Biodiversity of Arbuscular Mycorrhizal Fungi Associated with *Machilus* Species in Hong Kong

Submitted by

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The mycorrhizal symbiosis formed between plant root and the arbuscular mycorrhizal (AM) fungi (Glomales) is of great importance to the understanding of its potential influence on ecosystem processes. Up to now, the taxonomy of arbuscular mycorrhizal fungi is almost totally based on the morphology of spores from pot culture, little is known from the field. This study devotes efforts in collecting basic information from natural site concerning the diversity in AM fungi associated with three *Machilus* species in Hong Kong.

Machilus is a very important native genus of trees in Hong Kong and in South China, which represents one of the most dominant genera in the local natural secondary forest. The mycorrhizal association of *Machilus* species is, therefore, believed to play an important role on the structure and nutrient dynamics in the local forest communities.

This study described details in morphological characterization of AM fungi associated with 3 *Machilus* species and explore their functional role on phosphorus acquisition in pot-culture experiments. The results obtained - including edaphic physical characteristics of the site, density of AMF spores in soil, infection rate of roots, morphological characterization and identification of AMF spores, the effect of AMF on nutrient acquisition in pot-cultures - provide basic database of the diversity in the AM fungi associated with the *Machilus* species studied. Hopefully, this information will be valuable for further studies, such as the evaluation of biological and molecular characteristics of AM fungi, in order to elucidate the role of such symbiosis in the forest ecosystems.

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Table of Contents

LIST OF PLATES

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1 LITERATURE REVIEW AND BACKGROUND OF RESEARCH

1.1 Literature review

1.1.1 What is a mycorrhiza

Mycorrhizae are dual organs of absorption formed by the symbiotic fungi inhabiting healthy absorbing organs (roots, rhizomes or thalli) of most terrestrial, aquatic and epiphytic plants (Trappe, 1994). They are the most prevalent, and perhaps the most important symbiosis system found in the Plant Kingdom. In natural conditions, mycorrhizal fungi are essential to the health and growth of the host plants. They can benefit plant communities in a number of ways although the most important one is the enhancement of nutrient absorption from soil (Harley and Smith, 1983).

1.1.2 The importance of mycorrhiza

Perhaps the most important factor stimulating interest in mycorrhiza studies is the well-documented evidence indicating that they can promote growth of the host plants. The importance of the association of mycorrhizal fungi with tree roots arises from the fact that trees commonly have low rooting densities in soil (Bowen, 1981). Mycorrhizal fungi, with their extramatrical hyphae, can substantially extend the area of absorption beyond that of root hairs. This is especially important for the absorption of relatively immobile elements in soil such as phosphorus, copper and zinc. The hyphae associated with mycorrhizal plants can also ramify a greater soil volume and provide a greater absorptive surface than root hair on a non-mycorrhizal plant. In addition, mycorrhizal plants have been shown to have greater tolerance to toxic heavy metals, root pathogens, drought, high soil temperature, saline soils, adverse soil pH and transplant shock than non-mycorrhizal plants (Harley and Smith, 1983).

Because of these attributes, mycorrhizal plants are important in the re-establishment of plantations in inhospitable sites such as coal and copper mine wastes, borrow pits, and badly eroded locations.

There are currently seven known types of mycorrhizae: vesicular-arbuscular (VA) mycorrhiza (some authors call these arbuscular mycorrhizae (AM) because some of the fungi fail to form vesicles; the term AM will be adopted in this thesis.), ectomycorrhiza, ectendomycorrhiza, arbutoid mycorrhiza, ericoid mycorrhiza and orchid mycorrhiza. The distinct structural features, morphogenesis events during mycorrhizal formation, ecological significance and plantation practice of these seven mycorrhizae have been well documented in various reviews (Harley and Smith, 1983; Bonfante-Fasolo, 1984; 1987; Kottke and Oberwinkler, 1986; Peterson et al., 1994). They are categorized primarily by the taxonomic groups of the fungi involved in the mycorrhiza formation, as well as the alterations in the morphology of the fungi and roots during the development of the mycorrhiza (fungus-root) (Harley and Smith, 1983). Despite the variations among the seven types of mycorrhizae most share fundamental features that are associated with the interactions between symbionts. It is these processes, including chemotropism, recognition, compatibility and early alterations in the morphogensis of the symbionts that ultimately lead to an integrated, functioning structure (Peterson and Farquhar, 1994).

