castell stamp pad ink (Das and Kayang, 2008). Root segments of approximately 1-cm long stained samples were mounted on slides in lactoglycerol and examined for mycorrhizal structures under light microscope (Olympus 41209) to investigate different colonization patterns (the structures of hyphal, arbuscular, vesicular, and dark septate hyphal). The estimation of AMF and DSE colonization were done by magnified intersection method (McGonigle *et al.*, 1990).

Spore analysis

The spores were extracted by modified wet sieving and decanting method (Muthukumar *et al.*, 2006). The isolated spores were picked up with needle in polyvinyl alcohol-lactoglycerol under a dissecting microscope (Koske and Tessier, 1983) and also in mixed polyvinyl alcohol- lactoglycerol: Meltzer's reagent (1: 1, v: v) for identification. The complete and broken spores were examined using a compound microscope, Olympus.

Taxonomic identification of spores to species level was based on sporocarpic size, colour, and ornamentation and wall characteristics by matching original descriptions (<http://www.invam.caf.wvu.edu> & <http://www.lrz-muenchen.de/~schuessler/amphylo>). The photography of the root segments colonized by fungi and spores of AMF were done with the help of Leica EC 3 camera attached in Leica DM 1000 microscope (Switzerland). Spore density (SD), relative abundance (RA), isolation frequency (IF), species richness (SR), evenness (E), Simpson's diversity index (D), Shannon-Wiener index of diversity (H') and Sorenson's coefficient (Cs) were calculated (Zhao and Zhao, 2007).

Soil analysis

The soil samples were air dried after analysis of pH and moisture content. Then they were cleaned, ground, sieved with a 2-mm sieve, stored at 4°C and processed for further soil analysis. Soil texture was analyzed using sodium hexametaphosphate method (Allen *et al.*, 1974). For moisture content (%), 10 g sub sample of soil was oven dried and weight was determined. Measurement of the soil pH was done using microprocessor-based pocket pH tester 2 (Eutech Instruments). Available phosphorus of soil was determined following molybdenum-blue method (Allen *et al.*, 1974). The soil organic carbon was estimated using colorimetric method (Anderson and Ingram, 1993).

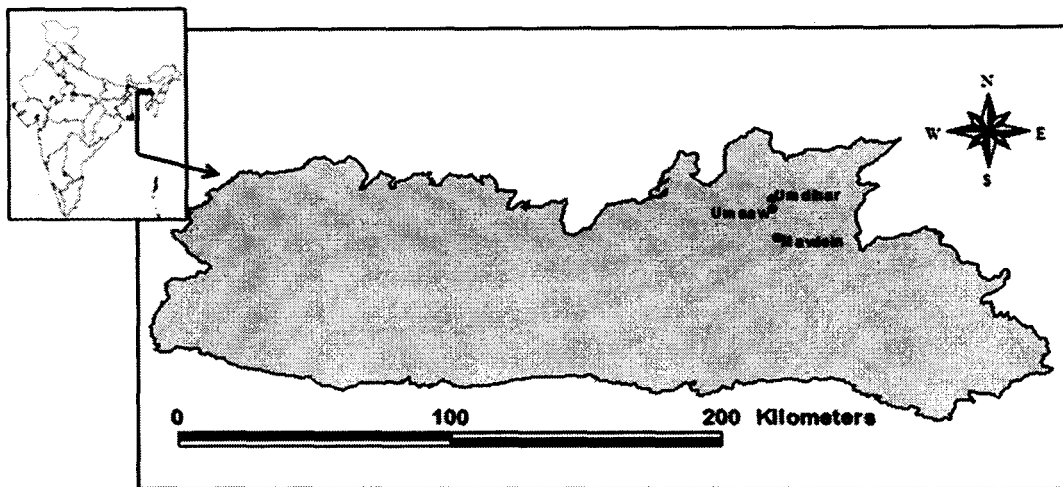


Figure 4.1: The three plantation sites of *Michelia champaca* in Meghalaya, northeast India

Data analysis

Standard errors of means were calculated. Analysis of variance (ANOVA) was carried out and the means were separated by Tukey test. Pearson correlation coefficients were computed between soil physico-chemical properties, mycorrhizal colonization, CBH and H of tree species and between relative abundance and species richness of AMF species. Principal component analysis (PCA) was used to determine variation in AMF abundance. PCA analysis was done with the help of software, PAST (Hammer *et al.*, 2001).

Results

The circumference breast height and height of *M. champaca* in the plantations of Umdihar, Umsaw, and Mawlein are 169.92 ± 2.53 ; 2293.33 ± 7.87 , 35.2 ± 0.9 ; 1486.92 ± 4.72 and 32.4 ± 2.02 ; 947.14 ± 3.09 cm, respectively. The soil physical and chemical properties are presented in Table 4.1.

The mycorrhizal structural colonization is presented in Table 4.2. *Paris* type of AMF morphology exists in the roots. There was no significant difference in total AMF and DSE colonization between the sites. However, AMF colonization was significantly higher ($p < 0.05$) than DSE (Figure 4.2). Spore density differed statistically in all the sites ($p < 0.05$). No significant correlation was found between mycorrhizal colonization, soil properties, CBH and H ($p < 0.05$).

Table 4.1. Physical and chemical properties of soil collected from three different sites of *Michelia champaca*

Sites	Moisture (%)	Texture (%)			pH	Organic carbon (%)	Available phosphorus ($\mu\text{g/g}$)
		Silt	Sand	Clay			
Umdihar	15.3 ± 0.5	12.20	83.77	4.03	6.13 ± 0.09	0.63 ± 0.023	166.67 ± 3.33
Umsaw	15.5 ± 1.4	14.21	69.61	16.19	6.13 ± 0.03	0.59 ± 0.012	163.33 ± 3.33
Mawlein	27.2 ± 0.4	19.52	75.19	5.29	6.07 ± 0.12	0.57 ± 0.005	176.67 ± 3.33

Table 4.2. Mycorrhizal structural colonization in the roots of *Michelia champaca*

Sites	Arbuscules (%)	Vesicles (%)	Hyphae (%)	Dark septate hyphae (%)	Spore density / 50 g soil (%)
Umdihar	9.91±1.23b	4.24±0.87b	52.49±2.84c	4.11±1.11b	537.66±65.83 d
Umsaw	21.99±1.57a	8.46±0.95b	58.95±2.01c	1.84±0.97b	1939.66±58.37f
Mawlein	17.42±1.31a	9.30±1.14b	50.91±2.84c	1.99±0.40b	1163.00±177.88g

Tukey test showing different alphabetical letters varies significantly ($p < 0.05$)

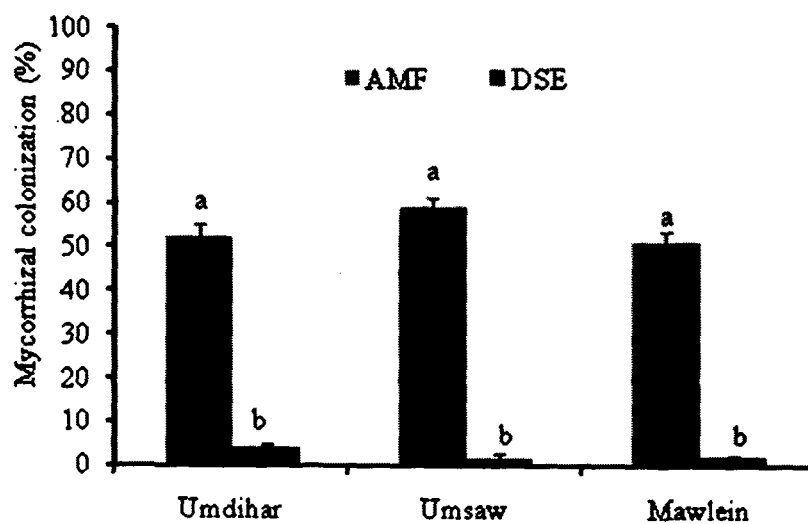


Figure 4.2: Mycorrhizal colonization in the roots of *Michelia champaca* from three sites

Tukey test showing different alphabetical letters varies significantly ($p < 0.05$)

Acaulospora, *Ambispora*, *Entrophospora*, *Gigaspora*, *Glomus*, *Pacispora*, and *Paraglomus* were extracted from three plantation sites of *M. champaca* (Figure 4.3&4.4). A total of 29 species were identified from all the soil samples (Table 4.3). Out of seven genera, four, five and six were isolated from Umdihar, Umsaw and Mawlein, respectively. *Glomus macrocarpum*, *G. multicaulis*, *G. constrictum*, *Acaulospora* sp 1 and *Pacispora chimonobambusae* were present in all the sites. The isolation frequencies of five, twelve and other 12 numbers of species were 100, 66.67 and 33.33 %, respectively. *Acaulospora* sp 1 is relatively more abundant in Umdihar than other species

and *G. macrocarpum* is comparatively abundant than other species in Umsaw and Mawlein.

Table 4.3. Relative abundance and isolation frequency of arbuscular mycorrhizal fungi from three plantation sites

AMF Species	Species abbreviation	Relative abundance (%)			Isolation frequency (%)
		Umdihar	Umsaw	Mawlein	
<i>Acaulospora</i> sp 1	A1	27.91	0.46	5.39	100.00
<i>A. bireticulata</i> Rothwell & Trappe	Ab	0.20	-	-	33.33
<i>A. rehmi</i> Sieverd. & Toro	Ar	-	0.26	0.29	66.67
<i>A. foveata</i> Janos & Trappe	Af	0.40	0.07	-	66.67
<i>A. lacunosa</i> Morton	Al	10.04	-	-	33.33
<i>A. tuberculata</i> Janos & Trappe	At	-	0.13	2.04	66.67
<i>A. cavernata</i> Blaszk.	Ac	-	-	0.29	33.33
<i>Ambispora</i> sp 1	Aml	-	-	0.15	33.33
<i>Entrophospora colombiana</i> Spain & Schenck	Ec	-	0.13	-	33.33
<i>Gigaspora</i> sp 1	Gi1	-	0.07	1.02	66.67
<i>Glomus</i> sp 1	G1	0.4	0.40	-	66.67
<i>Glomus</i> sp 2	G2	-	-	0.44	33.33
<i>Glomus</i> sp 3	G3	23.49	-	-	33.33
<i>G. aggregatum</i> Schenck & Sm. emend. Koske	Gag	0.20	-	-	33.33
<i>G. ambisporum</i> Smith & Schenck	Gam	6.02	-	-	33.33
<i>G. aureum</i> Oehl & Sieverd.	Gau	-	0.07	0.15	66.67
<i>G. constrictum</i> Trappe	Gcon	9.04	9.26	13.83	100.00
<i>G. fuegianum</i> (Speg.) Trappe & Gerd.	Gfu	-	-	0.15	33.33
<i>G. glomeratum</i> Sieverd.	Gglo	-	2.67	1.6	66.67
<i>G. intraradices</i> Schenck & Smith	Gin	-	0.07	1.31	66.67
<i>G. macrocarpum</i> Tul. & Tul.	Gmac	13.05	45.11	32.02	100.00
<i>G. microaggregatum</i> Koske, Gemma & Olexia	Gmic	-	-	0.58	33.33
<i>G. mosseae</i> (Nicol. & Gerd.) Gerd. & Trappe	Gmos	0.40	0.13	-	66.67
<i>G. multicaulis</i> Gerdemann & Bakshi	Gm	8.43	32.79	26.49	100.00
<i>G. taiwanense</i> (Wu & Chen) Almeida & Schenck	Gtaw	-	2.80	0.44	66.67
<i>G. tortuosum</i> Schenck & Smith	Gto	-	5.93	10.77	66.67
<i>Pacispora boliviana</i> Sieverd. & Oehl	Pb	-	-	1.60	33.33
<i>P. chimonobambusae</i> (Wu, Liu, Hwuang, Wang & Chao) Sieverd. & Oehl	Pc	0.20	0.07	0.58	100.00
<i>Paraglomus occultum</i> (Walker) Morton & Redecker	Po	0.20	-	0.87	66.67
Total species	29				

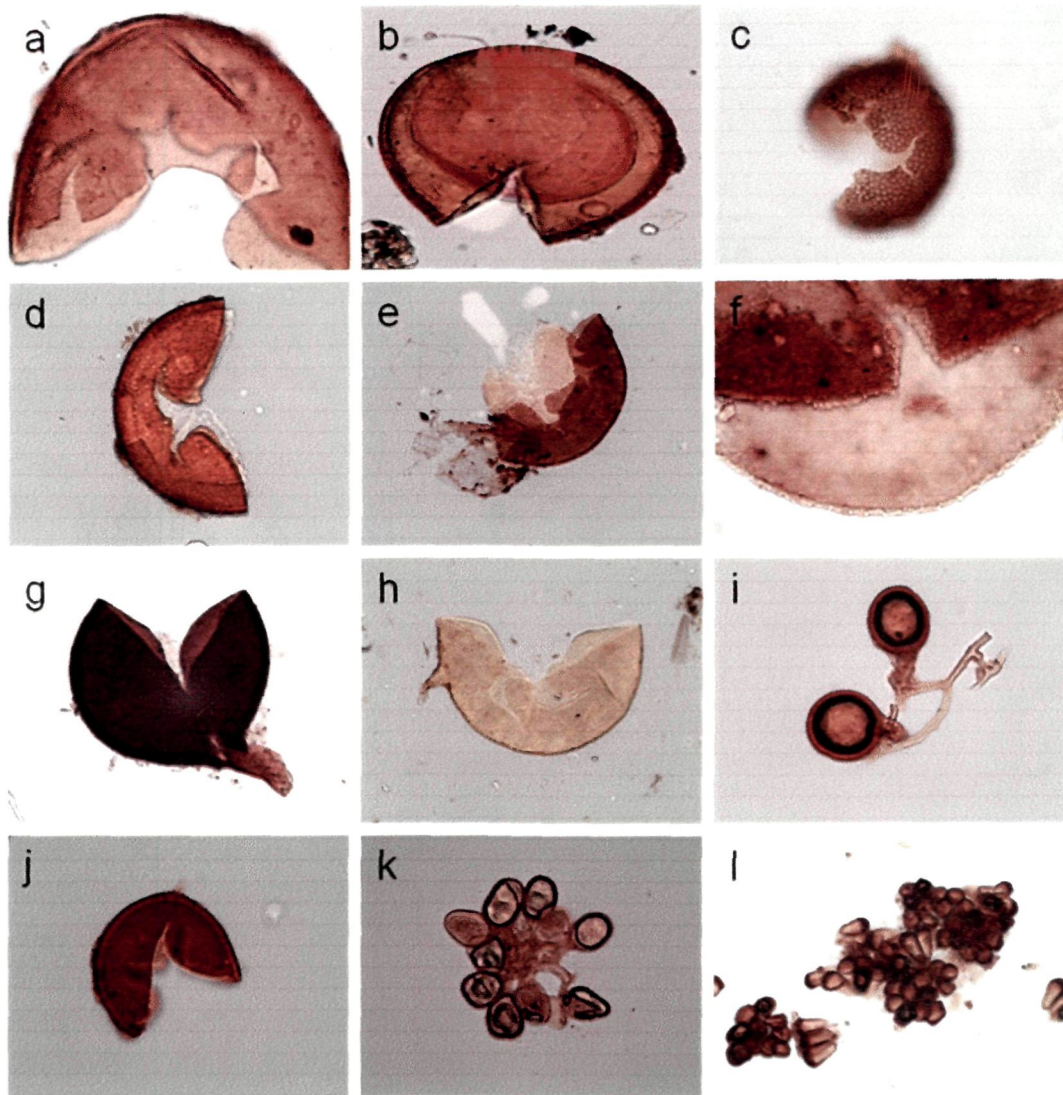


Figure 4.3. Arbuscular mycorrhizal spores. (a) *Acaulospora tuberculata* with cicatrix. Bar scale = 200 μm . (b) *A. lacunosa* with cicatrix and wall layers. Bar scale = 150 μm . (c) *A. rehmsii* with cicatrix. Bar scale = 100 μm . (d) *A. foveata*. Bar scale = 150 μm . (e) *A. cavernata* with saporiferous saccule. Bar scale = 200 μm . (f) *A. bireticulata*. Bar scale = 50 μm . (g) *Glomus constrictum* Bar scale = 150 μm . (h) *G. mosseae*. Bar scale = 200 μm . (i) *G. multicaulis*. Bar scale = 150 μm . (j) *Glomus* sp 1. Bar scale = 100 μm . (k) *G. feugianum*. Bar scale = 100 μm . (l) *G. taiwanense*. Bar scale = 200 μm .

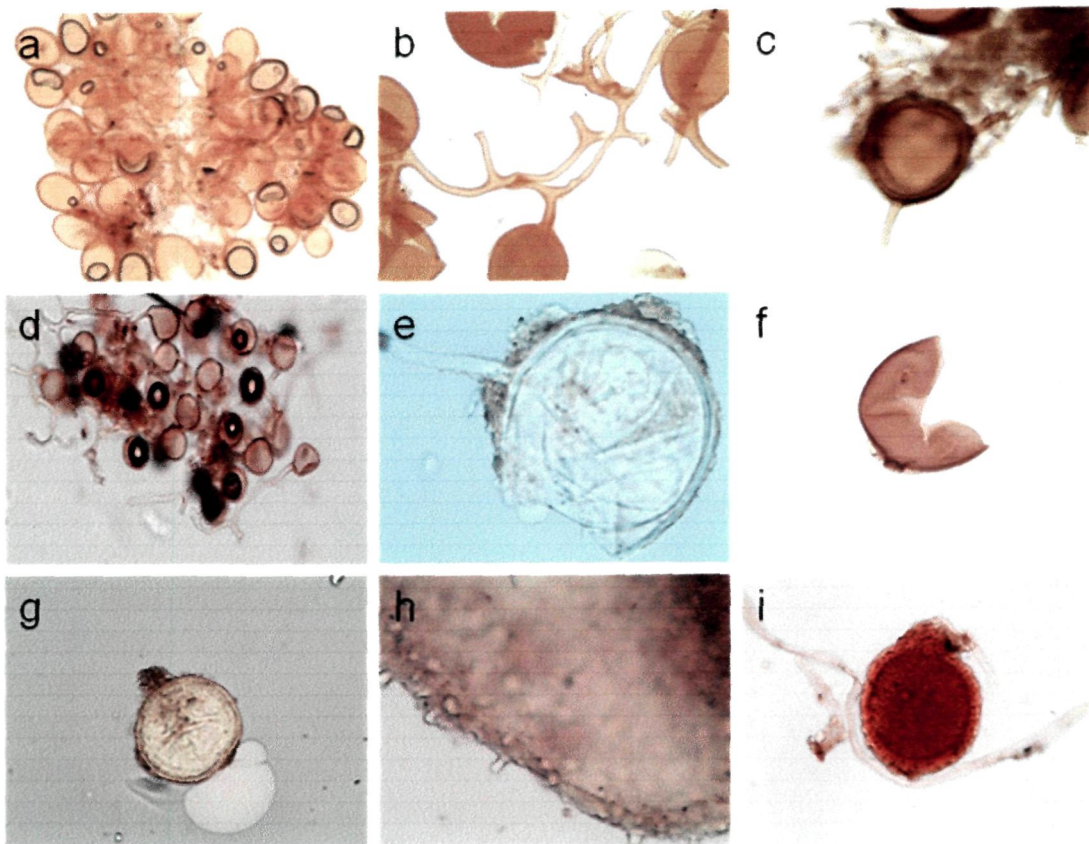


Figure 4.4. Arbuscular mycorrhizal spores isolated from the plantations. (a) *G. aureum*. Bar scale = 100 μm . (b) *G. macrocarpum*. Bar scale = 100 μm . (c) *G. glomeratum*. Bar scale = 50 μm . (d) *G. microaggregatum*. Bar scale = 250 μm . (e) *Paraglomus occultum*. Bar scale = 100 μm . (f) *Entrophospora colombiana*. Bar scale = 150 μm . (g) *Ambispora* sp 1. Bar scale = 100 μm . (h) *Pacispora chimonobambusae*. Bar scale = 50 μm . (i) Unidentified Bar scale =100 μm .

Ambispora sp 1, *Entrophospora colombiana* and *G. fuegianum* were lower in relative abundance. Significant positive correlation ($p = 0.001$) was found between relative abundance and species richness of AMF spores (Figure 4.5). *Glomus* exhibited high relative abundance and high species richness, whereas *Entrophospora* and *Ambispora* were the lowest in terms of abundance and species richness (Table 4.4). In addition, species richness increased with the increase in relative abundance as depicted in the plot (Figure 4.5).