Two types of mycorrhizae, namely arbuscular mycorrhizae and ectomycorrhizae, are extensively studied. The former are present in the majority of vascular plants, including most crops and horticulture species, and the latter are found in most timber-producing tree species (Peterson et al., 1984). This review will be focusing on the discussion of the diversity of AM fungi.

1.1.3 The AM fungi and their ecological significance

The growing interests in AM is demonstrated by the remarkable number of recent reviews devoted to the taxonomic, genetic, structural and physiological aspects of AM fungi (Walker, 1992; Gianinazzi and Schuepp, 1994; Robson et al., 1994). The most common mycorrhizal association is the arbuscular mycorrhizae (AM). They are present in about 90% of plant species and are found worldwide in virtually all habitats. They are widespread geographically (in arctic, temperate, and tropical plants) as well as, ecologically (from aquatic to desert environment) (Harley and Smith, 1983).

According to current terminology AM are endo-mycorrhizae because the endophytic fungus undergoes complex morphogensis inside the host root, with development of intracellular structures. AM is a complex system balancing and regulating both fungal and host growth. During its life cycle, the fungus produces spores that germinate and give rise to a vegetative mycelium that contacts the host root surface and produces a structure named appressoria. Hyphae will develop and they will initiate infection of root tissues and later give rise to inter- and intracellular hyphae, coils and vesicles. Only specific root tissues such as epidermal and cortical tissues are colonized whereas others, such as meristems or vascular tissues are resistant to mycorrhizal infection (Bonfante and Perotto, 1992).

The potential impact of AM fungi on the functioning of ecosystems is significant. AM are ubiquitous, with about 90% of terrestrial plant species forming the AM symbiosis. They are potential factors in determining the diversity in ecosystems, probably by modifying the structure and functioning of a plant community, in a complex and unpredictable way (Grime et al., 1987; Read, 1990). AM fungi, being an important component in the soil microbial biomass, can interact with land flora and are directly involved in essential processes at the plant-soil interface (Harley and Smith, 1983; McGee et al., 1989). The extraradicle AM

fungal hyphae increase the efficiency in phosphorus acquisition from soil for the plants (Koide, 1991) and in return, the plants donate the carbohydrates to the fungal partner (Smith and Smith, 1990). This carbohydrate can move from one plant to another through hyphae extension (Grime et al., 1987). Thus the AM fungi have the potential to influence both the carbon and phosphorus cycles at the ecosystem level.

Recently, the concern over both global and local losses in biodiversity and the effects that these might have on the environment impel great emphasis on understanding how ecosystems function and, in particular, the role that diversity (or biodiversity) plays in the ecosystems. Although AM fungi have been shown to play a significant role in the floristic diversity and structure of annual and perennial plant communities (Grime et al., 1987; Gange et al., 1990; Sanders and Koide, 1994), little or no attention has been paid to the diversity of AM fungi themselves. This is because experiments conducted in pots have indicated that AM fungi show little host specificity. Consequently, it was thought that there is little selection pressures that would favor extensive divergence arisen from mutualistic symbioses (Law, 1985). In support of this, the morphological diversity of AM fungi is, indeed low: approximately 152 species in six genera have been discussed so far (Walker and Trappe, 1993). Although these fungi show no specific variations in their range of host plants, a large variability can be found in their population biology, ecological specificity and symbiotic activity. Therefore, the low evolutionary divergence of AM Fungi seems to be inconsistent with their functional diversity in colonizing such a wide range of plants. It has been shown that different isolates of AM fungi can result in different effects on plant growth (Jakobsen et al., 1992). Some other results have shown that isolates indeed differ in their effects on plant growth but that these effects differ according to plant species (Sanders et al., 1996). These studies outline the potential importance of diversity of AM fungi in natural communities.

Understanding the real, rather than the potential, significance of AM fungal diversity in natural communities has posed a greater problem. For a given plant community we need to know how diverse the fungal community is, which plant roots are colonized by which AM fungi, whether there is seasonality in the patterns of colonization, whether any specificity between plants and AM fungi occurs and what the effect of those AM fungi will be on plants and ecosystem. However, together with the obstacles of identifying AM fungi in the roots of plants, the inability to grow the fungi in pure culture, problems in taxonomic classification, and a lack of basic information on the life histories of AM fungi, render much difficulties in the study of the ecological role of AM fungi in natural ecosystems. Advances in molecular techniques may provide more information about the genetic diversity of AM fungi that might help to explain some of the uncertainties mentioned above.

1.1.4 Taxonomy and classification of AM fungi

The basis for the taxonomy of the mycorrhizal fungi was provided by Thaxter (Thaxter, 1922). However, at that time, their trophic status was not known and both mycorrhizal and non-mycorrhizal fungi were classified together in one genus, *Endogone*. In 1953, the first link was made between "*Endogone*" (= *Glomus mosseae*) spores and the mycorrhizal habit of the fungus (Mosse, 1953). This was followed by the informal description of several 'spore types' (Mosse and Bowen, 1968), of which seven formed AM. In a monograph detailing the *Endogonaceae* in the Pacific Northwest (Gerdemann and Trappe, 1974), the family was further defined, and new species described. Five genera were included, namely *Endogone*, *Glomus*, *Sclerocystis*, *Gigaspora* and *Acaulospora*. Since then, two additional genera, *Entrophospora* and *Scutellospora*, have been erected. All genus, except *Endogone*, contain members capable of forming AM. More recently, *Endogone* was separated out and remained in its own family and order. All other genus, which are known or supposed to form AM,

were placed in their own order, *Glomales*, with three families: Glomaceae (genus: *Glomus* and *Sclerocystis*), Acaulosporaceae (genus: *Acaulospora* and *Entrophospora*) and Gigasporaceae (genus: *Gigaspora* and *Scutellospora*) (Morton and Benny, 1990).

Currently, approximate 152 species have been described in the Glomales on the basis of morphological characteristics of the spores, although not all are actually known to be mycorrhizal (Walker and Trappe, 1993). Although many of the named species were described from field-collected material (Walker and Trappe, 1993) and are therefore of unknown mycorrhizal status, it is assumed that they are all capable of forming arbuscular in roots. These fungi represent a phylogenetic group that probably became specialized very early in evolution. Fungi with a similar appearance to AM fungi have been recorded in fossils of some of the first land plants (Pyrozynski and Dalpe, 1989), thought to be approximately 400 million years old. There has been little divergence of AM fungi over this period of time (Law, 1988). Sequence analysis of the small-subunit rDNA of 12 current representative species of AM fungi is consistent with the fossil record that the divergence of the main genera of AM fungi occurred between 400 and 100 million years ago, followed by relatively little further divergence (Simon et al., 1993). It is thought that mutualistic interaction are stable and that the inhabitant symbionts are not subject to strong selection for further speciation (Cook, 1985; Law, 1985).

The current taxonomy system for *Glomales* is summarized in **Table 1** (Morton and Benny, 1990).

Table 1. The current taxonomy of the Glomales

Order: Glomales Morton and Benny
Suborder: Glomineae Morton and Benny
Family: Glomaceae Pirozynski and Dalpé
Genus: *Glomus* Tulasne and Tulasne
Genus: *Sclerosystis* (Berkeley and Broome) Almeida and Schenck
Family: Acaulosporaceae Morton and Benny
Genus: *Acaulospora* (Gerdemann and Trappe) Berch
Genus: *Entrophospora* Ames and Schneider
Suborder: Gigasporineae Morton and Benny
Family: Gigasporaceae Morton and Benny
Genus: *Gigaspora* (Gerdemann and Trappe) Walker and Sanders
Genus: *Scutellospora* Walker and Sanders

1.1.4.1 Use of fungal spores in classification of mycorrhiza

AM fungi have obligatory biotrophic status because they have so far resisted all attempts to be cultivated axenically (in pure culture). Consequently, the taxonomy and identification of these fungi has been largely and almost exclusively based on the distinct morphology of the large soil-borne spores, typically 80-500 μm in diameter and found near colonized roots. The major criteria used for species delineation are spore size, shape, colour, basal structure (including mode of occlusion), ornamentation, and wall (or wall-layer) structure (Morton et al., 1996). The relative importance of these criteria for taxonomic purposes remains controversial. This will become clearer as the species are being re-described (Rosendahl et al., 1994). Thus, unlike many plant and animal groups, the taxonomy and classification of AM fungi is not well established to the species level and it is virtually impossible to distinguish between genera or species when the fungus is associated with root tissues. In addition, the distribution, abundance and life history of AM fungi can hardly be observed without using any specialized or sophisticated techniques. With all the difficulties mentioned above, together with the difficulty in manipulating AM fungi in natural ecosystems without greatly modifying their environment in other ways, it is difficult to study their ecology,

evolution and the role within natural ecosystems. Despite this, some of the problems of identification, and possibly of taxonomy, can be overcome by using molecular techniques, and these techniques provide us an insight into the structure of natural communities of AM fungi. The techniques available have only recently been modified for specific use with AM fungi and some results, although preliminary already yielded interesting information that we will discuss below.