Table 4.4. Species richness and relative abundance of arbuscular mycorrhizal fungi associated with *Michelia champaca*

AMF species	Species richness	Relative abundance (%)
<i>Glomus</i>	9.7	89.14
<i>Acaulospora</i>	4.0	9.60
<i>Pacispora</i>	1.3	0.63
<i>Gigaspora</i>	0.7	0.26
<i>Paraglomus</i>	0.7	0.26
<i>Ambispora</i>	0.3	0.04
<i>Entrophospora</i>	0.3	0.07
Total	17	100

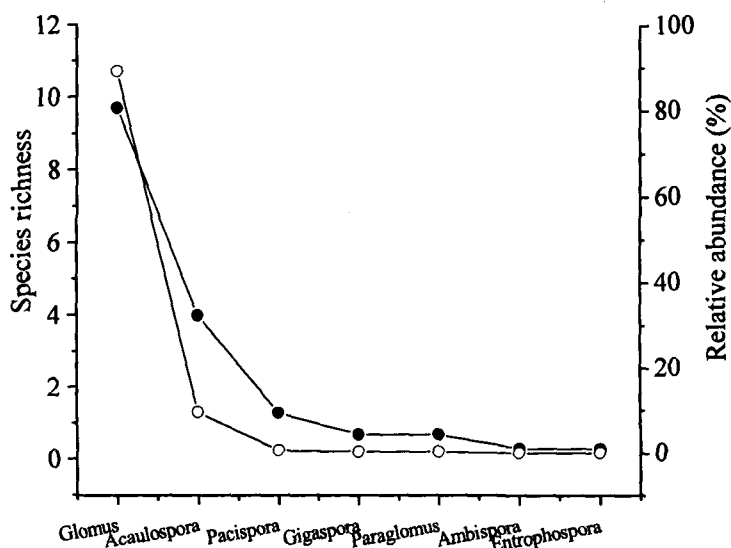


Figure 4.5: Relation between species richness and relative abundance of arbuscular mycorrhizal fungi

Sorenson coefficient varied between the sites. Moreover, high C_s was observed between nearest sites and the lowest value between distant sites. The dissimilar number of AMF species between Umdihar X Umsaw, Umsaw X Mawlein and Umdihar X Mawlein were 15, 11 and 22, respectively. The similar number of species between Umdihar X Umsaw, Umsaw X Mawlein and Umdihar X Mawlein were eight, 13 and six, respectively (Figure 4.6).

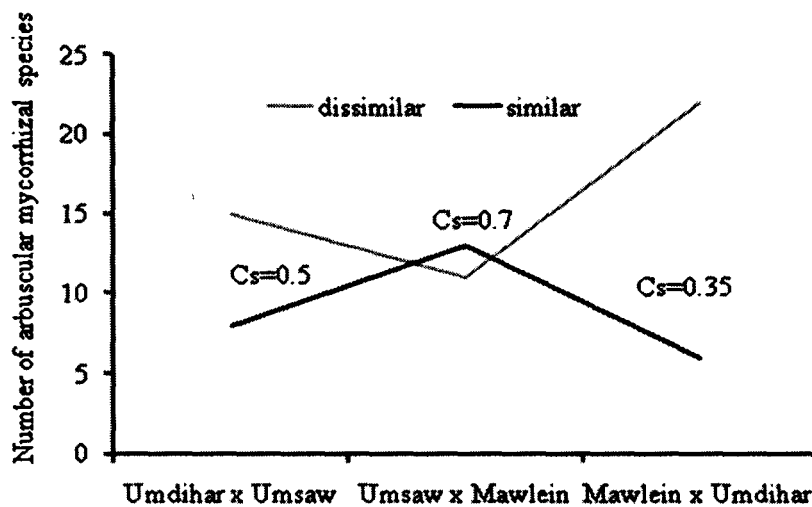


Figure 4.6: Sorenson coefficient (C_s) of arbuscular mycorrhizal fungi in the three plantations

The highest species richness was observed in the plantations of Mawlein (Table 4.5). Diversity index reveals that H' and E were high in Umdihar and Mawlein, however, D was high in Umsaw (Table 4.5). PCA plot showed the distribution of AMF species and host preference (Figure 4.7). The variability in the distribution existed between species, where *G. macrocarpum*, *G. multicaulis*, *G. constrictum* and *Acaulospora* sp 1 accounted for 78.57% of the total variation. The other species were clumped together in one spot, indicating no variation. PCA plot showed close relation of the four species to the sites. *G. macrocarpum* and *G. multicaulis* were closely related to Mawlein and Umsaw. *Acaulospora* sp 1 was closely correlated with Umdihar, while *G. constrictum* was

relatively associated with Mawlein and Umsaw. Moreover, the entire three axes showed that these four species were distributed in all the sites.

Table 4.5. Diversity index of arbuscular mycorrhizal fungi associated with *Michelia champaca*

Sites	Species richness	Shannon - Wiener index of diversity (H')	Simpson's index of diversity (D)	Evenness (E)
Umdihar	2.0	1.9	0.18	0.38
Umsaw	2.4	1.4	0.32	0.20
Mawlein	2.9	1.9	0.21	0.43

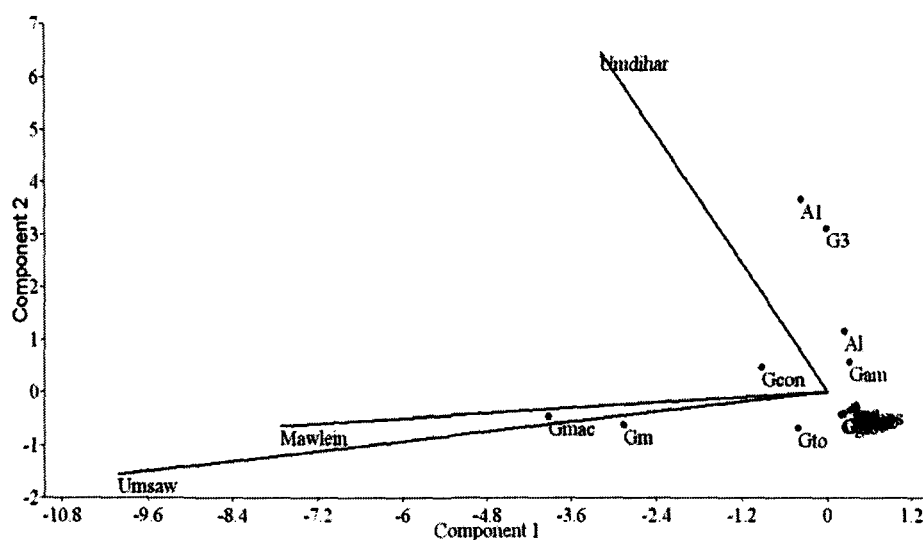


Figure 4.7: Array of the relative abundance of AMF species distribution in the three plantation sites, which were determined by PCA biplot. For other abbreviations, see Tab. 4.3.

Discussion

M. champaca was colonized by AMF and DSE in all the sites. Presence of DSE and AMF confirms with the findings of Muthukumar *et al.* (2006). Mycorrhizal colonization recorded in the present study was less than the earlier report (Muthukumar *et al.*, 2006). Moreover, our results showed that the AMF colonization was significantly higher than

DSE colonization. This may be because of the presence of coarse structure of the root, the characteristic feature of Magnoliales, which favors mycorrhizal infection (Baylis, 1975). The results suggest that mycorrhizal colonization of *M. champaca* in different plantations recorded in the investigation had a narrow range i.e., intermediate AMF colonization (50.91%–58.95%) and low in DSE (1.84%–4.1).

There was no correlation found between mycorrhizal colonization, soil properties, CBH and H. It implies that AMF colonization may be affected by the comprehensive interactions of several factors, such as the factors inherent to the host plant, climatic and edaphic factors, and effects of the soil community (Moreira *et al.*, 2006).

The spore number varied significantly between the sites. This might be due to production of AMF spores in the rhizosphere vicinity of surrounding herbaceous species (Kruckelmann, 1975). Presence of herbaceous community in Umsaw and Mawlein were observed but in Umdihar spore density and herbaceous community were comparatively lower. The herbaceous community was removed or not frequent in Umdihar, however, the spore density could be disturbed as the site was located very near to national highway No. 44. Moreover, in an undisturbed ecosystem, higher spore population was quite natural as the number of AMF spores and the population diversity were higher in native undisturbed forests than the disturbed and replanted areas (Moreira-Souza *et al.*, 2003). Spore population is affected by a wide range of soil, climatic, fungal and host factors (Anderson *et al.*, 1983; Howeler *et al.*, 1987). Plant phenology and root production are closely related to the patterns of spore production and spore quantity (Brundrett, 1991).

Out of seven genera, *Glomus* was the most widely distributed genera, followed by *Acaulospora* and *Pacispora*. *Glomus* sporulated abundantly regardless of the sites selected. Das and Kayang (2009) also reported the dominance of the *Glomus* from this region. They described the wider adaptation of the taxon in varied soil conditions. The

sporulation pattern of *Glomus* might bring about the dominance of the taxon. Spores of *Glomus* grow in cluster and sporulate more frequently while the spores of other genera like *Gigaspora* sporulated singly (Dhar and Mridha, 2006).

The altitudinal variation and distances of plantation sites could play an important role in the similarities of AMF species. Higher similarity coefficient (Cs) was found between the nearest sites (Umsaw and Umdihar) with a relatively low altitude, and lower between the distant sites (Umdihar and Mawlein) with a relatively high altitude. In contrast, the results of An *et al.* (2008) showed that highest similarity index was observed between the most distant sites and the lowest value was observed between the nearest sites.

In the present study, diversity attribute of *M. champaca* plants such as H' and E were higher in Mawlein and Umdihar than in Umsaw. The variation in the diversity attribute can be substantiated to the study of Allen and Boosalis (1983) where the diversity of mycorrhizal fungi associated with the same plants was found to vary.

G. macrocarpum, *G. multicaulis*, *G. constrictum*, *Acaulospora* sp 1 and *P. chimonobambusae* were most frequently distributed in the three sites, of which *G. macrocarpum*, *G. multicaulis*, *G. constrictum* and *Acaulospora* sp 1 were relatively abundant. However, *P. chimonobambusae* was less abundant than other four species. PCA plot with relative abundance of AMF species in all three sites showed that there was close relation between the sites and the highly abundant species. The distribution and relative abundance indicate that these four species may favour *M. champaca*.

Isolation, characterization and identification of the arbuscular mycorrhizal fungi associated with *Michelia champaca*

Introduction

The diversity of arbuscular mycorrhizal fungi (AMF) has been investigated in many ecosystems, including arable sites (Helgason *et al.*, 1998; Franke-Snyder *et al.*, 2001), conservation and forest lands (Helgason *et al.*, 1998; Zhao *et al.*, 2001), and saline and spoil soils (Wang and Liu, 2001). Usually, AMF diversity in farm or degraded soils has been shown to be lower than in soils supporting a diverse flora of native plants (Helgason *et al.*, 1998; Liu and Li, 2000). In addition, Genney *et al.* (2001) noted that the degree of AMF colonization was related to host density in the field. Whether the host can influence diversity of mycorrhizal fungi under controlled conditions is an important issue as different researchers often use different test plants for studies on AMF diversity (Helgason *et al.*, 1998; Frank-Snyder *et al.*, 2001). AMF trap cultures can be very helpful in unveiling fungal community members that are undetected in initial extraction of spores from field soil (Morton *et al.*, 1995). Although many AMF are thought to have a broad host range, the appropriate test plants for trap cultures should be evaluated to ensure maximum detection of fungal species in specific soils or site types.

Most of the 214 currently described species (www.amf-phylogeny.com) are characterized only by spore morphology and the majorities have not yet been cultured. Some AMF are broadly effective while others, though they colonize many host species, may promote the growth of only a few (Sylvia, *et al.*, 199; Bever, 2000). Therefore, the first consideration in inoculum production is the choice of the fungal isolates which are capable of growth promotion of a given plant. Thus preliminary trails on a variety of isolates are required to find out the most effective ones. Another biological consideration in the production of inoculum is the host plant upon which the fungus will grow (Ryan

and Graham, 2002). The degree of importance of this consideration also depends upon the growth system. Mass propagation of AMF varies greatly on root structure, and habitat of host plant (Bever *et al.*, 1996).

Producing microbial inocula is a complex procedure that involves not only the development of the necessary biotechnological expertise, but also the ability to respond to the specifically related legal, ethical, educational, and commercial requirements. This is particularly true in the case of obligate endosymbiotic microorganisms such as AMF, because satisfying the aforementioned requirements is closely associated with the particular method of inoculum production (Gianinazzi and Vosátka, 2004).

Basically, the formulation procedure consists of placing fungal propagules (root fragments colonized with AMF, fragments of fungal mycelium, and spores) in a given carrier (perlite, peat, inorganic clay, zeolite, vermiculite, sand, etc.) for a given application. Biological inoculants belong to diverse taxonomic groups varying considerably in physiology and, as a consequence, in their nutritional and environmental requirements. Therefore, the final configuration of the formulation will result from a more or less technologically complex procedure, determined by the microbe involved, the way of producing inoculum, and the target inoculum application. The fungi should be selected to be compatible with the target environment (Requena *et al.*, 1996; Estaún *et al.*, 2002; Vosátka and Dodd, 2002).

The reasons for the development of this agricultural biotechnology industry producing AMF inocula are multiple: (i) AMF are increasingly being considered as a natural plant health insurance (Gianinazzi and Gianinazzi-Pearson, 1988), and examples of their positive impact on plant development and health, land reclamation, and phytoremediation are continually increasing (Leyval *et al.*, 2002; Turnau and Haselwandter, 2002); (ii) there is higher awareness of biodiversity issues, including those

concerning soil microbial communities, and acceptance of these natural technologies as alternatives to agrochemicals (Barea, 2000; Gryndler, 2000); and (iii) society is demanding more sustainable means of production, with a consequent feedback to farmers and land conservationists.

However, to evaluate the efficacy of native isolate of AMF, there was need to isolate from natural soil.

Materials and Methods

Propagation of AMF spores through trap culture

Soils from the seedlings of *Michelia champaca* were collected from nursery of Social Forestry Division, Upper Shillong, Meghalaya. The spores were extracted by modified wet sieving and decanting method (Muthukumar *et al.*, 2006) from 25 g of soil. For single species culture, the isolated spores were picked up with needle on top of slide and were observed for similar morphology i.e., colour, size and ornamentation.

The method of propagating AMF through trap culture were followed from the method given by INVAM (International Culture Collection of arbuscular and vesicular – arbuscular mycorrhizal fungi). Seeds of *Paspalum notatum* Flüge procured from IGFR (Indian Grassland and Fodder Research Institute, Palampur, India) were surface-sterilized, using 10% solution of household bleach: 1 volume of bleach (containing 5 % sodium hypochlorite) plus 9 volumes of water. The seeds were soaked in the solution for approximately 5–10 minutes. Then seeds were germinated on double sterilized river bed sand: pine forest soil (1: 1 vol: vol) in 3 kg garden pots was used as trap plants.

The same spore morphotypes of isolates extracted from soil were incorporated on the root of germinated seedlings of *P. notatum* for monospecific culture and transferred in 500 ml disposable cups containing double sterilized soil and sand mixtures. In addition, a starter-culture of *Glomus intraradices* Schenck & Smith AM WG19 obtained from TERI

(The Energy and Resource Institute, India) were placed on double sterilized river bed sand: pine forest soil (1:1 vol: vol) on 3 kg garden pots which were inoculated by spreading 2-3 cm below the seeds with 20 g of supplied mycorrhizal inoculants. 50 seeds were placed in each pot. The set up was maintained in a well lighted area under a rain shelter to minimize contamination from microorganisms carried by rain or wind. The plants were watered whenever it was required. Two generation of spores were maintained in 500 ml disposable cups i.e., for 4 months each cycle (Figure 5.1a&b). After 8 months they were transferred in 3 kg pots containing spores from nursery for mass inoculum propagation of the two isolated species (Figure 5.1c&d). In addition, *G. intraradices* was maintained only for one cycle in 3 kg garden pots for mass inoculum propagation (Figure 5.1e).

Root processing

To confirm that mycorrhizal associations have formed, several roots were excavated containing fine branches after six weeks and for determination of mycorrhizal associations. The root samples were washed in tap water, processed and stained with black Faber Castell stamp pad ink (Das and Kayang, 2008). Root tissues were quantified for colonization under Olympus (41209) microscope by magnified intersection method (McGonigle *et al.*, 1990).

Spore analysis

The spores were extracted by modified wet sieving and decanting method (Muthukumar *et al.*, 2006). The isolated spores were picked up with needle in polyvinyl alcohol-lactoglycerol under a dissecting microscope (Koske and Tessier, 1983) and also in mixed polyvinyl alcohol- lactoglycerol: Meltzer's reagent (1: 1, v: v) for identification. The complete and broken spores were examined using a compound microscope, Olympus.

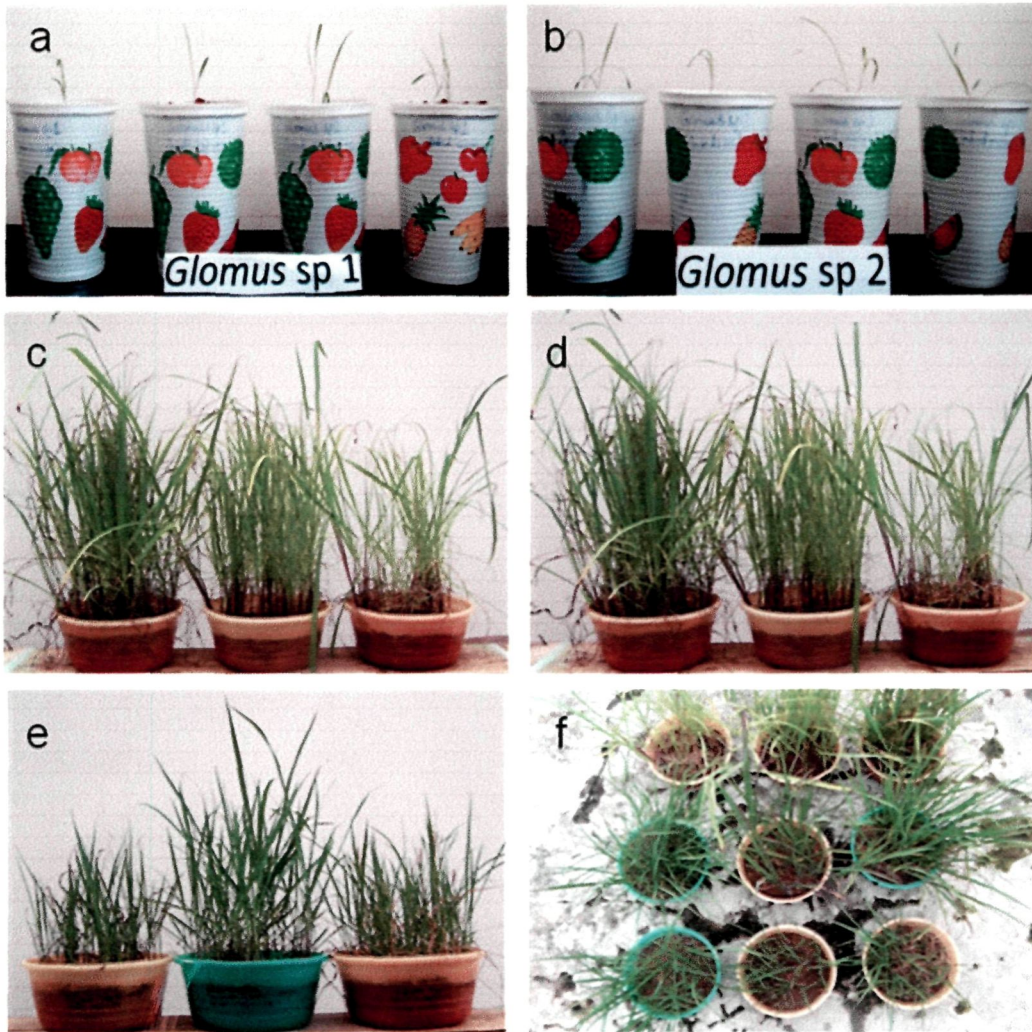


Figure 5.1: (a&b) *Paspalum notatum* used as trap plants for isolation of *Glomus* sp 1 and *Glomus* sp 2 in the first cycle. *P. notatum* used as the trap plants for AMF spore propagation. (c) *Glomus* sp 1 (d) *Glomus* sp 2 (e) *Glomus intraradices* and (f) Pots showing trap plants.

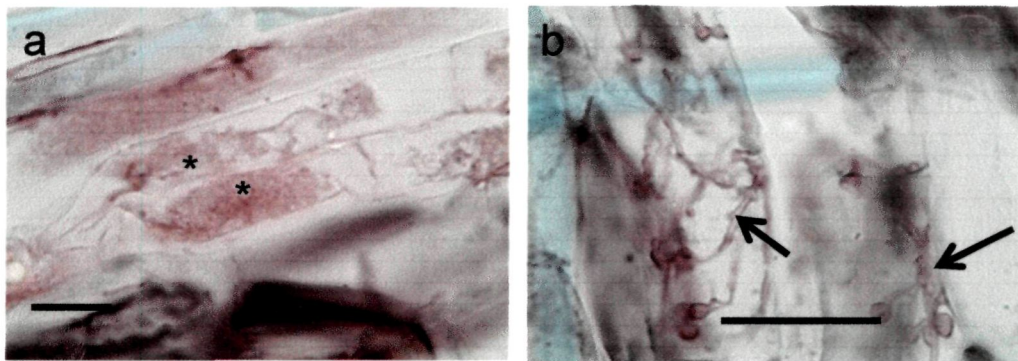


Figure 5.2: Arbuscular mycorrhizal colonization in the root of *Paspalum notatum*.

(a) Portion showing arbuscules (asterisk). Scale bar=100 μm . (b) Intracellular hyphal colonization (arrow). Scale bar=50 μm .