1.1.5 The biodiversity of AM fungi

Biodiversity, a term to describe variability between organisms or populations of organisms, has different facets depending on the level of diversity considered (species, population, ecosystem...etc.) or the organisms concerned (Barbault, 1992). Those aspects of glomalean fungi, when taken together, indicate that molecular, morphological and ecological levels of diversity are produced by different, and even independent processes (Morton and Bentivenga, 1994). The daunting task for systematists and other biologists is to make sense of this diversity.

1.1.5.1 Morphological diversity

Biodiversity can be exploited in many ways at scientific levels, the best known and the most ancient is undoubtedly in taxonomy and systematics. Currently, only the information of the morphological characters of mycelium and spores of glomalean organisms are available to hierarchically order species in a phylogenetic tree (Morton, 1990). Enough discrete variation is expressed in multinucleate spores to recognize over 150 species (Morton, 1988; Schenck and Pérez, 1990). This diversity is a remarkable evolutionary achievement because it is confined to subcellular levels of organization. It is evident that their exact identification is a fundamental requirement to understanding biodiversity and essential for monitoring changes

in natural managed or disturbed ecosystems. Diversity in AM fungi can be explored at this level by studying spore characteristics, ultrastructural features and infection patterns.

The most important taxonomical parameters of the spore characters include sporocarp occurrence, shape, colour and size; peridium occurrence and characteristics; spore colour, size and shape; spore wall number, colour, thickness and ornamentation; hyphal attachment, shape and type of occlusions. The comparative scanning electron microscope (SEM) and transmission electron microscope (TEM) investigations can be used to examine the ultrastructure of glomalean fungi to decide which wall features might contribute most to significant taxonomic variability (Morton and Benny, 1990).

Some fungi in the Glomales can differ in their pattern of colonization and the structures they form. After appresorium formation, infection can begin by the formation of either simple unbranched, inter- or intracellular hyphae with large hyphal loops or vesicles in the epidermal cells. However, such criteria based on mycorrhiza morphology have not been widely applied to fungal identification since variations are small and changes often depend on the host plant (Hetrick et al., 1985; Bonfante and Fontana, 1985; Gianinazzi-Pearson et al., 1991).

1.1.5.2 Biological diversity

Some of the intrinsic biological characters of the Glomales contribute to their taxonomy definition, whilst others provide a good indication of the variability for practical use of these fungi. The study of biological diversity should include the life cycle and developmental sequences of these biotrophic fungi as well as their symbiotic performance. Some factors that have to be considered are spore ontogeny and germination, infectivity and efficiency, biogeography, edaphic requirements and tolerance to agricultural practices.

Since AM fungi cannot be grown in pure culture, detailed ontogenetic studies would be a useful aid in distinguishing between different taxa and contribute greatly to a better understanding of the biology of these biotrophic organisms. More attention is given to the taxonomic characters of spore and sporocarp ontogeny because they are increasingly used in describing new species or studying established ones (Morton and Benny, 1990; Walker, 1992). Although AM fungi are obligate biotrophs, isolated spores can germinate *in vitro* (Mosse, 1962; Hepper, 1981). The characters of germinating ability, pattern of germination and quantity of mycelium produced are highly variable (Hepper and Smith, 1976; Pons and Gianinazz-Pearson, 1984; Giovannetti et al., 1991). Germination is an integral part of the life cycle of AM fungi and germination characters have importance for their taxonomy.