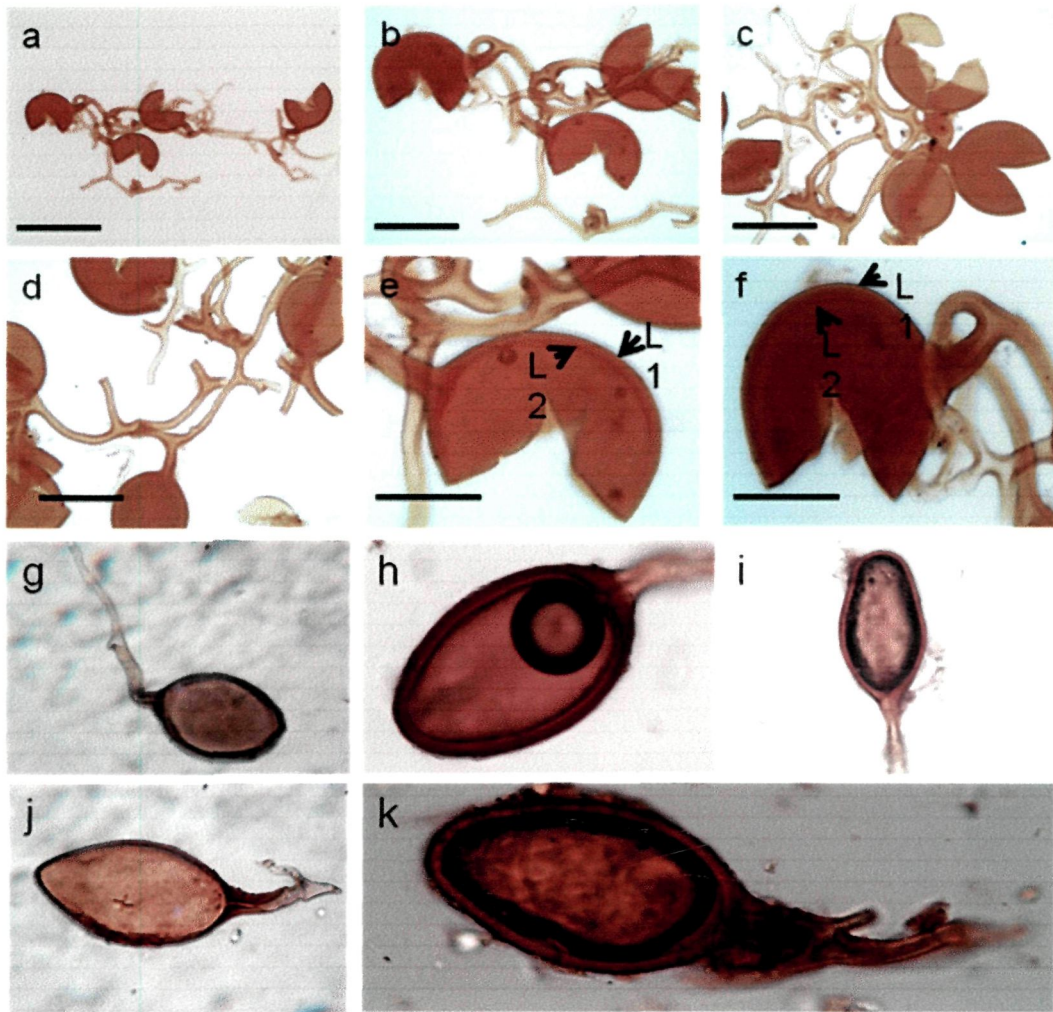


Figure 5.3: (a-f) Spores of *Glomus* sp 1 observed after isolation using trap plant culture. Scale Bar=200, 100, 100 and 100 μm. (e&f) *Glomus* sp 1 showing layer 1 (L1) and layer 2 (L2). Scale Bar=50 μm each. (g-k) Spores of *Glomus* sp 2 observed after isolation using trap plant culture. (a) Scale bar= 100 μm. Scale bar=50 μm all.

Taxonomic identification of spores to species level was based on sporocarpic size, colour, and ornamentation and wall characteristics by matching original descriptions (<http://www.invam.caf.wvu.edu> & <http://www.lrz-muenchen.de/~schuessler/amphylo>).

Inoculum

The plants were harvested after 12 months and the soil medium and roots from the pots were placed into a clean tray. The dried roots were cut with scissors, mix the fragments with the soil medium, and stored as inoculum in a refrigerator at 4°C packed in air tight zipped polythene bags.

Results

The single spore culture in the small pots was evaluated to possess successfully in case of *Glomus* sp 1 in the first cycle. However, in the first cycle of spores in *Glomus* sp 2 was found to be contaminated by a different morphotype of spore varying 50-80 µm in size. The second cycle showed single morphotype of *Glomus* sp 2. The transfer of both the types of spore in the third cycle in large pots was found to contain pure single spores and sporulation was in abundance. The density of spores in the third cycle was 752 spores of *Glomus* sp 1 per 25g of soil and 962 spores of *Glomus* sp 2 per 25g of soil. AMF structural colonization in trap plants (Figure 5.2) for both the isolated species of AMF were presented in Table 5.1 and 5.2. However, the colonization of both the species of AMF in the root of *P. notatum* did not differ significantly.

Table 5.1. Arbuscular mycorrhizal colonization of *Glomus* sp 1 in the root of trap plant

Spore cycle	Arbuscules (%)	Vesicles (%)	Hyphae (%)
1	30.8±1.22a	19.4±1.92b	80.4±1.99c
2	30.2±1.53a	21.4±1.93b	80.5±2.42c
3	31.2±1.32a	18.8±2.06b	78.9±2.86c

Fischer LSD test showing different alphabet differs significantly at $p < 0.05$

Table 5.2. Arbuscular mycorrhizal colonization of *Glomus* sp 2 in the root of trap plant

Spore cycle	Arbuscules (%)	Vesicles (%)	Hyphae (%)
1	30.4±1.13a	20.3±1.66b	82.1±1.47c
2	29.1±1.37a	21.3±2.14b	80.3±2.27c
3	30.2±1.16a	20.3±2.13b	80.4±2.12c

Fischer LSD test showing different alphabet differs significantly at $p < 0.05$

The two morphotypes *Glomus* sp 1 (Figure 5.3a-f) and *Glomus* sp 2 (Figure 5.3g-k) isolated from the trap culture technique are described as follows:

***Glomus* sp 1**

Sporocarps in 4-35 distributed spores 350 µm in crushed state, spores yellow subglobose; 110-115 diameter; mostly with one subtending hypha. Peridium not found. Additionally, the spore wall structure of *Glomus* sp 1 comprises two layers, of which outer layer is hyaline. Layer 1 semi flexible, hyaline, 1.83 µm thick, layer 2 smooth, yellow, 4.29 µm thick and Layers 1 and 2 do not react in Meltzer's reagent.

***Glomus* sp 2**

Spores found singly. Spores reddish brown; 50-100 diameter; mostly with one subtending hypha. Peridium not found. Additionally, the spore wall structure of *Glomus* sp 2 comprises of two layers i.e., L1 and L2. Spore oval. Layers 1 and 2 do not react in Meltzer's reagent.

Discussion

For mass inoculation to evaluate the isolate of such obligate symbionts, it is necessary to produce mass production of inoculum. However, at present, the technique generally employed for mass production of AMF inoculum is pot culture. Although, attempts to produce artificial media have met with little success or no success, the only method of large scale inoculum production is an association with host plant. *In vitro* culture of AMF

was achieved for the first time in the early 1960s (Mosse, 1962). Since this pioneering work, the use of Ri T-DNA-transformed *Daucus carota* L. (carrot) roots as the host has permitted increases in spore production of *Glomus mosseae* (Mugnier and Mosse, 1987), *Gigaspora margarita* (Bécard and Fortin, 1988) and *Glomus versiforme* (Declerck *et al.*, 1996). Isolation of the fungal symbiont from the plant root partner, using a two-compartment Petri dish, is the most successful way to date of achieving large production of spores (Fortin *et al.*, 1996; St-Arnaud *et al.*, 1996) of *Glomus intraradices*, making these propagules even more attractive for mass-production of inoculum. Nevertheless, in our study pot culture with soil and sand based inoculum was perhaps most appropriate because for evaluation of growth in tree species which could be utilized for afforestation programmes. Moreover, the inoculum requirement was in large scale for such a inoculation programmes.

Of the two species isolated, *Glomus* sp1 possibly resembles morphologically *Glomus macrocarpum*. This species was found to be highly abundant from the plantations and distributed in all the sites. Moreover, it was concluded in earlier chapter that it may facilitate the better growth of host plant. The other species was kept as *Glomus* sp 2. This species was found also in one the plantation site and was abundant in that site. The native AMF species possibly could be more effective than an exotic species. Therefore, an exotic species of *G. intraradices* was also raised along with the native isolates to evaluate the effect on the growth of *M. champaca*. Although AMF differ in their abilities to influence plant growth, it is currently not possible to categorize them along the parasite-mutualist continuum because not all infected plants seem to do "best" with the same AMF (Sanders and Fitter, 1992, van der Heijden *et al.*, 1998a, Kiers *et al.*, 2000, Klironomos, 2000). Even when a few co-occurring plant and AMF taxa are studied, there is a strong interaction between plant and AMF taxa in plant growth

response (Sanders and Fitter, 1992; van der Heijden *et al.*, 1998b). Terrestrial ecosystems contain many AMF and plant species that coexist in communities (Johnson *et al.*, 1991; Allen *et al.*, 1995; Sanders *et al.*, 1996; Helgasson *et al.*, 1998; Picone, 2000; Ergeton-Warburton and Allen, 2000), so the influence of AMF on growth of plants may be very complex. To date, there is little appreciation of the range of plant-growth responses that can occur within any AMF community. Yet, such information would be valuable for the development of plant community models because the variation in AMF-host response within a community is believed to be a driving force behind the structuring of AMF and plant communities (Grime *et al.*, 1987, Bever *et al.*, 1996, van der Heijden *et al.*, 1998b, Marler *et al.*, 1999, Smith *et al.*, 1999).

The isolation of the spores of AMF from the rhizosphere soil of seedlings from nursery resulted in two morphotypes i.e., *Glomus* sp 1 and *Glomus* sp 2.

Determination of the potential of the isolated arbuscular mycorrhizal fungi on the growth of *Michelia champaca*

Introduction

Arbuscular mycorrhizal fungi (AMF) are obligate symbionts that form associations with most the terrestrial plants (Smith and Read, 2008). Their effects on host plants can range from beneficial to antagonistic (Johnson *et al.*, 1997; Jones and Smith, 2004). The beneficial effects are mainly attributed to facilitation of nutrient acquisition, enhanced pathogen resistance, drought stress alleviation, and protection against herbivores (Newsham *et al.*, 1995, Smith and Read, 2008). The negative effects are likely to be complex but seem to be related to the balance between carbon costs and phosphorous uptake via AMF (Li *et al.*, 2008).

AMF were reported to increase the growth of some tropical trees. They increased seedling growth of 23 of 28 species from lowland tropical rain forest in Costa Rica under nursery conditions (Janos, 1980). AMF colonization of the tropical tree *Oubanguia alata* (Scytopetalaceae) was positively correlated with increase in phosphorus (P) uptake despite low P availability in Cameroon (Moyersoen *et al.*, 1998). AMF also improved the growth of the Brazilian pine *Araucaria angustifolia* (Araucariaceae) (Zandavalli *et al.*, 2004). There are also some reports on the improved growth of NTFP tree species after AMF inoculation in tropical forests. Muthukumar *et al.* (2001) reported that the inoculation of *Azadirachta indica* (Meliaceae) with AMF improved plant growth compared with control seedlings. Furthermore, the combination of inoculation of AMF with P solubilising and nitrogen (N)-fixing bacteria increased the growth of *A. indica*. Conversely, *A. excelsa* inoculated with AMF (without fertilizer) grew more slowly than controls at low P availability (Huat *et al.*, 2002). Kashyap *et al.* (2004) showed that the

inoculation of *Morus alba* (Moraceae) with AMF, *Azotobacter*, and indole butyric acid increased the survival of saplings.

Weber *et al.* (1995) reported that the inoculation of three Apocynaceae species with AMF almost doubled plant growth compared with control seedlings. Guadarrama *et al.* (2004) showed that the inoculation of the late pioneer tropical tree species *Stemmadenia donnell-smithii* (Apocynaceae) with AMF increased survival rate and biomass. Tawaraya *et al.* (2003) found the high natural AMF colonization of native species including *Gonystylus bancanus* (Thymelaeaceae) in the peat swamp forests of Kalimantan, suggesting a possibility of growth improvement of these species by AMF inoculation. AMF can accelerate the establishment of the planting stocks of *Dyera polyphylla* and *Aquilaria filaria*, thereby promoting their conservation ecologically and sustaining the production of these NTFPs economically (Turjaman *et al.*, 2006).

The rapid production of forest tree seedlings of high quality in nurseries is important for reversing the current degradation of natural forests, woodlands and scrublands in the tropics. Indigenous tree species are often slower growing than exotics and are more difficult to raise in nurseries. A recent study (Michelsen, 1992) indicates that nursery conditions in the tropics are not always optimal for quality seedling production. The soils used to raise seedlings may originate from an area without vegetation, a sub soil or may have been stored, resulting in reduction in microbial populations including AMF. Some earlier studies (Bagyaraj *et al.*, 1989; Cuenca *et al.*, 1990; Reena and Bagyaraj, 1990; Young, 1990; Wilson *et al.*, 1991; Michelsen, 1993; Vasanthakrishna *et al.*, 1994) used AMF in nurseries. Host preference has been reported in many forest tree species like *Casuarina equisetifolia* (Vasanthakrishna *et al.*, 1995); *Tectona grandis* (Rajan *et al.*, 2000); and a few medicinal plant species like *Phyllanthus amarus* and *Withania somnifera* (Earanna, 2001), *Garcinia indica* (Lakshmipathy *et al.*,

2003) *A. angustifolia* (Zandavalli *et al.*, 2004) and *Coleus forskohlii* (Sailo and Bagyaraj, 2005), *Ploiarium alternifolium* and *Calophyllum hosei* (Turjaman *et al.*, 2008), and *Eucalyptus globulus* (Arriagada *et al.*, 2009).

Hence, in the present investigation, attempts were undertaken to screen and select an efficient AMF for inoculating *Michelia champaca* for its propagation.

Materials and Methods

Plant materials and growth conditions

During August, 2009, seeds of *M. champaca* were collected from Agronomy Division, ICAR (Indian Council of Agricultural Research), Umiam in Ribhoi District of Meghalaya, northeast India (N25°38'; E91°52'; 850 m.a.s.l). In September, 2009, seeds (Figure 6.1) were disinfected with 1% sodium hypochlorite; seeds were then soaked in distilled water for two days. The arils (Figure 6.1) of seed were removed to enhance maximum germination (Candiani *et al.*, 2004) and 8-10 seeds were placed on double sterilized river bed sand: pine forest soil (1:1 vol: vol) in 200 ml disposable plastic containers. The germination set up were kept in B.O.D. incubator at 25°C under white fluorescent tubes (photoperiod 12 h) and watered whenever it was required to keep the soil mixture moist. After one month, about 90 % of the seeds were observed to germinate (Figure 6.2) and further allowed to grow for another one month so that all the seedlings attain more or less equivalent growth (Figure 6.3).

Trap culture

Soils from the seedlings of *M. champaca* L. were collected from nursery of Social Forestry Division, Upper Shillong, Meghalaya. The spores were extracted by modified wet sieving and decanting method (Muthukumar *et al.*, 2006) from 25 g of soil. For one species culture, the isolated spores were picked up with needle on top of slide and were observed for similar morphology i.e., colour, ornamentation and wall characteristics. The

method of propagating AMF through trap culture were followed from the method given by INVAM (International Culture Collection of arbuscular and vesicular – arbuscular mycorrhizal fungi). Seeds of *Paspalum notatum* Flügge procured from IGFRI (Indian Grassland and Fodder Research Institute, Palampur, India) were surface-sterilized, using a 10% solution of household bleach: 1 volume of bleach (containing 5 % sodium hypochlorite) plus 9 volumes of water. The seeds were soaked in the solution for approximately 5–10 minutes.

The seeds were then germinated on double sterilized river bed sand: pine forest soil (1:1 vol: vol) in 3 kg garden pots was used as trap plants. The trap culture technique used for isolation of AMF spore yield two morphotypes: *Glomus* sp 1 and *Glomus* sp 2. For bulk production of inoculum, 50-60 seeds of *P. notatum* procured from were placed on double sterilized river bed sand: pine forest soil (1:1 vol: vol) on 3 kg garden pots which were inoculated by spreading 2-3 cm below the seeds with 20 g of supplied mycorrhizal inoculants *Glomus intraradices* Schenck & Smith AM WG19 collected from TERI (The Energy and Resource Institute, India) and the two isolated native species.

The germinated seedlings of *M. champaca* were transferred in sterilized 2 kg garden bags containing same sand and soil mixture. On November, 2009, inoculation was done with *G. intraradices* by adding 10 g of dried crushed inoculum raised with *P. notatum* all around the root of *M. champaca*, dug with glass rod of about 12 cm deep and seedling was placed in it (Figure 6.3). The set up was maintained in green house and watered whenever it was required every week. The non mycorrhizal plants were grown only in sterilized sand and soil. After 180 and 270 days plants were harvested for plant growth and root structure assessment.



Figure 6.1: (a-d) Seeds of *Michelia champaca*. (e-g) Arrilated seeds of *Michelia champaca*



Figure 6.2: (a-f) Germination of seeds of *Michelia champaca*

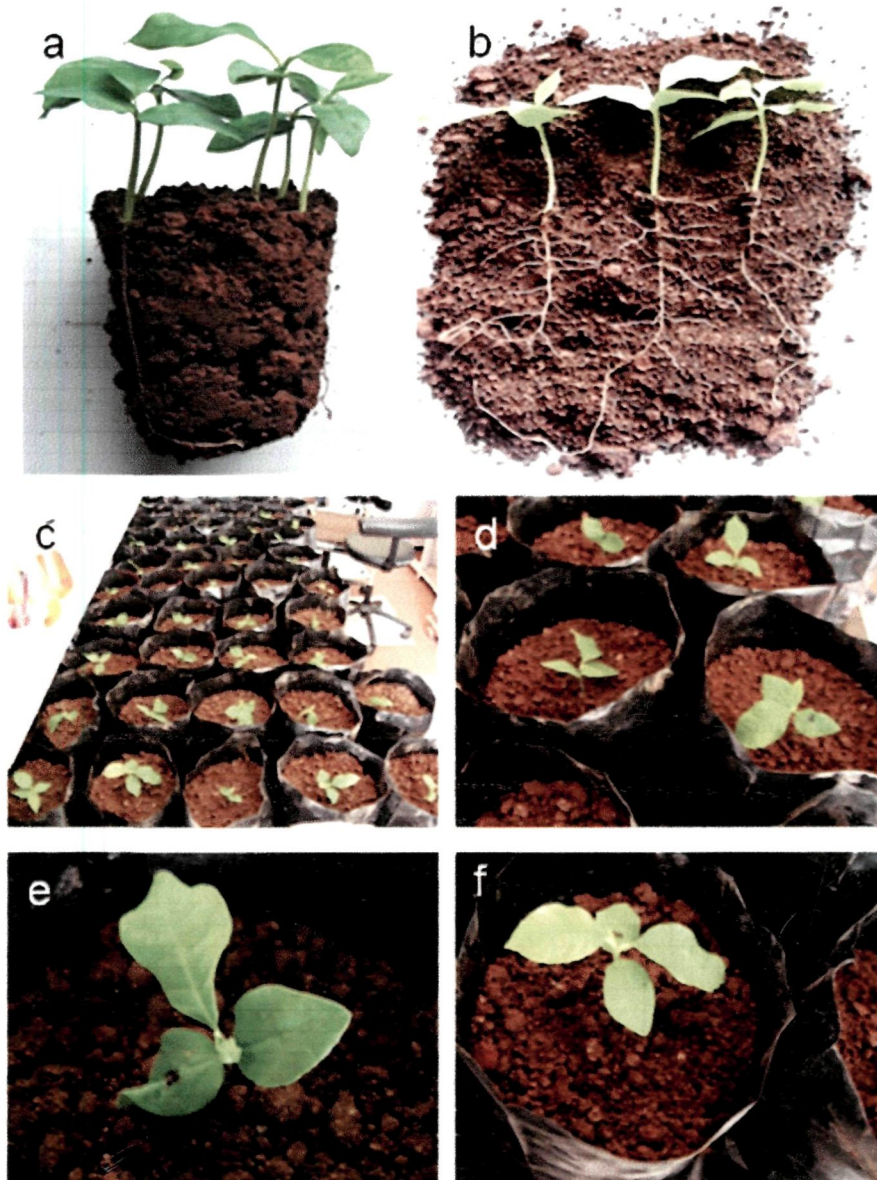


Figure 6.3: (a&b) Germination and uniform growth of plants before inoculation. (c-f) Plants after inoculation in laboratory.

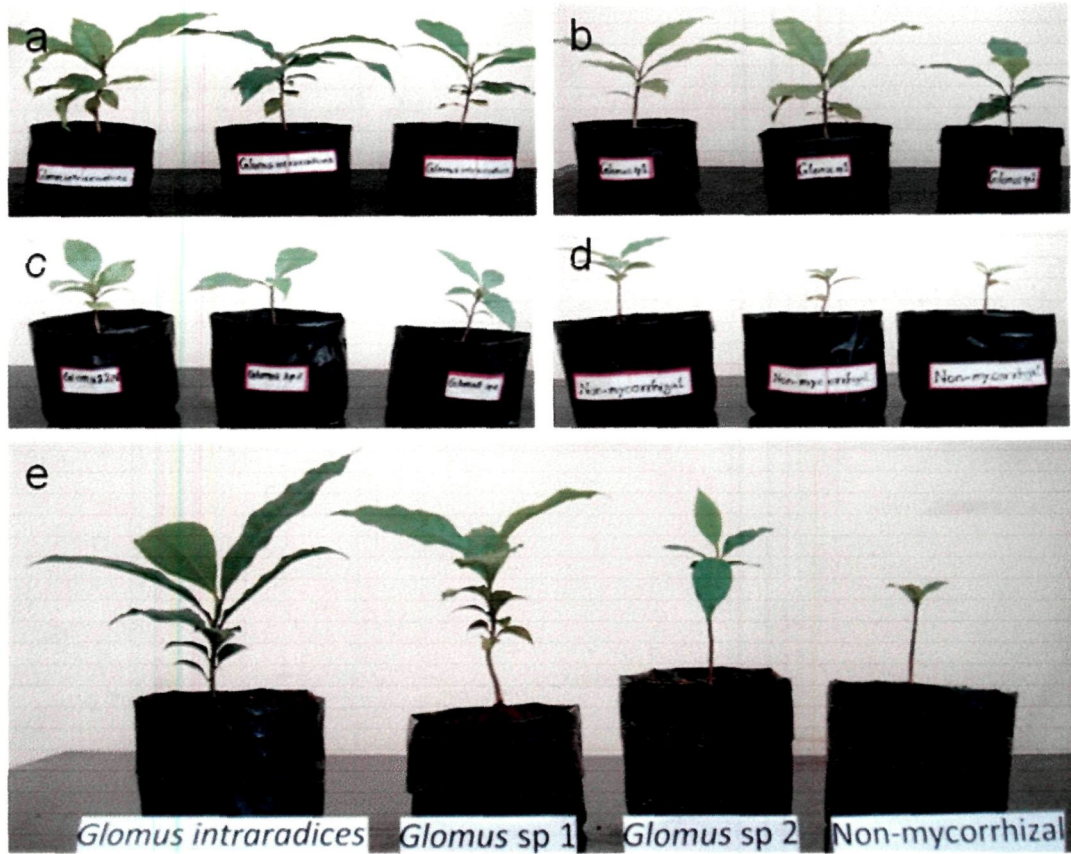


Figure 6.4: Mycorrhizal and non-mycorrhizal seedlings of *Michelia champaca*. (a) Plants growing with inoculum of *Glomus intraradices*. (b) Plants inoculum of *Glomus* sp 1. (c) Plants growing with inoculum of *Glomus* sp 2. (d) Non-mycorrhizal plants. (e) Comparison of mycorrhizal and non-mycorrhizal seedlings.

Data analysis

Means and standard errors were calculated for the aboveground and belowground plant parameters as well as mycorrhizal colonization in mycorrhizal inoculated seedlings and trees from plantations were calculated. Analysis of variance (ANOVA) was conducted to analyse the difference between growth parameters in mycorrhizal and non mycorrhizal plants and means were separated using *post-hoc* test (Fischer's LSD). All the statistical analysis was done using Statistica software 9.0.

Results

Mycorrhizal and non-mycorrhizal seedlings of *M. champaca* after 280 days of AMF inoculation was depicted (Figure 6.4). The comparative photograph of mycorrhizal and non-mycorrhizal seedlings was presented (Figure 6.4). There was significant influence of *G. intraradices* and *Glomus* sp 1 inoculation on the shoot dry weight, shoot length, and leaf area of *M. champaca* after 180 days of inoculation.

Overall, there was no effect on AMF inoculation on the root dry weight, root/shoot dry weight ratio, roots length, stem girth, root to shoot ratio and number of leaves of the plants (Table 6.1). There were significant differences between mycorrhizal (*G. intraradices* and *Glomus* sp1) and non-mycorrhizal plants in shoot dry weight, shoot length, and leaf area (Table 6.2). *G. intraradices* exhibit more significant differences in growth parameters than *Glomus* sp1. *Glomus* sp 2 did not show significant variation in growth parameters than non-mycorrhizal ones.

There was significant influence of *G. intraradices* inoculation on the shoot dry weight, shoot length, stem girth, leaf area and number of leaves of *M. champaca* after 270 days of inoculation. Moreover, there was significant influence of *Glomus* sp 1 inoculation on the shoot dry weight, shoot length, stem girth, leaf area, root dry weight

and root/shoot ratio of *M. champaca* after 270 days of inoculation. However, there was no effect on AMF inoculation on the roots length of the plants (Table 6.3).

Table 6.1. One way ANOVA results of growth features between mycorrhizal and non-mycorrhizal plants of *Michelia champaca* after 180 days

Variables	<i>Glomus intraradices</i>		<i>Glomus sp 1</i>		<i>Glomus sp 2</i>	
	F	<i>p</i> <	F	<i>p</i> <	F	<i>p</i> <
Shoot dry weight (g)	213.0	0.05	62.8	0.05	-	N.S.
Root dry weight (g)	-	N.S.	-	N.S.	-	N.S.
Root to shoot ratio	-	N.S.	-	N.S.	-	N.S.
Shoot length (cm)	222.0	0.05	43.9	0.05	-	N.S.
Root length (cm)	-	N.S.	-	N.S.	-	N.S.
Stem girth (mm)	-	N.S.	-	N.S.	-	N.S.
Number of leaves	-	N.S.	-	N.S.	-	N.S.
Leaf area (cm ²)	10.6	0.05	36.6	0.05	-	N.S.

Table 6.2. Growth properties of mycorrhizal (M) and non-mycorrhizal (NM) plants of *Michelia champaca* after 180 days

Types	Shoot dry weight (g)	Root dry weight (g)	Root to shoot ratio	Shoot length (cm)	Root length (cm)	Stem girth (mm)	Number of leaves	Leaf area (cm ²)
Non-mycorrhizal	0.16± 0.01 a	0.08± 0.02a	0.54± 0.13a	8.70± 0.52a	19.00± 2.09 a	2.33± 0.17a	7.33± 0.67a	2.80± 0.15a
<i>Glomus intraradices</i>	0.36± 0.01 b	0.10± 0.001a	0.27± 0.02b	17.93± 0.34b	23.77± 3.73 a	2.67± 0.17a	8.67± 0.33a	6.43± 1.10b
<i>Glomus sp 1</i>	0.33± 0.02 b	0.09± 0.01a	0.29± 0.03b	16.03± 0.98b	22.10± 2.06 a	2.50± 0.29a	8.00± 0.58a	5.40± 0.40b
<i>Glomus sp 2</i>	0.18± 0.01 a	0.09± 0.001a	0.50± 0.05a	8.53± 0.64a	17.87± 1.47 a	2.17± 0.17a	7.33± 0.33a	2.80± 0.10a

Fischer LSD test showing different alphabet differs significantly at *p* < 0.05

Table 6.3. One way ANOVA results of growth features between mycorrhizal and non-mycorrhizal plants of *Michelia champaca* after 270 days

Variables	<i>Glomus intraradices</i>		<i>Glomus sp 1</i>		<i>Glomus sp 2</i>	
	F	p <	F	p <	F	p <
Shoot dry weight (g)	9.87	0.05	17.7	0.05	-	N.S.
Root dry weight (g)	-	N.S.	16.4	0.05	-	N.S.
Root to shoot ratio	-	N.S.	45.9	0.05	-	N.S.
Shoot length (cm)	30.7	0.01	25.0	0.01	-	N.S.
Root length (cm)	-	N.S.	-	N.S.	-	N.S.
Stem girth (mm)	10.1	0.05	76.3	0.05	-	N.S.
Number of leaves	17.8	0.05	-	N.S.	-	N.S.
Leaf area (cm ²)	37.2	0.05	48.2	0.05	19.3	0.05

Table 6.4. Growth properties of mycorrhizal (M) and non-mycorrhizal (NM) plants of *Michelia champaca* after 270 days

Types	shoot dry weight (g)	Root dry weight (g)	Root to shoot ratio	Shoot length (cm)	Root length (cm)	Stem girth (mm)	Number of leaves	Leaf area (cm ²)
Non-mycorrhizal	0.19± 0.02a	0.12± 0.02a	0.64± 0.02a	12.63± 0.69a	19.03± 2.67a	2.33± 0.17a	6.67± 0.88a	2.91± 0.16a
<i>Glomus intraradices</i>	1.30± 0.35b	0.62± 0.28b	0.45± 0.10b	23.67± 1.87b	24.77± 1.52a	4.31± 0.60b	11.33± 0.67b	47.30± 7.27b
<i>Glomus sp 1</i>	1.12± 0.22b	0.53± 0.10b	0.47± 0.02b	20.93± 1.51b	24.17± 0.99a	4.47± 0.18b	8.67± 1.20a	47.13± 6.37b
<i>Glomus sp 2</i>	0.26± 0.02a	0.12± 0.01a	0.48± 0.06b	14.47± 0.50a	17.43± 1.34a	2.51± 0.14a	7.00± 2.08a	5.45± 0.56c

Fischer LSD test showing different alphabet differs significantly at $p < 0.05$

There were significant differences between mycorrhizal (*G. intraradices* and *Glomus sp1*) and non-mycorrhizal plants on the shoot dry weight, shoot length, stem girth, leaf area root dry weight and root/shoot dry weight ratio (Table 6.4). *Glomus sp1* exhibit more significant differences in growth parameters than other *Glomus sp 2*. *Glomus sp 2* showed significant variations in leaf area than non-mycorrhizal ones. *G. intraradices* exhibit significant higher number of leaves compared to others. The

variation in aboveground and belowground features is depicted in the graph (Figure 6.5-6.7).

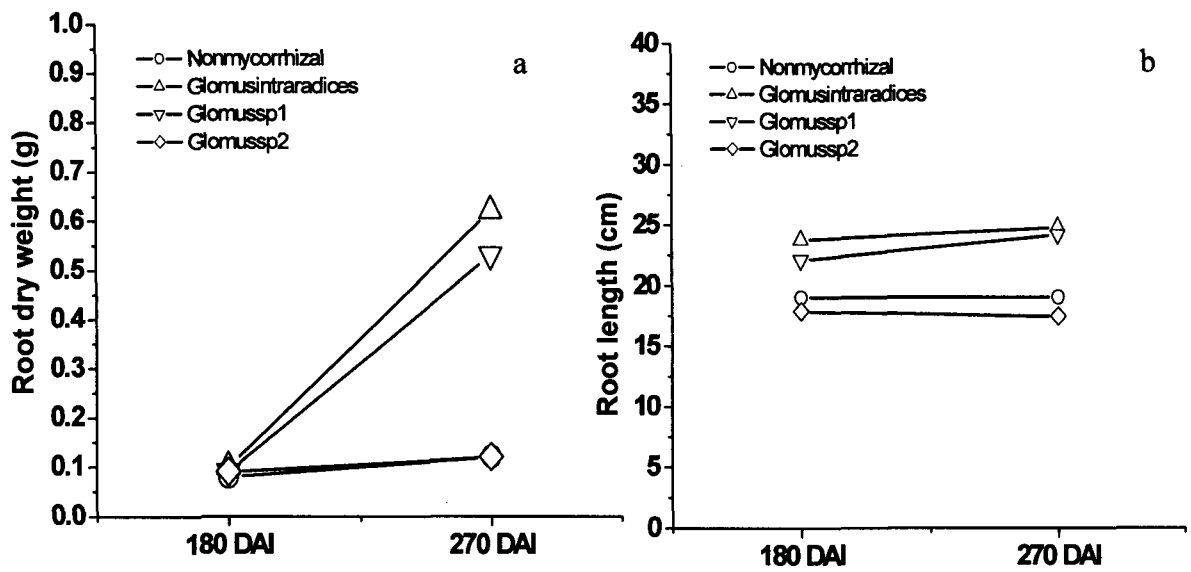


Figure 6.5: Difference in aboveground features of *Michelia champaca* inoculated with AMF isolates and non-mycorrhizal plants (a) and shoot length difference of AMF isolate (b)

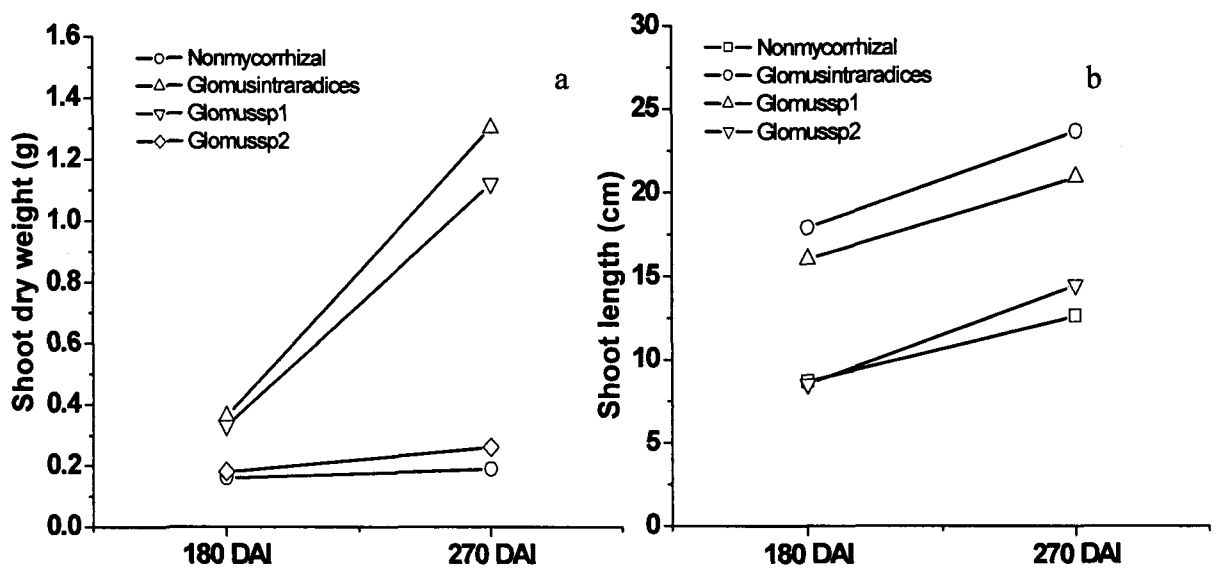


Figure 6.6: Difference in belowground features of *Michelia champaca* inoculated with AMF isolates and non-mycorrhizal plants (a) and root length difference of AMF isolate (b)

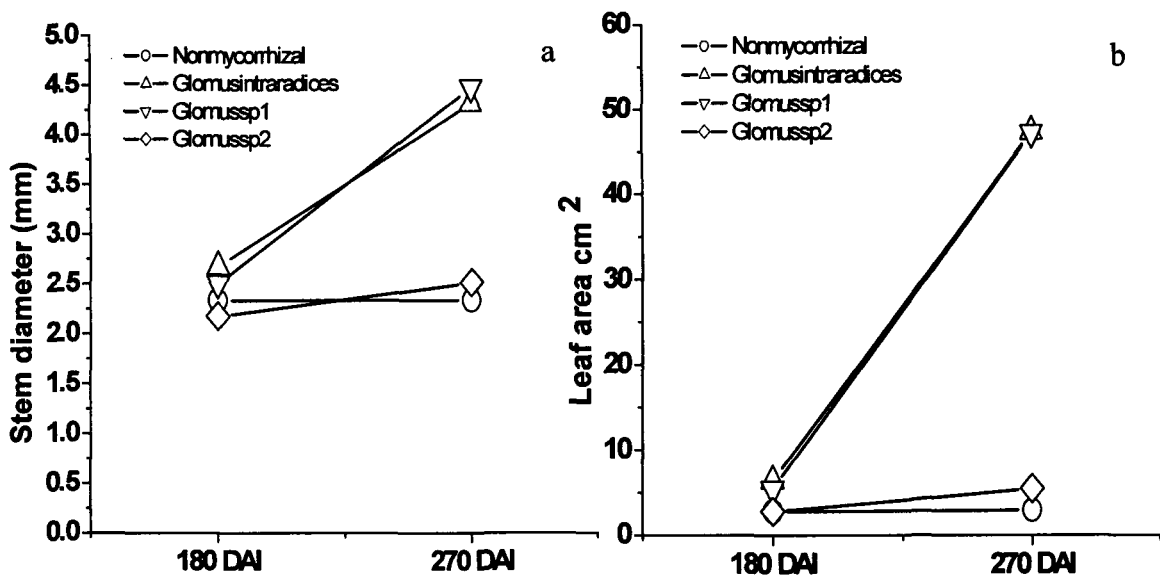


Figure 6.7: Graph showing stem diameter difference of AMF isolate (a) leaf area difference of AMF isolates (b)

Discussion

The growth feature enhancement by mycorrhizal fungi is the first report in *M. champaca*. AMF significantly enhances growth improvement in the host plant in the present investigation. Improvement of plant growth with inoculation of AMF has been reported in a number of forest tree species (Vasanthakrishna *et al.*, 1995; Rajan *et al.*, 2000) and medicinal plants (Earanna 2001; Boby and Bagyaraj, 2003). The main effect of mycorrhizal fungi in improving plant growth is through improved uptake of nutrients, especially phosphorus due to the exploration by the external hyphae of the soil beyond root hair zone when phosphorus is depleted (Gerdemann, 1975). Increased phosphorus uptake has been attributed not only to increased surface area of absorption but also to enhanced hyphal translocation (Hattingh *et al.*, 1973). Enhanced plant biomass and P uptake because of AMF inoculation has been reported by earlier workers in forest tree species and a few medicinal plants (Vasanthakrishna *et al.*, 1995; Sailo and Bagyaraj, 2005). This study was in accordance with the study of colonization by *G. clarum* and *G.*

decipiens increased plant growth, shoot nutrient concentrations, and survival rates of *Dyera polyphylla* and *Aquilaria filaria* seedlings 180 days after transplantation under greenhouse conditions (Turjaman *et al.*, 2006).

Infectivity of AMF can be affected by many soil properties, such as P content (Bolan and Abbott, 1983) and pH (Clark, 1997). The difference in infectivity of native and introduced fungal species has been investigated. Abbott and Robson (1981) noted that the native *Glomus* species was easily replaced by three of the inoculant fungi in York soil but not in Merredin soil, indicating that introduced fungal species may have higher infectivity in a certain soil. In the experiments conducted by Yao *et al.* (2008), *G. versiforme* was inoculated as the introduced AMF species. Regardless of the sites and plant species, the colonization by *G. versiforme* was always higher than that by the native AMF. This may suggest the wide adaptation of *G. versiforme* to many soil types or some properties favoring this fungal species presence in the soil (Yao *et al.*, 2008). Although AMF lack absolute host specificity, as far as the colonization is concerned, the growth responses of plants to a given AMF species differ widely (Sanders, 2003). Moreover, native AMF greatly promoted the growth of one species but not the other (Yao *et al.*, 2008). In our experiments, native AMF differed widely from introduced AMF in promoting the growth of host plants. The contribution of native AMF to plant growth, when compared with introduced AMF, also varied greatly, depending on the plant-fungi combination. Klironomos (2003) found that the performance of native AMF in promoting native plant growth was more extreme than that of exotic AMF.

Although, the results of this study generally agree with previous reports on the positive growth response of tree seedlings to AMF where indigenous AMF were found to be ineffective or less effective (Bagyaraj *et al.*, 1989; Reena and Bagyaraj, 1990) compared to exotics. However, in some of the very few previously reported trials with

tropical trees in unsterile soils, mycorrhizal inoculation failed to improve tree seedling growth (Cornet and Diem, 1982; Cuenca *et al.*, 1990).

Studies on neem and other plant species do indicate that inoculation with AMF (Habte *et al.*, 1993; Phavaphutanon *et al.*, 1996) can reduce fertilizer requirement in plant production. Although, such a cost: benefit ratio was not tested in this study, the results clearly indicate that microbial inoculations can substantially reduce fertilizer requirement in the host seedling production.

Plants get benefited from mycorrhizal symbiosis mainly due to the increased absorptive surface provided by fungal hyphae in their root system (Smith and Read, 1997). Growth of mycorrhizal and non-mycorrhizal plants, therefore, may respond differentially to soil nutrient concentration, as observed in several experiments (e.g. Bougher *et al.*, 1990; Titus and del Moral, 1998). Mycorrhizal seedlings, in this experiment, had higher shoot height, root length, stem diameter, leaf area and biomass. Significant enhancement in root and shoot growth of nursery-raised seedlings may be due to increasing supply of nutrients (Giri *et al.*, 2005), carbohydrate partitioning (Graham *et al.*, 1997) and toxicity mediating ability (Dong *et al.*, 2008) of mycorrhizal fungi.

Pérez and Urcelay (2009) reported plant species belonging to different functional types differed in their response to AMF. As predicted, when grown alone, the deciduous shrub *Acacia caven* was positively affected while the annual ruderal forb *Bidens pilosa* was not significantly affected, although a slight negative effect on shoot mass was observed. It has been suggested that woody species may derive more benefits from mycorrhizal fungi than plants with shorter life spans and that this benefit could be related to root system structure (Brundrett, 1991; Peat and Fitter, 1993). This could be also attributed to our study where woody host plant AMF inoculation significantly affected its growth. In the forb and shrub species studied here, root/shoot ratio was consistently

decreased by arbuscular mycorrhizal colonization but not affected by plant density. These results contrast with other reports that competition, but not AMF, positively affected root/shoot ratio (Ayres *et al.*, 2006). In our study root/shoot ratio increased from 180 days to 270 days of inoculated plants.