The symbiotic performance of AM fungi and the final mycorrhizal phenotype is a result of the interaction of two main factors, namely infectivity and efficiency, both of which depend on the fungal and the plant determinants. AM fungi have a very different levels of mycorrhizal colonization in terms of the root length infected (Gianinazzi and Gianinazzi-Pearson, 1986; Sieverding, 1991). This variability appears to be determined by factors linked to spore germination behavior, and pre- and post-infection events. Parameters relating to the efficiency of AM fungi depend very much on what is expected from the outcome of the symbiosis. In terms of the host plant, this can be better growth or survival (fitness), improved mineral nutrition, or increased tolerance biotic or abiotic stresses (Bethlenfalvay and Linderman, 1992). There are many reports of inter- and intraspecific differences in the effectiveness of AM fungi for plant growth and protection (Harley and Smith, 1983; Gianinazzi and Gianinazzi-Pearson, 1986; Thomas et al., 1989; Sieverding, 1991). However, the response range can be affected by host plant or soil conditions (Abbott and Robson, 1984; Sieveding, 1991) and the physiological bases of variation are virtually unknown. And there are a lot of questions that future researches need to be addressed.

While AM fungi can be found in most terrestrial ecosystems, they are more abundance in temperate and tropical grasslands, shrubs, deserts, as well as tropical forests (Read, 1991). Variation in the occurrence of many glomalean fungi is probably related to their edaphic requirement but, again, there is a lack of data on the ranges of soil variables under which specific glomalean species occur. Several edaphic factors have been shown to affect spore germination, root colonization and efficiency of AM fungi under experimental conditions (Hetrick, 1984). Although variations in the behavior of AM fungal species are known to exist with respect to soil factors (soil pH, heavy metals, texture, moisture, temperature, nutrient levels, salinity, etc.), the significance of these for fungal biodiversity in native habitats is poorly understood. There is evidence that human disturbance and agricultural practices such as the use of chemical fertilizers, pesticide applications, cropping sequences or soil disturbance may have variable effects on infectivity, efficiency and species composition of AM fungi (Schenck and Kinloch, 1980; Walker et al., 1982; Gianinazzi-Pearson, et al., 1985; Dodd and Jeffries, 1989; Dodd et al., 1990; Sieverding, 1991). Knowledge of how soil management and agricultural practices affect the dynamics and diversity of AM fungal communities is a pre-requisite to managing these symbiotic microorganisms in agricultural systems.

1.1.5.3 Molecular diversity

Molecular characters include DNA sequences, restriction fragment-length polymorphisms of DNA regions, isoenzymes, glycoproteins, and fatty acids. As previously discussed, the taxonomy and identification of AM fungi is almost exclusively based on the distinct morphology of their spores and it is virtually impossible to distinguish between genera or species when fungi are within root tissues. Analysis of molecular features offers an

alternative approach for accurate identification, for establishment of more precise phylogenetic relationships and for detection *in planta*.

Various regions of the nuclear rDNA have been widely used in systematics studies of eukaryotic groups, including fungi (Bruns et al., 1991). Nucleotide sequences of a highly conserved region of the 18S rDNA have been compared in isolates of selected glomalean species (Simon et al., 1992). Variable fragments detected from comparative sequence analysis may be potentially useful to elucidate the relationships among organisms within a species.

Molecular characters based on proteins maybe analyzed using electrophoresis, immunology or direct amino acid sequencing (Hillis and Moritz, 1990). Attempts have been made to characterize AM fungi by analysis of total or enzymically-active proteins extracted from spores and separated by electrophoresis. Proteins or polypeptides from spores or sporocarps of different fungi show a large variability both in quantity and electrophoretic patterns. Identification and purification of specific proteins from known species or isolates could lead to the production of specific antibodies for population studies of AM fungi, both inside and outside roots.

Fatty acid analysis has not been applied extensively to fungi, so very little information concerns glomalean fungi is available in this aspect.

Molecular techniques offer a wide range of possibilities to develop specific probes for AM fungi, not only for phylogenetic relationships and studies of their biodiversity at different levels of organization (ecosystem, population, species, isolate), but also for their accurate identification in soil and *in planta*. However, as far as this group of obligate symbiosis is concerned, molecular approaches can only be exploited efficiently if they are used together with available morphological and biological characteristics.