AMF affect carbon partitioning in the species or, alternatively, that an important fraction of the carbon allocated to build roots is taken up by the fungi in colonized roots. Even though, these findings support the idea that AMF are important in the carbon economy of plants (Smith and Read, 2008) although the mechanisms are not well known yet. Inoculation of two AMF increased the early growth and nutrient concentrations of *D. polyphylla* and *A. filaria*. Although AMF colonization was observed in native tree species grown in the tropical rain forest of Southeast Asia (Smits, 1994; Moyersoen *et al.*, 2001), studies about the role of AMF in the tropical rain forest of Southeast Asia are rare (Alexander *et al.*, 1992) compared to those of Africa and Latin America. *D. polyphylla* and *A. filaria* are important tree species in rain forests of Southeast Asia because these species provide non-timber forest products (NTFPs). Moreover, inoculation of AMF would be useful for conservation of *A. filaria* because this species is among the list of species that are considered to be threatened (Turjaman *et al.*, 2006). Likewise, *M. champaca* is a commercially important tree species important for its valuable flowers and timber. Chemical fertilizer was commonly applied to these species in nurseries on the commercial scale. Heavy application of chemical fertilizer decreases AMF colonization and thereafter diminishes growth improvement by AMF. If AMF inoculum could be produced at small cost, inoculation of AMF can reduce the application of chemical fertilizer without decrease in AMF colonization. The survival rate of seedlings is a key measure of success in reforestation and afforestation. In northeast India, loss of tropical

and sub-tropical forests was extensive due to population growth, logging, and land demand for various development projects.

Today, reforestation and afforestation programs aim to cover three million hectares during a 5-year period to convert degraded forest into sustainable forest production units. This means that more than three billion seedlings (1 ha=1,000 seedlings) are needed. There is a 10% difference in survival rate between AMF colonized seedlings and control seedlings. The estimated profit from this 10% difference is US \$ 90 million (price of one seedling=US \$ 0.3) (Turjaman *et al.*, 2006). This high profit is tantamount to saving in seedling stocks, labor cost, and fertilizer cost in nurseries. Increased survival rates were noted in tropical tree species with AMF colonization, such as *Ochroma pyramidale*, *Luehe seemanii* (Kiers *et al.*, 2000), *Heliocarpus appendiculatus*, and *S. donnell-smithii* (Guadarrama *et al.*, 2004). Field trials with AMF inoculation are required to monitor the growth and survival rates of host species. Inoculation techniques may be adopted by a large scale nursery jointly with reforestation programs, thereby aiding in the recovery of the population of such cultivated tree species as *M. champaca*. From the results of this study, we suggest that AMF can accelerate the establishment of *M. champaca*, thereby ecologically promoting nature conservation and economically sustaining the harvest and production of NTFPs.

Our results suggest that of the native two species isolated, *Glomus* sp 1 influenced the growth of *M. champaca*. Moreover, the exotic species (*G. intraradices*) also improved the growth of host plant. However, *Glomus* sp 1 improved the belowground features after 270 days of inoculation whereas exotic species enhanced aboveground features.

Root structure characteristics of *Michelia champaca* in association with arbuscular mycorrhizal fungi

Introduction

Arbuscular mycorrhizal fungi (AMF) form symbiotic associations with majority of terrestrial land plants. Two major morphological classes of AMF symbiosis were identified by and since these appeared to be plant-related they were named after the plants in which they were originally observed. The two classes, which differ in fungal structures, were named *Arum* and *Paris* mycorrhizas (Gallaud, 1904).

It is well known that responsiveness to mycorrhizal colonization depends on root system properties (Brundrett, 1991). It has been shown that annual forbs exhibit higher specific root length, which is usually associated with rapid rates of root elongation, high relative growth rate, and high nutrient uptake capacities. In contrast, perennials are generally associated with traits that suggest a more conservative strategy like higher root tissue density, larger diameter, and lower root nutrient content, typical of long lived roots (Roumet *et al.*, 2006). Thus, different plant functional types (PFTs), defined as groups of species that differ in their morpho-functional traits (Díaz and Cabido, 1997; Lavorel *et al.*, 1997), may show distinct patterns of growth response to mycorrhiza and plant density.

Most cultivated plants produce *Arum*-type mycorrhizal colonization consisting of intercellular hyphae and arbuscules, whereas many trees and forest herbs form *Paris*-type intracellular hyphae, coils and arbusculate coils. The *Arum*-type has generally been regarded as the most common due to the fact that most experimental studies use crop plants. Brundrett and Kendrick (1990) in fact suggested that the *Paris*-type might be just as common in natural communities as the *Arum*-type. In a review of the literature, Smith and Smith (1997) compiled a list of plant families with the type of mycorrhizal

colonization observed in them and showed that *Paris*-type AMF were more common, with 39 families compared with 26 with *Arum*-type, and 21 with either intermediate or both morphologies. 'Intermediate' morphology was assigned when both structural types were recorded in the same plant species and both when they were observed in different species or genera within the same family.

However, there is scarce information on root morphology and mycorrhizal anatomy from Magnoliaceae. Hence, the work was pursued to assess the morphology and mycorrhizal anatomy of the root of *Michelia champaca*.

Materials and Methods

Plant growth conditions

During August, 2009, seeds of *M. champaca* were collected from Agronomy Division, ICAR (Indian Council of Agricultural Research), Umiam in Ribhoi District of Meghalaya, northeast India (N25°38'; E91°52'; 850 m.a.s.l). In September, 2009, seeds were disinfected with 1% sodium hypochlorite; seeds were then soaked in distilled water for two days. The arils of seed were removed to enhance maximum germination (Candiani *et al.*, 2004) and 8-10 seeds were placed on double sterilized river bed sand: pine forest soil (1:1 vol: vol) in 200 ml disposable plastic containers. The germination set up were kept in B.O.D. incubator at 25°C under white fluorescent tubes (photoperiod 12 h) and watered whenever it was required to keep the soil mixture moist. After one month, about 90 % of the seeds were observed to germinate and further allowed to grow for another one month so that all the seedlings attain more or less equivalent growth. For bulk production of inoculum, 50-60 seeds of *Paspalum notatum* Flüge procured from IGRI (Indian Grassland and Fodder Research Institute, Palampur, India) were placed on double sterilized river bed sand: pine forest soil (1:1 vol: vol) on 3 kg garden pots which were inoculated by spreading 2-3 cm below the seeds with 20 g of mycorrhizal inoculants

Glomus intraradices Schenck & Smith AM WG19 collected from TERI (The Energy and Resource Institute, India).

The germinated seedlings of *M. champaca* were transferred in sterilized 2 kg garden bags containing same sand and soil mixture. On November, 2009 inoculation was done with *G. intraradices* by adding 10 g of dried crushed inoculum raised with *P. notatum* all around the root of *M. champaca*, dug with glass rod of about 12 cm deep and seedling was placed in it. The set up was maintained in green house and watered whenever it was required every week. The non mycorrhizal plants were grown only in sterilized sand and soil. After 180 and 270 days plants were harvested for root structure assessment.

Sampling

Roots from another three plants each from mycorrhizal and non mycorrhizal were measured for root orders features. The primary centrifugal segment based root ordering scheme has been applied to plant root systems. This scheme has been used extensively in describing patterns of root growth and in modeling the growth of plant root systems (Berntson, 1997).

For AMF anatomy and morphology studies, three mycorrhizal inoculated seedlings were harvested from greenhouse for assessment of AMF morphology. The roots were cut and made into 1 cm approximately and all the three sub samples were made into composite samples. From natural plantations, the root samples were collected from Mawlein in Ribhoi District of Meghalaya, northeast India (N25°42'; E91°53'; 828 m.a.s.l). The roots at depths of 0-20 cm around the tree traced from the trunk, at four different points were collected. Then washed with tap water to remove adhering soil and fixed in FAA for light microscopy and AMF morphology. Three root tips of approximately of 2 cm each were hand sectioned transversely with double sided razor

blade and fixed in glutaraldehyde for scanning electron microscopy (SEM). Another three root tips of 2 cm from three mycorrhizal seedlings were collected from green house which were hand sectioned transversely with double sided razor blade and further treated immediately for confocal laser scanning microscopy (CLSM).

Light microscopy and AMF colonization

The washed root samples were processed and stained with black Faber Castell stamp pad ink (Das and Kayang, 2008). Root segments of approximately 1 cm long stained samples were mounted on slides in lactoglycerol and examined for mycorrhizal structures under light microscope (Olympus 41209) to investigate different colonization patterns (vesicular, arbuscular and hyphal) and AMF morphology (Dickson, 2004). The root segments colonized by fungi were photographed with the help of Leica EC 3 camera attached in Leica dm 1000 microscope.

Scanning electron microscopy

The transverse sections of fresh roots were fixed in 3 % glutaraldehyde for 24 hours in 4°C in refrigerator. The root sections were washed thrice in 0.1 M sodium cacodylate buffer. The roots were than dehydrated with acetone series. Final dry treatment was given with the tetra methyl silane. The specimens were mounted in brass stubs and coating of the root sections with gold was done for final examination in the microscope. The root segments were examined under scanning electron microscope, Jeol, JSM 6360 (Japan).

Confocal laser scanning microscopy

For CLSM, roots were treated with berberine and aniline blue (Brundrett *et al.*, 1988). The specimens were examined using confocal laser scanning microscopy (Leica TCS SP 5, DM6000B, Germany) using conditions suitable for DAPI fluorescence.

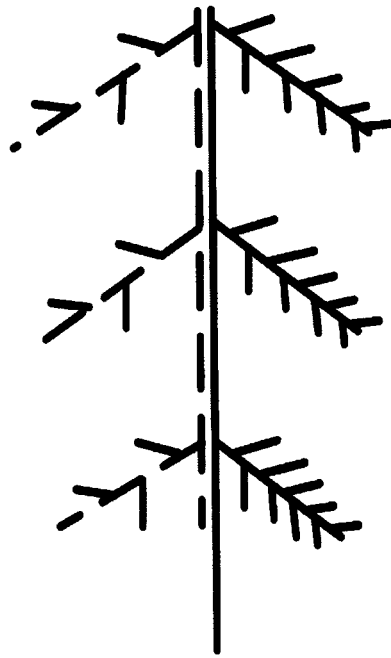


Figure 7.1: Schematic diagram of mycorrhizal root (—) and non-mycorrhizal root (---) showing length and number of first and second order root system of *Michelia champaca*. The mean primary root length (cm) does not differ significantly between plant types. Mean first order length (cm) with $SE\pm 0.4$ and mean second order length (cm) with $SE\pm 0.3$ of mycorrhizal root. Mean first order length (cm) with $SE\pm 0.7$ and mean second order length (cm) with $SE\pm 0.4$ of non-mycorrhizal root. Nine numbers of second orders mycorrhizal roots were presented compared to 3 in non-mycorrhizal root which differs by a factor of 3:1 considering the significant relation between mycorrhizal and non-mycorrhizal plants.

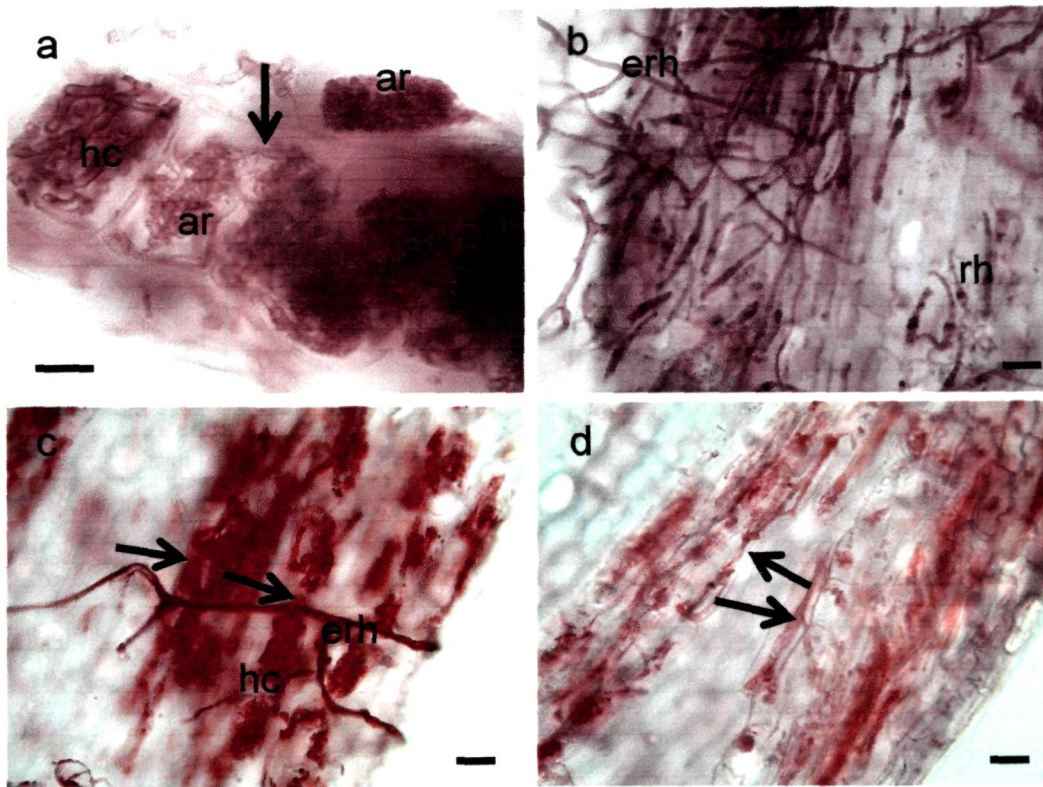


Figure 7.2: Light microscopy images of mycorrhizal colonization of root of *Michelia champaca* from plantations (a) and plants from greenhouse (c&d). Root portion showing hyphal coils [hc] and intracellular arbuscules [ar]. Scale bar=100 μ m (b) Root segment showing extraradical hyphae [erh] and root hairs [rh]. Scale bar=200 μ m (c) Portion of root showing intracellular hyphal coils and extraradical hyphae. Scale bar=200 μ m (d) Root segment showing intercellular hyphae with penetrating hyphae into the cells. Scale bar=200 μ m.

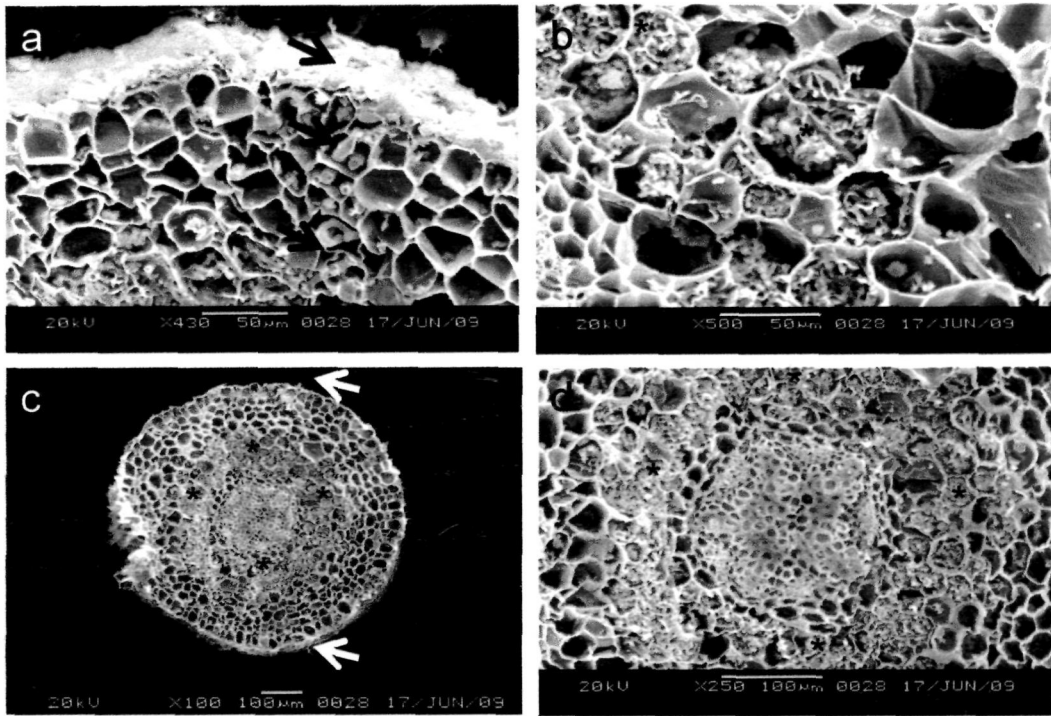


Figure 7.3: Scanning electron microscope images of root sections of trees from plantation of *Michelia champaca* (a-d) (a) T.S. of root segment showing colonization progress from root surface to the outer cortex and towards inner cortex. (b) Arbuscules and coils in the cortex. (c&d) T.S. of root section exhibiting inner cortical colonization. Arbuscules (asterisk) and hyphae at the root surface (arrow).

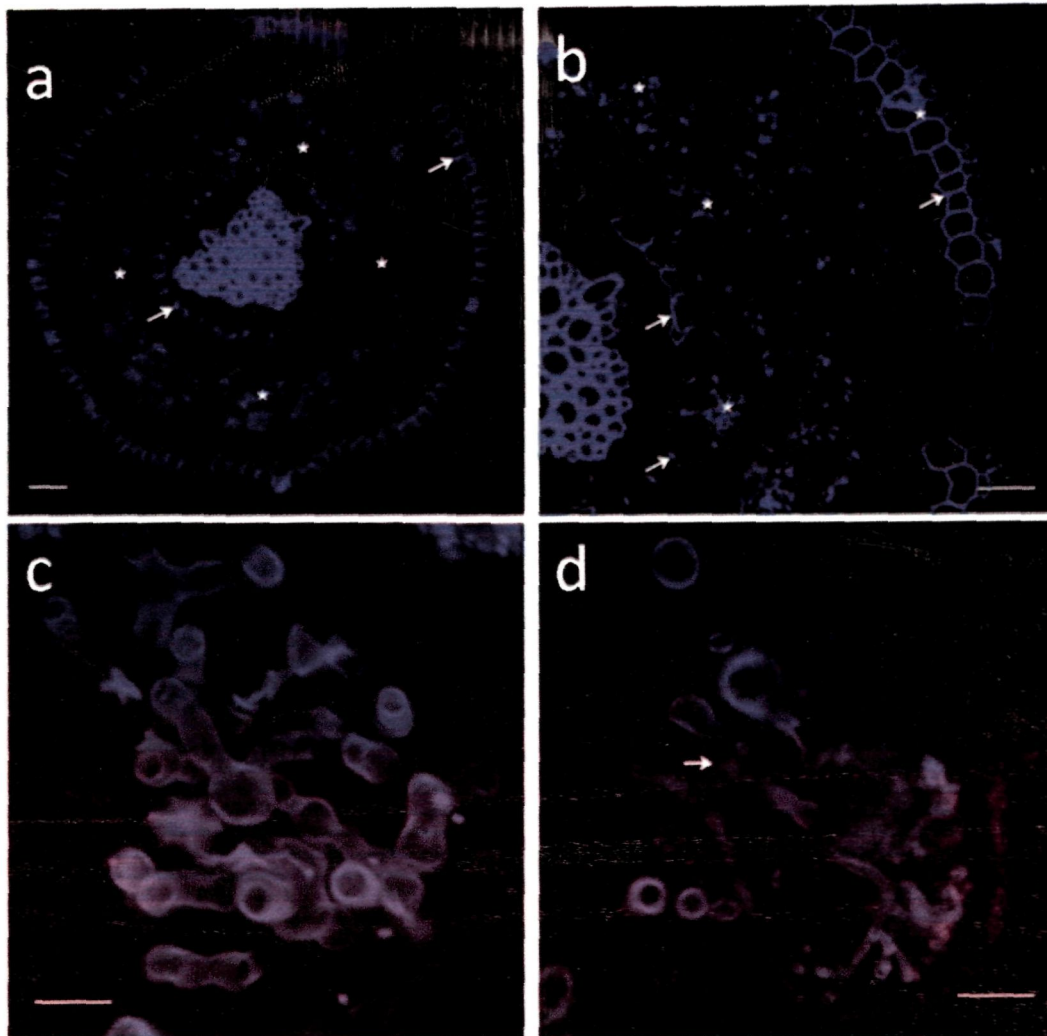


Figure 7.4: Confocal Laser Scanning Microscope image of root segments of seedlings of *Michelia champaca* from net house (a-d). (a) T.S. of root with inner cortical colonization by AMF (asterisk), casparian strips (arrow) and xylem fluorescing. Scale bar=700 μ m (b) Portion of root showing casparian strips in endodermis and hypodermis; fluorescing xylem and fungal hyphae. Scale bar=350 μ m (c) Intracellular hyphal coil. Scale bar=50 μ m (d) Intracellular arbuscule. Scale bar=50 μ m.

Data analysis

Means and standard errors were calculated for belowground plant parameters as well as mycorrhizal colonization in mycorrhizal inoculated seedlings and trees from plantations were calculated. Analysis of variance (ANOVA) was conducted to analyze the difference between growth parameters in mycorrhizal and non mycorrhizal plants and means were separated using *post-hoc* test (Fischer's LSD). All the statistical analysis was done using Statistica software 9.0.

Schematic diagrams representing the root structure in terms of mean root order length and number of root order were constructed. The diagram represents the mean with standard errors of root order length for mycorrhizal and non-mycorrhizal plants in two dimensional preparations. The numbers of root order were kept different considering the significant relation between mycorrhizal and non-mycorrhizal plants.

Results

There were two root orders observed in both mycorrhizal and non-mycorrhizal plants observed after 180 days. There were significant differences in length and dry weight between orders of both types of plants. However, there were no significant differences in diameter of first and second order roots of non-mycorrhizal plants (Table 7.1). Moreover, length and diameter of root orders of non-mycorrhizal plants were comparatively higher than mycorrhizal plants. In addition, there were no significant differences in the number between orders of mycorrhizal plants. However, the number of second order mycorrhizal roots was significantly higher than non-mycorrhizal roots. The diagrammatic presentation of mycorrhizal and non-mycorrhizal root system is depicted in Figure 7.1. There were three root orders observed in both mycorrhizal and non-mycorrhizal plants observed after 270 days (Table 7.2).