1.1.5.4 Ecological diversity

Until recently, methods for studying AM fungal diversity and the ecology of the symbiosis have been wholly reliant on the morphology of the spore phase. However, the use of spore data alone for the assessment of ecosystem diversity has long been recognized as unsatisfactory (Walker et al., 1982; Klironomos et al., 1993; Rolden-Fajardo, 1994). Situations, such as the presence of non-sporulating fungi, spore types that are visually isomorphic but are in fact distinct, or the presence of spores in soil being assumed to reflect the situation within root, can lead to misleading conclusions. In addition, data derived from observation of spore numbers reflect past events in the symbiosis. How this information relates to the contemporary situation is at best uncertain. The study of spores alone, therefore, is an insufficient method for the study of the ecology of mycorrhizal symbiosis and is likely to underestimate the actual diversity. Therefore, a better establishment of the levels and relationships between the morphological diversity of AM fungi and their biological, genetic and functional diversity is essential to the understanding their ecological diversity.

1.1.6 Molecular Techniques available for studying genetic diversity of AM fungi

Molecular biology techniques represent alternative tools for detailed studies of the genetic variability and phylogenetic relationships in the Glomales, and for obtaining specific probes for rapid identification of the fungi within host tissues.

It is difficult to describe all the molecular techniques available for ecological studies. The following is only a review of those which have been used, or likely to be used in the future, for studying AM fungi. For many organisms, a DNA fingerprint for identification or for the estimation of genetic diversity can be achieved by using restriction fragment length polymorphism (RFLP) analysis. This technique can only be used when a large amount of DNA can be obtained from individual organisms. Because AM fungi can not be maintained

in pure culture, obtaining sufficient DNA for RFLP is difficult. Consequently, it is better to employ polymerase chain reaction (PCR), which amplifies relatively small amounts of DNA, for identifying AM fungi or for studying their genetic diversity.

A universal molecular phylogeny has been based largely on sequences of the small subunit ribosomal DNA and regions of other ribosomal DNA (Winker and Woese, 1991). For elucidating the phylogenetic relationships of fungi, the 18S and 5.8S genes, along with flanking regions known as internal transcribed spacers (ITS), can be amplified and sequenced (White et al., 1990). Universal primers have been generated from comparative studies of conserved regions of different fungi (Simon et al., 1992). The 18S and 5.8S genes evolve relatively slowly and are useful for studies of distantly related organisms. The ITS region evolves faster than the 18S and 5.8S genes and are therefore useful in differentiating between species. The advantage of using these regions of DNA is that multiple copies of the genes are present in all organisms.

Another technique, known as random amplified polymorphic DNA (RAPD-PCR) is also available for studies of genetic diversity. The technique is potentially more sensitive in detecting genetic differences between individuals, and by screening large numbers of arbitrary primers, the desired sensitivity can be found for detecting genus. species or individual genotype differences. RAPD-PCR has been successfully used to identify spores at the level of species and to differentiate between isolates of the same species (Wyss and Bonfante, 1993). So far the technique has only been used in spores isolated from pot cultures and not been used for studying the diversity of natural AM fungal communities.

1.1.7 Summary and future directions

Knowledge and conservation of diversity in symbiotic AM fungi is of crucial importance for their efficient use in environmental conservation and sustainable plant production systems. A multidisciplinary approach is needed to identify the significant differences between populations, species or isolates. **Table 2** (Giovannetti and Gianinaai-Pearson, 1994) summarises some of the morphological, biological, and molecular aspects of AM fungi that may contribute to defining and understanding biodiversity in this group of symbiotic microorganisms.

Table 2. Some sources of biodiversity in AM fungi

Morphological characters	Biological characters	Molecular characters
Spores	Ontogeny of spores	Protein profiles
Wall ultrastructure & cytochemistry	Spore germination	Isozyme polymorphism
Infection patterns	Symbiotic efficiency	DNA analysis
	Biogeography	
	Edaphic requirements	
	Environmental tolerance	

An understanding of the mechanisms which promote and maintain genetic diversity in the AM fungi is crucial, not only to further advances in ecological and evolutionary studies but also to studies of molecular basis of the regulation of the symbiosis. Moreover, future studies should also aim to fill the gaps in current knowledge of links between genetic diversity and distribution of AM fungi in natural ecosystems, and their functional diversity. And also

much of descriptive work of species or communities should be carried out to form a foundation for realistic experimental investigation into their ecology.