Table 7.1. Root order features of mycorrhizal (M) and non-mycorrhizal (NM) plants of *Michelia champaca* after 180 days.

Root order	Length (cm)		Dry weight (g)		Diameter (mm)		Number	
	M	NM	M	NM	M	NM	M	NM
1	4.98±	5.59±	0.04±	0.031±	0.82±	0.73±	36.33±	25.0±
	0.4a	0.71a	0.005a	0.005a	0.07a	0.02ac	7.33ab	3.21ab
2	1.26±	1.71±	0.01±	0.006±	0.51±	0.63±	48.0±	16.76±
	0.27b	0.43b	0.002b	0.002b	0.01b	0.06bc	11.01a	5.86b

Fischer LSD test showing different alphabet differs significantly at $p < 0.05$

Table 7.2. Root order features and colonization (%) of mycorrhizal plants of *Michelia champaca* after 270 days.

Root order	Arbuscules (%)	Vesicles (%)	Hyphae (%)	Height (cm)	Diameter (mm)	Number of root
1	20.0	4.1	47.0	7.0	1.1	56.0
	±3.79a	±1.23a	±6.57a	±0.73a	±0.03a	±4.04a
2	38.0	16.0	84.0	3.6	0.8	48.0
	±3.36b	±1.79b	±5.64b	±0.41b	±0.04b	±2.40a
3	31.0	9.2	64	2.1	0.7	11.0
	±5.37b	±3.00b	±10.42b	±0.34b	±0.03b	±2.03b

Fischer LSD test showing different alphabet differs significantly at $p < 0.05$

The mycorrhizal colonization in second and third orders were significantly higher than first order. However, height and diameter of first order was significantly higher than second and third orders. There was significant correlation noticed between hyphal and vesicles colonization and between height and diameter of root order (Table 7.3).

Roots collected from plantation sites exhibit *Paris* type of AMF morphology in all the roots examined (Figure 7.2a). In addition, intermediate type of AMF morphology was also observed in few segments (Figure 7.2a). Root hairs were not observed from the plantations sites. The colonization proceeds from epidermis and passes through 4th-6th layers to colonize the inner cortex (Figure 7.3a). 10th-15th layers of cortex were observed

where from 5th-7th layers onwards inner cortical layers colonization was observed. The colonization was found all around vascular bundle in the inner cortex (Figure 7.3b).

Casparian strips in xylem, endodermis and exodermis is clearly shown (Figure 7.4 a&b). AMF colonization was also confined to the inner cortex surrounding the vascular tissue (Figure 7.4a&b). In the plants grown in greenhouse inoculated with AMF, root hairs were observed along with extraradical hyphae (Figure 7.2 b). *Paris* type AMF morphology was observed in the roots with intracellular hyphal coils (Figure 7.2 c&3e); *Arum* type was detected in a few segments (Figure 7.2 d) as well as intermediate type (Figure 7.4d). 6th-8th cortical layers were found of which three layers onwards colonization was encountered.

Table 7.3. Correlation between root order features and AMF colonization

	Arbuscules (%)	Vesicles (%)	Hyphae (%)	Height (cm)	Diameter (mm)	Number
Arbuscule (%)	1.0	0.98	0.98	-0.76	-0.8	-0.29
Vesicles (%)		1.0	0.99*	-0.61	-0.66	-0.08
Hyphae (%)			1.0	-0.64	-0.69	-0.12
Height (cm)				1.0	0.99*	0.84
Diameter (mm)					1.0	0.8
Number						1.0

*Correlation significant at $p < 0.05$

Discussion

This is the first study of plant growth assessment relative to root morphology and structure in comparison with mycorrhizal and non mycorrhizal seedlings from Magnoliaceae. Vozzo and HacsKaylo (1964) compared root morphology and mycorrhizal anatomy of *Liriodendron tulipifera* from natural or cultivated habitats with non-mycorrhizal plants grown in the greenhouse in sterilized soil. In addition, mycorrhizal

colonization and growth in *Zea mays* and *L. tulipifera* was compared as reported earlier (Gerdemann, 1965). However, 89 Brazilian forest species were studied for root size, root hair and mycorrhizal colonization (St. John, 1980); moreover, 78 tropical native woody species from Brazil were assessed for root morphology and seedling growth by AMF (Zangaro *et al.*, 2005). The anatomy and mycorrhizal colonization were examined by branch order in 23 Chinese temperate tree species of both angiosperms and gymnosperms forming ectomycorrhizal and AMF associations (Guo *et al.*, 2008). Nevertheless, there were meager reports available from the family Magnoliaceae.

Nutrient acquisition by root is influenced more by root length than mass and root length increases with decreases in root diameter (Eissenstat, 1992). Evolutionary advances in root morphology result in greater control of fungi and their confinement to smaller zones within plants, culminating in the reduced organs of plants with exploitative mycorrhiza (Brundret, 2002). AMF colonization did not affect the tap root length, the average root diameter, and the basal root growth angle in spite AMF species exerted differential influence on the plant growth. Contrastingly, AMF colonization significantly reduced the total root length, the root volume, the root surface area, but promoted the formation of lateral roots of high order (Yao *et al.*, 2009). In the present investigation, the root system in terms of weight and length is proportional in both types of plants which support significant more shoot biomass in mycorrhizal plants than non-mycorrhizal plants. In this study, number of roots of second order in non-mycorrhizal plant was significantly lower than mycorrhizal plant and length and diameter of non-mycorrhizal root was comparatively higher. The modifications in terms of insignificant longer and thick diameter roots in non-mycorrhizal seedlings which may be not be sufficient for proper nutrient acquisition than in mycorrhizal plants where short and additional number of roots exhibit more surface area and nutrient acquisition may be achieved by extensive

hyphal system of AMF in mycorrhizal plants. Generally, AMF strengthens the absorption function of root systems by partial substitution of the outspread fungal hyphae. Carbon economy and maximal uptake capacity of root systems are the strategies of mycorrhizal plants (Liu, 2009). Previous data showed that AMF altered root morphology primarily when phosphorus was limited, but the ways in which the root morphology changed were not consistent (Liu, 2009). Mycorrhiza effects on root morphology were commonly attributed to improvement in phosphorus uptake by AMF, but AMF effects on hormone production may also be responsible (Berta *et al.*, 1993).

Different mycorrhizal dependency of tree species is related to characteristics of root and root hair. Root hairs were observed in AMF inoculated plants. The possible development of root hairs in this condition may be of the period in non-mycorrhizal condition after germination till inoculation. Moreover, hairs were also observed in non-inoculated roots.

This is the first study showing three types of AMF morphology in a single plant tree species. The root mycorrhizal morphology reveals *Paris*, *Arum* and intermediate type in mycorrhiza inoculated seedlings. However, *Paris* type morphology was reported earlier (Muthukumar *et al.*, 2006; Das and Kayang, 2010a). The intracellular hyphal coils and intracellular arbusculate coils indicates *Paris* type and intercellular hyphae with penetrating arbuscules into the cortical cells specify *Arum*- type AMF morphology (Dickson, 2004). However, in our study intracellular hyphae with penetrating arbuscules in adjacent cell were considered it to be intermediate type; moreover, Dickson (2004) reported intracellular arbuscule with hyphae in the same cell as intermediate type 2. The coincidence of different AMF morphology within the same root is consistent with the previous report (Bonfante-Fasolo and Fontana, 1985; Kubota and Hyakumachi, 2004; Kubota *et al.*, 2005). Bonfante-Fasolo and Fontana (1985) reported co-occurrence of

Arum and *Paris*-type morphologies in the same root system, but *Paris*- type morphology was less. This is contrast to the study of Kobato *et al.* (2005) where in tomato and cucumber both morphological types were well represented. *Lycopersicon esculentum* also formed both *Arum*- and *Paris* type (Kubota and Hyakumachi, 2004) but with high occurrence of *Paris*-type AMF morphology which is consistent with our study.

The root order modification such as number of root order in mycorrhizal and non-mycorrhizal was conspicuous. Moreover, arbuscular mycorrhizal structure reveals three types of AMF morphology in this tree species.

Quantification of root cortical cell and arbuscular mycorrhizal fungal colonization in *Michelia champaca*

Introduction

In the multifaceted world of symbioses, arbuscular mycorrhizal fungi (AMF) represent a unique interaction between two eukaryotes: an obligate biotrophic fungus and its host plant—leading to an overall improvement of the fitness of the interacting partners (Karandashov and Bucher, 2005). AMF improve plant nutrient uptake thanks to the fine exploration of the rhizosphere by the hyphae, which in return receive plant carbohydrates that are essential for the completion of the fungal life cycle. AMF are considered to be intractable organisms by taxonomists because they are asexual, obligatorily biotrophic, multinucleate and unculturable microbes; therefore species definition and recognition remain open to discussion (Rosendahl, 2008). However, advances in molecular analyses have allowed their identification also in basal groups of plants (Russell and Bulman, 2005; Ligrone, *et al.* 2007; Winther and Friedman, 2008) and stimulated original views on the roles that AMF might play in ecosystems (Selosse, 2005; Leake *et al.*, 2008).

AMF can be described as a living interface located between the plant and its soil environment. At a closer look, the concept of interface is crucial for the whole system of AMF interaction. In their extraradical phase, AMF enlarge the nutrient absorptive surface zone around the root, increasing the plant and soil nutrients interface (Leake *et al.*, 2004, Harrison *et al.*, 2002, Smith, 2002; Ortas *et al.*, 2004). During their intraradical growth, AMF develop an extended contact area with the root cell, which changes structurally depending on the intercellular or intracellular location of the fungus. Therefore, two interfaces or exchange surfaces are formed: an outer interface, between extraradical hyphae and soil, and an inner interface, between intraradical fungal structures and the host plant cells. These two biological surfaces have profound morphological and

functional differences. On the one hand the role of external hyphae is to explore the neighboring soil through maximal extension; at the same time the outer interface is actively acquiring nutrients and water from the environment used in the metabolism of both the fungus and the plant. This interface can be described as a continuously extending surface (Smith *et al.*, 2003), with unidirectional transport capabilities.

Only two cell types, epidermal and cortical, can be 'mycorrhizal cells'. Meristems and differentiating tissues are never colonized as well as the endodermis, the vascular tissues, and specialized cortical cells, such as idioblasts or those containing raphides or phenols. The mycorrhization process can be divided into two main steps: epidermis colonization and cortex colonization. During epidermis colonization ranging from molecular dialogue between the partners to actual fungal penetration across the epidermal cell the final direction of fungal development is vertical, towards the center of the root (Genre and Bonfante, 2005).

Previously described techniques to measure the proportion of root length colonized by AMF give observer-dependent measures of colonization which cannot be used to compare, quantitatively, roots examined by different researchers. A modified method was described to estimate AMF colonization on an objective scale of measurement, involving inspection of intersections between the microscope eyepiece crosshair and roots (McGonigle *et al.*, 1990). To overcome observer dependent measures of colonization we have investigated to evaluate the root quantitatively three-dimensionally.

Materials and Methods

Growth conditions

The seeds of *Michelia champaca* were collected from Agronomy Division, ICAR (Indian Council of Agricultural Research), Umium in Ribhoi District of Meghalaya, northeast

India (N25°38'; E91°52'; 850 m.a.s.l). In September, 2009, seeds were disinfected with 1% sodium hypochlorite; seeds were then soaked in distilled water for two days. The arils of seed were removed to enhance maximum germination (Candiani *et al.*, 2004) and 8-10 seeds were placed on double sterilized river bed sand: pine forest soil (1:1 vol: vol) in 200 ml disposable plastic containers. The germination set up were kept in B.O.D. incubator at 25°C under white fluorescent tubes (photoperiod 12 h) and watered whenever it was required to keep the soil mixture moist. After one month, about 90 % of the seeds were observed to germinate and further allowed to grow for another one month so that all the seedlings attain more or less equivalent growth.

The germinated seedlings of *M. champaca* were transferred in sterilized 2 kg garden bags containing same sand and soil mixture. On November, 2009 inoculation was done with *Glomus intraradices* Schenck and Smith AM WG19 collected from TERI (The Energy and Resource Institute, India) by adding 10 g of dried crushed inoculum raised with *Paspalum notatum* all around the root of *M. champaca*, dug with glass rod of about 12 cm deep and seedling was placed in it. The set up was maintained in green house and watered whenever it was required every week. The non mycorrhizal plants were grown only in sterilized sand and soil. After 180 and 270 days plants were harvested for root colonization assessment.

Root processing and sectioning

Roots from plants inoculated with *G. intraradices* were used in this study. Root samples were taken from the stem base, and have a diameter of 0.5-2.0 mm. Fresh samples were washed and cut into smaller pieces before dehydrating in alcohol and xylene series. The subsamples were embedded in paraffin wax for further microtome processing (Johansen, 1940). Longitudinal and transverse sections were done for study the segments. The roots were made into thin sections of 10 µm. Longitudinal sections and transverse sections

were taken from same root segment. Sections were stained with toulidene blue O and mounted in DPX. The root segments colonized by fungi were photographed with the help of Leica EC 3 camera attached in Leica dm 1000 microscope. The photos were taken and the print A4 copies were calculated for cortical cell and fungal colonization count.

For calculation of cortical cell and fungal colonization in cortex the following equations were used:

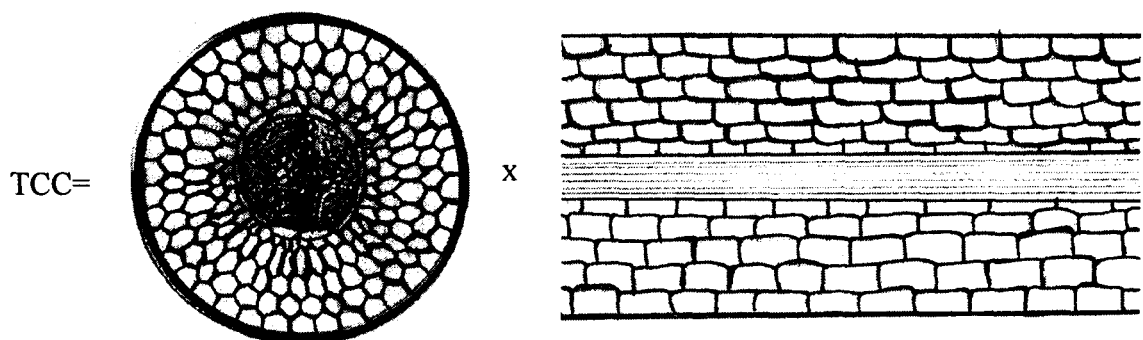


Figure 8.1: Schematic diagram showing counting in longitudinal and transverse sections

$$TCC=LSCC \times TSCC$$

TCC-Total cortical cell count; LSCC-Number of cell in longitudinal section; TSCC-Number of cell in transverse section

$$TFC=FLSC \times FTSC$$

TFC-Total fungal colonization; FLSC-Number of AMF structure in longitudinal section; FTSC-Number of AMF structure in transverse section

$$PFC= (TFC/TCC) \times 100$$

PFC-Percentage of AMF structural colonization

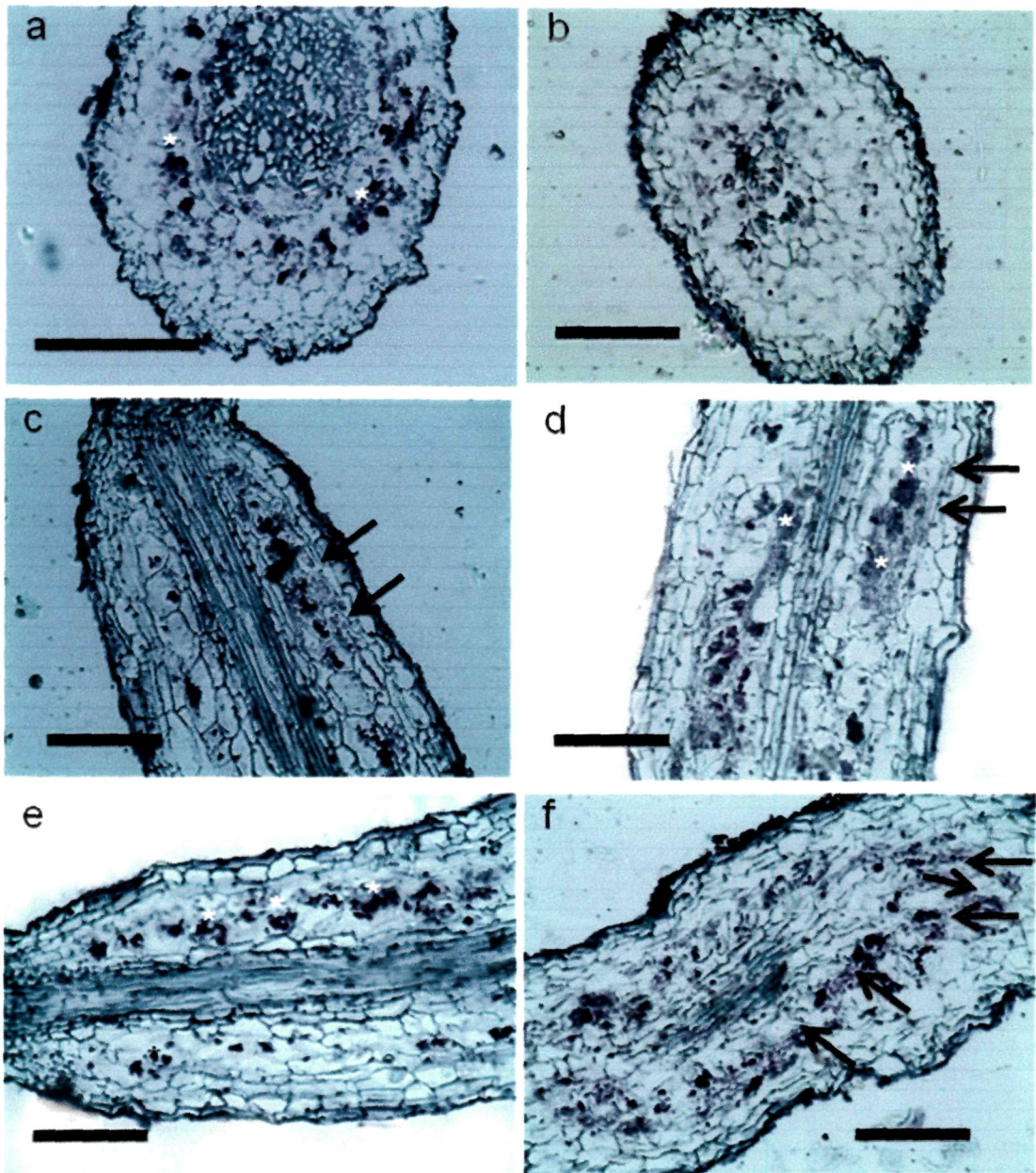


Figure 8.2: (a&b) Transverse sections of root of *Michelia champaca* of 0.4 mm. (c-f) Longitudinal sections of root of *M. champaca*.

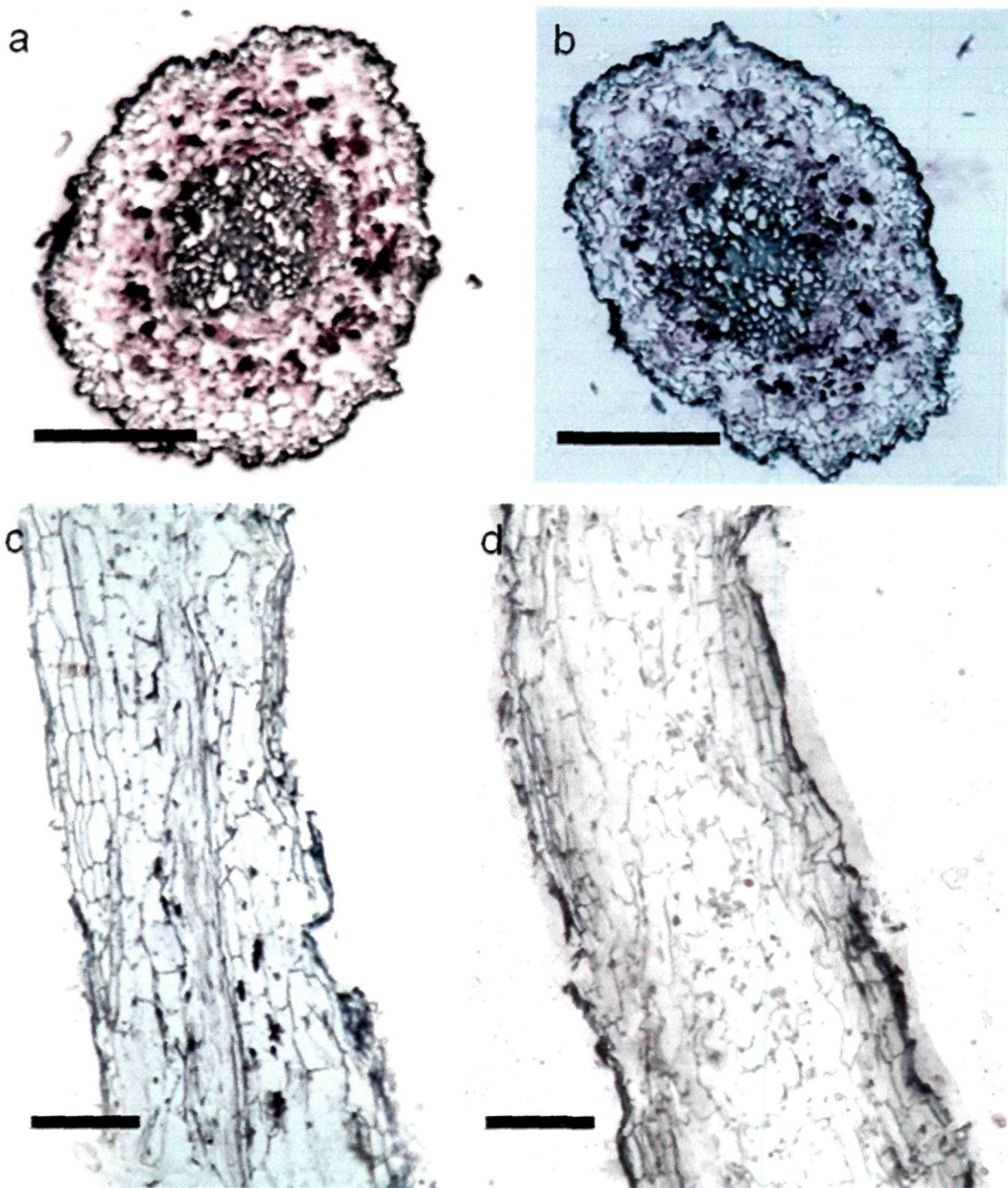


Figure 8.3: (a&b) Transverse sections of root of *Michelia champaca* of 0.5 mm. (c&d) Longitudinal sections of root of *M. champaca*.

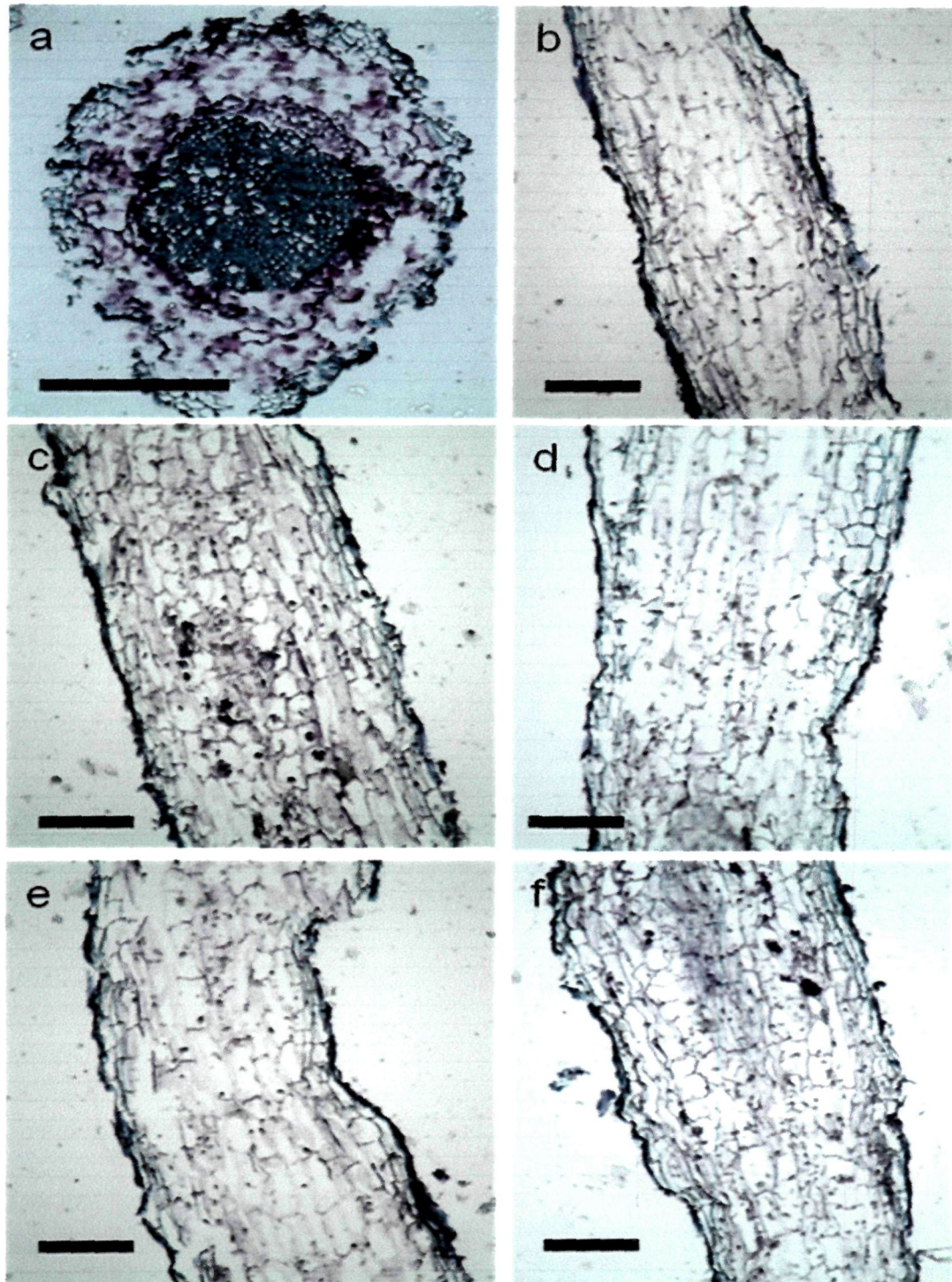


Figure 8.4: (a&b) Transverse sections of root of *Michelia champaca* of 0.6 mm.
(c&d) Longitudinal sections of root of *M. champaca*.

Results

The sections revealed inner colonization of the cortex in longitudinal and transverse sections. All the three size reveal arbuscular mycorrhizal structure such as arbuscules, hyphal coils and hyphae (Figure 8.1). The colonization was observed to be in transverse and longitudinal section. However, outer cortical region of longitudinal section was not having colonization (Figure 8.2,8.3&8.4). Highest number of cells was observed in 0.6 mm of diameter of root. Nevertheless, colonization was maximum in 0.4 mm of root segment (Table 8.1). Total number of cell was maximum in 0.6 mm of diameter of root and total number of AMF structural colonization was maximum in 0.4 mm of root segment. Moreover, percentage of AMF structural colonization was found to ranged from 2.9-0.7% (Table 8.2). The variation in log number of cells and log AMF colonization in three diameters of root is presented in Figure 8.5.

Table 8.1. Cortical cell count and AMF colonization count in 0.5 mm of root segment

Diameter (mm)	LSCC	TSCC	FLSC	FTSC
0.4	5933.33±168.26	3800±273.01	1300.00±110.15	500±69.28
0.5	6393.33±104.14	5133±148.92	640±103.92	360±30.55
0.6	6540.00±173.21	5687±116.81	673±128.75	360±52.92

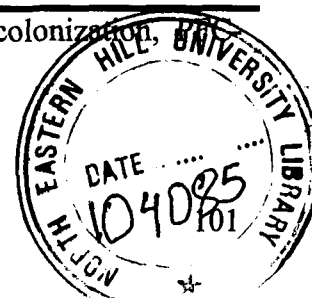
LSCC-number of cell in longitudinal section, TSCC-number of cell in transverse section, FLSC-number of AMF structure in longitudinal section, FTSC-number of AMF structure in transverse section

Table 8.2. Total cortical cell count and AMF colonization count/cm root segment

Diameter (mm)	TCC	TFC	PFC
0.4	22546666.67	650000.00	2.9
0.5	32819111.11	230400.00	0.7
0.6	37190800.00	242400.00	0.7

TCC-total number of cell, TFC-total number of AMF structural colonization

Percentage of AMF structural colonization



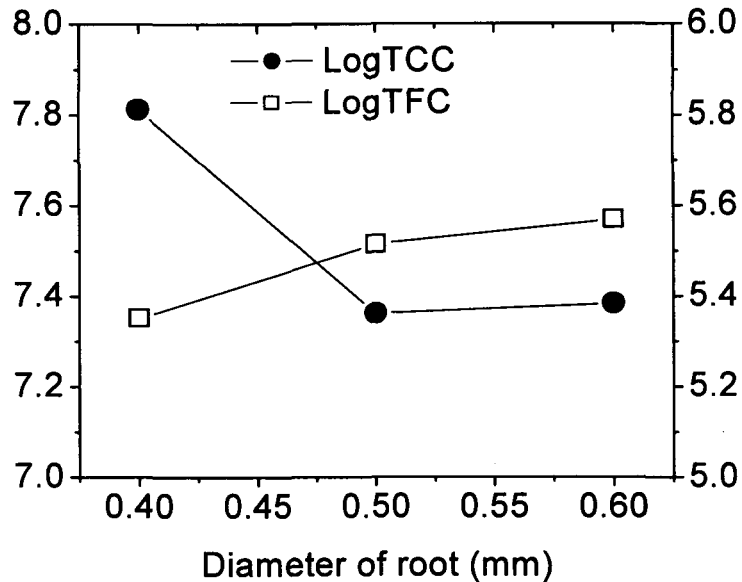


Figure 8.5: Log number of cells and AMF colonization per cm root segment in three diameters of root where TCC-total number of cell, TFC-total number of AMF structural colonization

Discussion

Previously described methods to quantify the proportion of root length colonized by arbuscular mycorrhizal fungi give observer-dependent measures of colonization which cannot be used to compare, quantitatively, roots examined by different researchers. A modified method was described to estimate AMF colonization on an objective scale of measurement, involving inspection of intersections between the microscope eyepiece crosshair and roots at magnification x 200; it is referred to as the magnified intersections method (McGonigle *et al.*, 1990). In the present method, the calculation is a quantitative three-dimensional description of cortex and AMF colonization count, where percentage fungal colonization is calculated from total number of cells in cortex and total number of arbuscular mycorrhizal structures present.

Any individual subjective estimate of a proportion is likely to be inaccurate, but a set of estimates gave a mean close to the actual value. Most observers either

overestimated or underestimated both proportions. By being consistent in this way an observer will correctly detect relative levels of colonization across experimental treatments, but these data will be observer-dependent and so should not be directly compared across experiments conducted by different researchers. The grid-line intersect method (Giovannetti and Mosse, 1980) and analogous methods using the compound microscope are likely to generate observer-dependent data because of the type of interpretations required during the analysis. The data obtained for the grid-line intersect method support this contention. However, our method is not dependent on the observer but the data were evaluated after the images are taken and calculated from the print photographs.

The magnified intersections method provides an objective measure of colonization, AC, which has been shown here to vary little from observer to observer (McGonigle *et al.*, 1990). Nevertheless, our method is not observer dependent, therefore it would not vary. To increase precision it would be advisable to increase the number of subsamples per root sample, and not the number of intersections per subsample or the number of observers per subsample. Arbuscules are the principle sites of transfer of phosphorus from endophyte to plant (Hayman, 1983), and so knowledge of the proportion of root length containing arbuscules is likely to be of use in physiological studies. However, it is the mycorrhizal hyphae which extend beyond the root and ramify into the surrounding soil that are important for nutrient uptake (Harley and Smith, 1983). The degree of colonization of roots by mycorrhizal hyphae might be expected to give the best indication of the extent of development of the external mycelium and so, in turn, for the capacity of the association to benefit the plant. The grid-line intersect method does not estimate the 'true' proportion of the root length colonized (Giovannetti and Mosse, 1980; Kormanik and McGraw, 1982). The magnified intersections method similarly

cannot estimate the 'true' percentage colonization, for which it would be necessary to distinguish between mycorrhizal and other hyphae at all intersections. Since it is difficult to estimate the proportion of root length containing mycorrhizal fungi (arbuscules, vesicles and hyphae), we believe it preferable to adopt a more reliable estimate, namely the proportion of root length containing arbuscules, or AC (McGonigle *et al.*, 1990). The main drawback to the magnified intersections method is that it takes up to one hour per subsample in total to mount the roots on microscope slides and score the colonization at x 200 magnification. Magnified intersection method suggests for using a multi-channel electronic counter helps to speed the analysis because the eyes do not need to look away from the microscope to record the status of each intersection. The magnified intersections method should be of use when objective assessment of the extent of 'formation of arbuscules, vesicles and hyphae are required. An important advantage of our method is the elimination of the need for a subjective interpretation of whether hyphae seen in the absence of arbuscules are mycorrhizal. The method also permits direct comparisons of colonization data from one researcher to another.

However, it was time consuming owing to the laborious and lengthy method for processing the root sections, although, it is difficult to have hand section longitudinally as compared to transverse section. The time can be reduced by taking hand section and longitudinal view could be used from the routine method of staining protocols. However, the transverse sections requires less time for processing and less concentrated sodium hydroxide solution can be used, as it also stain permeability will be efficient and fast. Moreover, this method allows fewer samples from one segment one transverse and one longitudinal view can explain with accuration the information about the total number of cortical cells and number of fungal occupying the cell. It is an approach to calculate and quantify root colonization by fungi from total cell as arbuscular mycorrhizal structure is

known to occupy each cell for instance arbuscules which are the most precious structure and important sites for phosphorus release and carbon uptake. Hence, calculating root segments on the basis of observer-dependent measures of colonization is not a total picture to evaluate the colonization percentage which has inherent discrepancies. Our method is far more an accurate approach for three dimensional quantification of root colonization.

The results suggest that with an increase in the number of cell, there is decrease in the colonization percentage of cell. The method of AMF colonization in the cortical cell is proposed to be accurate and may be applicable in all the plants species for calculating cortical cell and AMF colonization.

General Discussion

Mycorrhizal colonization recorded in the present study was less than the earlier report (Muthukumar *et al.*, 2006). Moreover, our results showed that the AMF colonization was significantly higher than DSE colonization. This may be because of the presence of coarse structure of the root, the characteristic feature of Magnoliales, which favors mycorrhizal infection (Baylis, 1975). The common presence of DSE along with AMF and the apparent healthiness of roots indicate that these are not pathogenic.

In our study, there was no correlation found between mycorrhizal colonization, soil properties, CBH and H. There is accumulated and established evidence on the difficulty of making generalizations about infection patterns in AMF. Variability can be extreme, since these parameters are influenced by factors inherent to the host plant, by climatic and edaphic factors, by effects of the soil community and by the interactions of all of these. Although root colonization and sporulation seem to be correlated, at least in some cases, with plant phenology and physiology, it has also been shown that the community of AMF may determine host plant community's association and production (Moreira *et al.*, 2006). The spore number varied significantly between the sites. This might be due to production of AMF spores in the rhizosphere vicinity of surrounding herbaceous species (Kruckelmann, 1975). Occurrence of herbaceous community in Umsaw and Mawlein were observed but in Umdihar spore density and herbaceous community were comparatively lower. The herbaceous community was removed or not frequent in Umdihar, however, the spore density could be disturbed as the site was located very near to national highway No. 44. Furthermore, in an undisturbed ecosystem, the number of AMF spores and the population diversity were higher in native undisturbed forests than the disturbed and replanted areas (Moreira-Souza *et al.*, 2003). Spore population is affected by a wide range of soil, climatic, fungal and host factors (Anderson

et al., 1983; Howeler *et al.*, 1987). In the present study, diversity attribute of *M. champaca* plants such as H' and E were higher in Mawlein and Umdihar than in Umsaw. The variation in the diversity attribute can be substantiated to the study of Allen and Boosalis (1983) where the diversity of mycorrhizal fungi associated with the same plants was found to vary. Disturbance might also be responsible for distribution of AMF. Climatic seasons seem to be more influential on distribution and abundance of mycorrhizal spores. Mycorrhization of forest plants have recently been considered as the substitute of chemical fertilization regarding environment pollution and disease control for better management of tropical forests. More studies are being emphasized to select the suitable indigenous AMF strains for the establishment and managing the forests and to make the foresters and people conscious about the role of mycorrhiza as a tool to maintain and manage the forests environmentally friendly (Dhar and Mridha, 2006).

These significant non-random associations between AMF and host plants might suggest some degree of AMF host preference. The distinct colonizing strategies of various AMF are taxonomically based at the family level, and therefore current AMF taxonomy has a true functional basis. Non-random differences in distribution among different AMF species and genera in the field, it is also likely that the preferences of different AMF with host plant in our study sites might be reflected at the species or family level. Many factors can influence AMF distribution and community structure, such as climatic and edaphic factors, spatial and temporal variation, vegetation, host-specificity between fungi and plants, disturbance, and differential sporulation ability of AMF taxa (Zhao and Zhao, 2007).

Glomus sporulated abundantly regardless of the sites selected. Das and Kayang (2009a&b) also reported the dominance of the *Glomus* from this region. They described the wider adaptation of the taxon in varied soil conditions. The sporulation pattern of

Glomus might bring about the dominance of the taxon. Spores of *Glomus* grow in cluster and sporulate more frequently while the spores of other genera like *Gigaspora* sporulated singly (Dhar and Mridha, 2006).

Trap cultures were established using Bahia grass (*Paspalum notatum*) as the host plant in our study. Trap cultures, using host plants grown in soil diluted with sterile sand, are most commonly used to isolate AMF. This pot culturing method usually results in the isolation of more species than other methods. It provides additional information on fungal diversity that complements spore occurrence data obtained using the same soil samples and may provide valuable new information about the biology of AMF (Liu and Wang, 2003). Inoculum production on a commercial scale has always used widespread host plants such as *Allium cepa*, *Cenchrus ciliaris*, *Panicum maximum*, *P. notatum*, *Sorghum halepense*, *Trifolium subterraneum* and *Zea mays* in which spores develop within 3-4 months (Chellappan *et al.*, 2001). AMF colonization and subsequent spore formation and production depend upon the type of host as well as the duration of infection of these symbiotic organisms. Generally, with increase in the growth period after infection, root colonization of host increase. However, this increase in colonization and period do not have greater bearing on spore production. It is the host type, which is more important for spore production. Therefore, the relationship between colonization and rate of colonization with growth period do not vary greatly in different hosts. Larger plant size has been associated with increased levels of sporulation, probably because larger plants often have more extensive root system than smaller plants, allowing greater mycorrhizal colonization and sporulation (Chaurasia and Khare, 2005). In ecosystem studies and glasshouse experiments, host plants and soil factors can influence both diversity and overall levels of mycorrhizal formation and sporulation. For instance, high levels of phosphorus in soil and plant are able to inhibit mycorrhiza formation and influence the

diversity of AMF in field soil. Variation in spore production could not be explained by mycorrhizal colonization level. The root length colonized by a single species of AMF on a host plant is not necessarily correlated with the spore number produced on the same plant which supports our investigation that AMF colonization and spore density do not correlate. Sporulation may have been further influenced by the presence of other species or by the different soil characteristics. This may explain why there was no correlation between root length colonized and the spore numbers formed in this investigation. The differences in spore numbers produced with different trap plants might contribute to the variation in host plant root type and morphology, carbon biomass, nutrient and endogenous hormone level. These factors might be expected to influence the richness of AMF isolated from soil in trap cultures (Liu and Wang, 2003).

In our study, *Glomus* sp1 possibly resembles morphologically *G. macrocarpum*. This species was found to be highly abundant from the plantations and distributed in all the sites. Moreover, it was concluded that it may facilitate the better growth of host plant. The other species was *Glomus* sp 2. This species was found also in one the plantation site and was abundant in that site.

The production of seedlings of fast growing species such as *Acacia*, *Eucalyptus* and *Gmelina* in commercial nursery scales requires application of large amounts of chemical fertilizers, which entails high cost. Without fertilizer application, intensively managed tree plantations generally have low harvest. Moreover, fertilization cost is highest for degraded forest lands (Mackensen and Foster, 2000). However, heavy fertilization often reduces the formation of some AMF (Johnson, 1993; Titus and Leps, 2000). If the production cost using AMF inoculum is lower than that using fertilizer, AMF inoculation can replace or reduce the amount of chemical fertilizers used in nurseries and in the field, without loss of efficiency of AMF colonization. Studies on tree

species have indicated that AMF inoculation reduces the amount of chemical fertilizers required for seedling production (Habte *et al.*, 1993; Siqueira *et al.*, 1998; Muthukumar and Udaiyan, 2006). Likewise, *M. champaca* is a commercially important tree species important for its valuable flowers and timber. Chemical fertilizer was commonly applied to these species in nurseries on the commercial scale. Heavy application of chemical fertilizer decreases AMF colonization and thereafter diminishes growth improvement by AMF. If AMF inoculum could be produced at small cost, inoculation of AMF can reduce the application of chemical fertilizer without decrease in AMF colonization. The survival rate of seedlings is a key measure of success in reforestation and afforestation. In northeast India, loss of tropical and sub-tropical forests was extensive due to population growth, logging, and land demand for various development projects.

The reforestation and afforestation programs aim to cover three million hectares during a 5-year period to convert degraded forest into sustainable forest production units. (Turjaman *et al.*, 2006). Increased survival rates were noted in tropical tree species with AMF colonization, such as *Ochroma pyramidale*, *Luehe seemanii*, *Heliocarpus appendiculatus*, and *Stemmadenia donnell-smithii*. Field trials with AMF inoculation are required to monitor the growth and survival rates of both species. Inoculation techniques may be adopted by a large scale nursery jointly with reforestation programs, thereby aiding in the recovery of the population of such endangered tropical tree species as *Aquilaria filaria* (Turjaman *et al.*, 2006). In our study, this plant is utilized as a highly valued timber plant which is evaluated for uniform growth with AMF inoculation and can be supplied to the growers with healthy growth than to the uninoculated ones.

According to Mosse and Hayman (1980), colonization of the roots with AMF prior to the introduction of seedlings to the field provides the plant with advantages for establishment. Prior to transplanting seedlings, the inoculum density should be evaluated

to determine the relative importance or necessity of inoculation. Generally, more seedlings are needed when the seedlings are not inoculated with AMF, because field conditions are much more extreme than nursery conditions. The use of AMF-inoculated seedlings might solve this problem: it would reduce significantly the cost of seedlings required for reforesting vast areas such as peat-swamp forests (Turjaman *et al.*, 2008).

There is significant influence in the growth of *M. champaca* under AMF inoculation. Moreover, improvement of plant growth with inoculation of AMF has been reported in a number of forest tree species (Rajan *et al.*, 2000; Vasanthakrishna *et al.*, 1995) and medicinal plants (Earanna 2001; Boby and Bagyaraj, 2003). The main effect of mycorrhizal fungi in improving plant growth is through improved uptake of nutrients, especially phosphorus due to the exploration by the external hyphae of the soil beyond root hair zone when phosphorus is depleted (Gerdemann, 1975). However, root hairs were observed in the plants grown in greenhouse in both inoculated and non-inoculated plants. The possible development of root hairs in this condition may be of the period in non-mycorrhizal condition after germination till inoculation. Moreover, hairs were also observed in non-inoculated roots. Increased phosphorus uptake has been attributed not only to increased surface area of absorption but also to enhanced hyphal translocation (Hattingh *et al.*, 1973). Enhanced plant biomass and P uptake because of AMF inoculation has been reported by earlier workers in forest tree species and a few medicinal plants (Vasanthakrishna *et al.*, 1995; Sailo and Bagyaraj, 2005). This study was in accordance with the study by Turjaman *et al.*, (2006) where colonization by *G. clarum* and *G. decipiens* increased plant growth, shoot nutrient concentrations, and survival rates of *Dyera polyphylla* and *A. filaria* seedlings 180 days after transplantation under greenhouse conditions.

The difference in infectivity of native and introduced fungal species has been investigated. In our investigation, *G. intraradices* was introduced which influences better growth than indigenous fungi i.e., *Glomus* sp 1 and *Glomus* sp 2. Abbott and Robson (1981) noted that the native *Glomus* species was easily replaced by three of the inoculant fungi in York soil but not in Merredin soil, indicating that introduced fungal species may have higher infectivity in a certain soil. In the experiments conducted by Yao *et al.* (2008), *G. versiforme* was inoculated as the introduced AMF species. Regardless of the sites and plant species, the colonization by *G. versiforme* was always higher than that by the native AMF. This may suggest the wide adaptation of *G. versiforme* to many soil types or some properties favoring this fungal species presence in the soil (Yao *et al.*, 2008). Although AMF lack absolute host specificity, as far as the colonization is concerned, the growth responses of plants to a given AMF species differ widely (Sanders, 2003). Moreover, native AMF greatly promoted the growth of one species but not the other (Yao *et al.*, 2008). The contribution of native AMF to plant growth, when compared with introduced AMF, also varied greatly, depending on the plant-fungi combination. This result is similar to that of Klironomos (2003). He found that the performance of native AMF in promoting native plant growth was more extreme than that of exotic AMF.

Plants get benefited from mycorrhizal symbiosis mainly due to the increased absorptive surface provided by fungal hyphae in their root system (Smith and Read, 1997). Growth of mycorrhizal and non-mycorrhizal plants, therefore, may respond differentially to soil nutrient concentration, as observed in several experiments (Bougher *et al.*, 1990; Titus and del Moral, 1998). Mycorrhizal seedlings, in this experiment, had higher shoot height, root length, collar diameter, leaf area and biomass. Significant enhancement in root and shoot growth of nursery-raised seedlings may be due to

increasing supply of nutrients (Giri *et al.*, 2005), carbohydrate partitioning (Graham *et al.*, 1997) and toxicity mediating ability (Dong *et al.*, 2008) of mycorrhizal fungi.

Mycorrhiza effects on root morphology were commonly attributed to improvement in phosphorus uptake by AMF, but AMF effects on hormone production may also be responsible (Berta *et al.*, 1993). The influence of root for nutrient acquisition is more by root length than mass and root length increases with decreases in root diameter (Eissenstat, 1992). Evolutionary advances in root morphology result in greater control of fungi and their confinement to smaller zones within plants, culminating in the reduced organs of plants with exploitative mycorrhiza (Brundret, 2002). In the present investigation, the root system in terms of weight and length is proportional in both types of plants which support significant more shoot biomass in mycorrhizal plants than non-mycorrhizal plants. In this study, number of roots of second order in non-mycorrhizal plant was significantly lower than mycorrhizal plant and length and diameter of non-mycorrhizal root was comparatively higher. The modifications in terms of insignificant longer and thick diameter roots in non-mycorrhizal seedlings which may be not be sufficient for proper nutrient acquisition than in mycorrhizal plants where short and additional number of roots exhibit more surface area and nutrient acquisition may be achieved by extensive hyphal system of AMF in mycorrhizal plants. AMF colonization did not affect the tap root length, the average root diameter, and the basal root growth angle in spite AMF species exerted differential influence on the plant growth. Contrastingly, AMF colonization significantly reduced the total root length, the root volume, the root surface area, but promoted the formation of lateral roots of high order (Yao *et al.*, 2009). Generally, AMF strengthens the absorption function of root systems by partial substitution of the outspread fungal hyphae. Carbon economy and maximal uptake capacity of root systems are the strategies of mycorrhizal plants. Previous data

showed that AMF altered root morphology primarily when phosphorus was limited, but the ways in which the root morphology changed were not consistent (Liu, 2009).

The intracellular hyphal coils and intracellular arbusculate coils indicates *Paris* type and intercellular hyphae with penetrating arbuscules into the cortical cells specify *Arum*-type AMF morphology (Dickson, 2004). The root mycorrhizal morphology reveals *Paris*, *Arum* and intermediate type in mycorrhiza inoculated seedlings. However, *Paris* type morphology was reported earlier (Muthukumar *et al.*, 2006; Das and Kayang, 2010a). However, in our study intracellular hyphae with penetrating arbuscules in adjacent cell were considered it to be intermediate type; moreover, Dickson (2004) reported intracellular arbuscule with hyphae in the same cell as intermediate type 2. The coincidence of different AMF morphology within the same root is consistent with the previous report (Bonfante-Fasolo and Fontana, 1985; Kubota *et al.*, 2005; Kubota and Hyakumachi, 2004). Bonfante-Fasolo and Fontana (1985) reported co-occurrence of *Arum* and *Paris*-type morphologies in the same root system, but *Paris*- type morphology was less. This is contrast to the study of Kobato *et al.*, (2005) where in tomato and cucumber both morphological types were well represented. *Lycopersicon esculentum* also formed both *Arum*- and *Paris*-type (Kubota and Hyakumachi, 2004) but with high occurrence of *Paris*-type AMF morphology which is consistent with our study.

A recent detailed study, with *L. esculentum* however, again suggests that colonization type is not only under plant control but that fungal identity plays an important role (Cavagnaro *et al.*, 2001). In this plant, Cavagnaro *et al.* (2001) showed that while three fungal species (*G. intraradices*, *G. mosseae*, and *G. versiforme*) formed *Arum*-type structures, the other three (*G. coronatum*, *Gi. margarita* and *Scutellospora calospora*) formed *Paris*-type coils with no visible intercellular phase.

Summary

Arbuscular mycorrhizal fungi (AMF) are associated with majority of land plants in a symbiosis. The economic and ecological importance of these ancient biotrophic plant symbionts is therefore obvious. AMF transfer inorganic nutrients and water to the plant and receive carbohydrates in exchange. By driving this bidirectional nutrient transport between soil and plants, they are highly relevant for global phosphorus (P), nitrogen (N) and CO₂ cycles. Moreover, they affect directly and indirectly the diversity and productivity of land-plant communities by their central role at the soil–plant interface. They can also improve host plant pathogen resistance and drought stress tolerance (Krüger *et al.*, 2009).

Generally, several plant species have consistently high levels of mycorrhiza, some have intermediate, or variable levels of mycorrhiza or numerous are not mycorrhizal. The obligate AMF associations are typical of plants with coarse, slow growing, long-lived, and relatively thick roots (Brundrett, 2002). The primitive angiosperm and all the gymnosperms had short, thick fine roots, which had lower specific root length (Pregitzer *et al.*, 2002). Plants in the Magnoliaceae are considered part of the ancestral angiosperm complex. The family Magnoliaceae *sensu stricto* comprises approximately 240 species of evergreen and deciduous trees and shrubs which are distributed widely in the tropical, subtropical and temperate zones of southern and eastern Asia, southeastern North America, and northern South America (Shi *et al.*, 2000). The coarse structure of the root typified by the order Magnoliales are especially dependent on AMF for mineral uptake (Baylis, 1975).

There were few preliminary assessment of AMF and its morphology was examined in a few species from Magnoliaceae. However, there is lack of report on studies of species having coarse root structure from Magnoliaceae between non-

mycorrhizal and mycorrhizal plants. Keeping in view the inadequacy, we have investigated the plants with the following objectives: (i) Assessment of arbuscular mycorrhizal colonization in *Michelia champaca* L. (ii) Diversity of arbuscular mycorrhizal fungi from the plantations of *M. champaca*. (ii) Isolation, characterization and identification of the arbuscular mycorrhizal fungi associated with *M. champaca*. (iii) Determination of the potential of the isolated arbuscular mycorrhizal fungi on growth of *M. champaca*. (iv) Root structure characteristics of *M. champaca* in association with arbuscular mycorrhizal fungi. (v) Quantification of root cortical cell and arbuscular mycorrhizal fungal colonization in *M. champaca*.

The findings of the study are summarized below:

- The assessment of mycorrhizal colonization conducted in the seedlings of *M. champaca* collected from nursery. The roots were stained with Trypan Blue, Acid Fuchsin and Stamp pad ink for the quantification of colonization. Among, all the stains used, the root was found to predominantly colonization by AMF. Nevertheless, stamp pad ink was found to be appropriate as other standard stains used normally for colonization assessment. The ink was found to be suitable and effective on this species as compared to standard methods. This is the first kind of report on the use of stamp pad ink as the staining reagent. The method with black stamp pad ink still further reduces health risks. The low cost availability of this dye is also reliable to carry out experimental research and teaching exercises in the developing country like India. Moreover, this very simple technique often results in high quality images of visible competitive interactions at early stages of the plant growth by arbuscular mycorrhizal fungal colonization and symbiosis that may be used in a routine way for staining of other root colonizing fungi in different plant species.

- AMF and dark septate endophyte (DSE) colonization were investigated in three different plantation sites (Umdihar, Umsaw and Mawlein) of Meghalaya, northeast India. Isolation and identification of the AMF spore were conducted to evaluate the AMF diversity and host preference in terms of AMF species distribution and abundance in the plantation sites. Results showed that AMF colonization was significantly higher than dark septate endophyte colonization ($p < 0.05$). AMF and DSE colonization had a narrow range of colonization, varying from 50.91%–58.95% and 1.84%–4.11%, respectively. Spore density varied significantly in all the sites ($p < 0.05$). Out of 29 species identified from 7 genera, the species from *Glomus* was found to be highly abundant. Sorenson coefficient (Cs) ranged from 0.35–7.0. Species richness varied from 2.0–2.9 in the sites. Total species richness was significantly correlated with total relative abundance ($p = 0.001$). The distribution, abundance and principal component analysis plot suggest that *Glomus macrocarpum*, *G. multicaulis*, *G. constrictum* and *Acaulospora* sp 1 were the most host preferred species which possibly may favour the host with proper nutrient acquisition and growth.
- To evaluate the efficacy of native isolate of AMF, there was need to isolate from natural soil. Therefore, the trap plant culture was established with *Paspalum notatum* to isolate AMF. The single spore culture was found to possess successfully in case of *Glomus* sp 1 in the first cycle. Nevertheless, in the first cycle of spores in *Glomus* sp 2 was found to be contaminated by a different morphotype of spore varying in 50-80 μm in size. However, the second cycle showed single morphotype of *Glomus* sp 2. The transfer of both the types of spore in the third cycle in large pots was found to contain pure monospecific spores and sporulation was in abundance. The density of spores in the third cycle was 752 spores of *Glomus* sp 1 per 25g of soil and 962

spores of *Glomus* sp 2 per 25g of soil. The trap culture technique used for isolation of AMF spore reveals two morphotypes: *Glomus* sp 1 and *Glomus* sp 2.

- In the present investigation, attempts were also undertaken to screen and select an efficient AMF for inoculating *M. champaca* for its propagation. For this reason, *Glomus* sp 1, *Glomus* sp 2 and *Glomus intraradices* were used to evaluate the efficacy of the isolate. There was significant influence of *G. intraradices* and *Glomus* sp 1 inoculation on the shoot dry weight, shoot length, and leaf area of *M. champaca* after 180 days of inoculation. Overall, there was no effect on AMF inoculation on the root dry weight, root/shoot dry weight ratio, roots length, stem girth, root to shoot ratio and number of leaves of the plants. There were significant differences between mycorrhizal (*G. intraradices* and *Glomus* sp1) and non-mycorrhizal plants in shoot dry weight, shoot length, and leaf area. *G. intraradices* exhibit more significant differences in growth parameters than *Glomus* sp1. *Glomus* sp 2 did not show significant variation in growth parameters than non-mycorrhizal ones.
- There was significant influence of *G. intraradices* inoculation on the shoot dry weight, shoot length, stem girth, leaf area and number of leaves after 270 days of inoculation. Moreover, there was significant influence of *Glomus* sp 1 inoculation on the shoot dry weight, shoot length, stem girth, leaf area, root dry weight and root/shoot ratio of *M. champaca* after 270 days of inoculation. However, there was no effect on AMF inoculation on the roots length of the plants. There were significant differences between mycorrhizal (*G. intraradices* and *Glomus* sp1) and non-mycorrhizal plants on the shoot dry weight, shoot length, stem girth, leaf area root dry weight and root/shoot dry weight ratio. *G. intraradices* exhibit more significant differences in growth parameters than *Glomus* sp1. *Glomus* sp 2 showed significant

variation in leaf area than non-mycorrhizal ones. *G. intraradices* exhibit significant higher number of leaves compared to others.

- The results suggest that of the native two species isolated, *Glomus* sp 1 influenced the growth of *M .champaca*. Moreover, the exotic species (*G. intraradices*) also improved the growth of host plant. However, *Glomus* sp 1 improved the belowground features after 270 days of inoculation whereas exotic species enhanced aboveground features.
- There were two root orders observed in both mycorrhizal and non-mycorrhizal plants observed after 180 days. There were significant differences in length and dry weight between orders of both types of plants. However, there were no significant differences in diameter of first and second order roots of non-mycorrhizal plants. Moreover, length and diameter of root orders of non-mycorrhizal plants were comparatively higher than mycorrhizal plants. In addition, there were no significant differences in the number between orders of mycorrhizal plants. However, the number of second order mycorrhizal roots was significantly higher than non-mycorrhizal roots. There were three root orders observed in both mycorrhizal and non-mycorrhizal plants observed after 270 days. The mycorrhizal colonization in second and third orders was significantly higher than first order. However, height and diameter of first order was significantly higher than second and third orders. There was significant correlation noticed between hyphal and vesicles colonization and between height and diameter of root order.
- Roots collected from plantation sites exhibit *Paris* type of AMF morphology in all the roots examined. In addition, intermediate type of AMF morphology was also observed in few segments. Root hairs were not observed from the plantations sites. The colonization proceeds from epidermis and passes through 4th-6th layers to

colonize the inner cortex. 10th-15th layers of cortex were observed where from 5th-7th layers onwards inner cortical layers colonization was observed. The colonization was found all around vascular bundle in the inner cortex.

- In the plants grown in greenhouse inoculated with AMF, root hairs were observed along with extraradical hyphae. *Paris* type AMF morphology was observed in the roots with intracellular hyphal coils; *Arum* type was detected in a few segments as well as intermediate type. 6th-8th cortical layers were found of which three layers onwards colonization was encountered. The presence of casparian strips in xylem, endodermis and exodermis is clearly shown. AMF colonization was also confined to the inner cortex surrounding the vascular tissue.
- The root order modification such as number of root order in mycorrhizal and non-mycorrhizal was conspicuous. Moreover, arbuscular mycorrhizal structure reveals three types of AMF morphology in this tree species.
- The sections revealed inner colonization of the cortex in longitudinal and transverse sections. All the three of diameter of root size reveal arbuscular mycorrhizal structure such as arbuscules, hyphal coils and hyphae. The colonization was observed to be in transverse and longitudinal section. However, outer cortical region of longitudinal section was not colonized. Highest number of cells was observed in 0.6 mm of diameter of root. Nevertheless, colonization was maximum in 0.4 mm of root segment. Total number of cell was maximum in 0.6 mm of diameter of root and total number of AMF structural colonization was maximum in 0.4 mm of root segment. Moreover, percentage of AMF structural colonization was found to be maximum in 0.4 mm root diameter.

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Academic qualification:

Sl. No	Examination Passed	Division with % of marks	Subject	Year	Board/ University	Distinction achieved
1.	SSLC	II (54.4)	Maths, science, Eng., etc	1996	MBOSE	-----
2.	HSSLC	I (60.8)		1999	MBOSE	-----
3.	B.Sc	I (76.6)	Phy,Che,Bio	2002	NEHU	First position
4.	M.Sc	I (75.1)	Botany (Honours)	2004	NEHU	First position
5.	NET-JRF	-----		2005	UGC	-----
6.	PhD	Pursuing	Botany Life Sciences Microbiology	2006	NEHU	-----

1. Awards

- A) B.Sc (Botany) = 1st Class 1st – Gold Medalist
- B) M.Sc (Botany) = 1st Class 1st – Gold Medalist
- C) NET-JRF UGC-2004 Dec
- D) BEST Poster Presentation-77th Annual Session National Academy of Sciences
- E) Best Poster Presentation-96th Indian Science Congress

2. Member of Association of Microbiology of India

3. Presently faculty in Botany Department, Tripura University

Publication in peer-reviewed Journals:

1. ***Das, P.** and Kayang, H. (2008). Stamp pad ink, an effective stain for observing arbuscular mycorrhizal structure in roots. **World journal of agriculture sciences**. 4: 58–60.
2. **Das, P.** and Kayang, H. (2009). Arbuscular mycorrhizal fungi association with *Blechnum orientale* Linn. in pine forest and anthropogenically disturbed areas of northeast India. **Archives of agronomy and soil sciences**. 55: 623–632.
3. **Das, P.** and Kayang, H. (2009). Arbuscular mycorrhizal fungi from northeast India. **Journal of Agricultural Technology**. 5: 291-298.
4. **Das, P.,** Chettri A., and Kayang, H. (2009) Habitat Preference of *Auriscalpium vulgare* Gray Inhabiting Slash and Burn Affected Khasi Pine Cones of India. **Our Nature**. 7: 32-38.
5. Barik, S.K. Lakadong N.J., Baishya, R., Chettri, A., **Das, P.,** Kayang, H. and Marbaniang, D. (2009). A new record of *Monotropia hypopitys* L., A myco-heterotrophic plant from India. **Journal of the Bombay Natural History Society**. 106: 127-128.
6. ***Das, P.** and Kayang, H. (2010). Mycorrhizal colonization and distribution of arbuscular mycorrhizal fungi associated with *Michelia champaca* L. under plantation system in northeast India. **Journal of forestry research**. 21: 137-142.
7. **Das, P.** and Kayang, H. (2010) Arbuscular mycorrhizal fungi and dark septate endophyte colonization in bamboo from northeast India. **Frontiers of agriculture in China**. 4: 375–382.
8. **Das, P.** and Kayang, H. (2010) Association of dark septate endophytes and arbuscular mycorrhizal fungi in potato under field conditions in the northeast region of India. **Mycology**. 3: 171–178.

(Publications in * symbol are included in thesis)

Proceedings:

1. **Das, P.** and Kayang, H. (2009). Occurrence of arbuscular mycorrhizal fungi in natural forest and arable land of Meghalaya, northeast India. **Proceeding of the workshop cum training programme on “Recent advances in microbial technology and molecular biology.”** Department of Forestry, NERIST, Arunachal Pradesh.

Seminars / Symposia / Workshops attended:

1. Workshop on "Science and technology for the better quality of life." (2006) Organized by Atomic Energy, Science Seminar Hall, NEHU, Meghalaya.
2. Participated in National Symposium on "Issues and Challenges for Conservation of Plants and Ecosystems in India" (2006) Department of Botany, NEHU, Meghalaya.
3. Workshop on "GIS & RS Application in Environmental Studies." (2007) Department of Geography, NEHU, Meghalaya.
4. Workshop on "Introductory statistical analysis of Biological data" (2007) Department of Statistics, NEHU, Meghalaya.
5. Training programme on "Recent Advances in Microbial Biotechnology & Molecular Biology." (2008) Department of Forestry, NERIST, Arunachal Pradesh.
6. Training course on "Bioinformatics: Recent trends in sequence analysis" (2007) Bioinformatics Centre, NEHU, Meghalaya.
7. Poster presentation in "National Academy of Sciences 77th Annual Session and Symposium on Novel Approaches for Food and Nutritional Security" (2007) Central Food Technological Research Institute, Mysore.
8. Participated in "National Symposium on Frontiers in Biocomplexity and Biodiversity of Plants." (2008) Department of Botany, NEHU, Meghalaya.
9. Poster presentation in "International Symposia on Microbial Biotechnology: Diversity, Genomics and Metagenomics." (2008) Delhi University, Delhi.
10. Oral presentation in Conference on "International Society on Biotechnology: Diversity, Genomics and Metagenomics." (2008) Sikkim Manipal University, Sikkim.
11. Oral presentation in "Recent Advances in Microbial Biotechnology & Molecular Biology." (2008) Department of Forestry, NERIST, Arunachal Pradesh
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