1.2 Background of the research

1.2.1 Biodiversity of mycorrhiza in the forests of Hong Kong

Hong Kong has more than 800 km^2 of hilly areas in a total area of just over 1000 km^2. Most part of these hilly areas are forest areas that have little economic value. Although the territory lies within the tropics, it enjoys a variety of weather unusual for tropical countries and it is under the influence of a modified type of subtropical monsoon climate with hot wet summers (average 120 inches of rainfall per year) and cool dry winters (So and Chiu, 1986). Long before the Second World War, human activities destroyed all the original vegetation in Hong Kong, where the climatic climax was believed to be subtropical evergreen monsoon forests. The reforestation programs in Hong Kong started at around 1945. All the forests now in Hong Kong are, therefore, the secondary forests including the plantation forests and the naturally succession forests.

Owing to the unique geographic and climatic situation of Hong Kong, a vast diversity of macro- and micro- flora can survive and flourish in the territory. Since 1970s, great advances have been made in the study of Hong Kong's ecosystems and the designation of conservation areas and protected species. However, biological information, especially in mycorrhizal studies, in Hong Kong is still scarce. Although the importance of AM fungi in nutrient uptake by plants has been extensively studied (Sanders et al., 1974; Gianinazzi-Pearson and Gianinazzi, 1986), little is known about their diversity, especially at the specific- and sub-specific level. The study of diversity among AM fungi is partly hampered by the inconsistent

or incomplete use of limited morphological data in species descriptions. More studies on the variations of AM fungi at different levels of their organisation are needed. Conservation and efficient utilisation of their diversity are of crucial importance for sustainable plant production systems. In this work, we concentrate on the study of the biodiversity of AM fungi associated with *Machilus* species in Hong Kong.

1.2.2 The establishment of *Machilus* in local forest communities

Machilus species (Family: Lauraceae) are very important native tree species in Hong Kong. They can produce both berry fruits as food for the wild life and high quality wood for the human use. They flourish naturally in local forests and can also be established well in plantations, especially the three species selected for studied in this thesis: *M. thunbergii*, *M. oreophila*, and *M. ichangensis*. The three species are extensively used in local afforestation programs (personal communication with Agriculture & Fisheries Dept. of Hong Kong). Traditionally, the trees used in afforestation programs are usually hardy, relatively quick growing exotics. Common examples include Acacia from Taiwan, Slash Pine from USA and Brisbane Box from Australia. The simple structure, low plant diversity and use of exotics make young plantations a poorer habitat for wildlife than natural secondary forest of similar age. In contrast, older plantations often have a richer understorey of native trees and shrubs that can greatly enhance their attractiveness to wildlife. Therefore, planting mixtures of species and using natives whenever possible would increase the value of plantations to the wildlife. In recent years, there has been an increase in the use of local species, for example Machilus, Chestnut, Schima, and Tallow-trees in plantations.

1.2.3 *Machilus* species and their mycorrhizae

The root morphology of *Machilus* is very special. It is round and thick with heavily pigmented epidermal tissue and has almost no root hairs. Such morphology suggested that

they might rely on some auxiliary features for nutrient absorption. Mycorrhizal association may be one of the probable solutions, since there is a tendency, with respect to phosphorus uptake, for mycorrhizal dependency of plants to be negatively related in the root parameters such as root length and surface area (Baylis, 1975). As a matter of fact, it has been reported that *Machilus* species form endomycorrhiza associations with AM fungi in both natural forests (Zhuang and Chan, 1997) and in nurseries (Zhao and Chan, 1998). Since AM association is found in more than 90% of the terrestrial plants (Harley and Smith, 1983), their occurrence in *Machilus* species in Hong Kong is expected, though surprisingly few studies have been previously reported in these important native trees except in some field surveys (Chan and Griffiths, 1992; Zhuang and Chan, 1997).

Owing to the tight schedule for my MPhil. study and the large quantity of time-consuming fieldwork, it was necessary to limit the study area to one single site. Tai Po Kau Nature Reserve was chosen to be the study site because it was easily accessible and it has a very healthy stand of naturally succeeded *Machilus* forest. In this work, field survey, morphological characterisation of the mycorrhiza found in the *Machilus* trees and pot culture experiments have been carried out to investigate the biodivesity of AM fungi associated with *Machilus* species.

1.3 Aims of the research

The present research devotes efforts in collecting basic information of the diversity in AM fungi associated with three *Machilus* species: *M. thunbergii*, *M. oreophila*, and *M. ichangensis* in Hong Kong. The overall goal of this work is to analyze the morphological, biological, ecological and physiological aspects of diversity in the AM fungi. The specific aims of this research are as follows:

(1) To understand the basic mycorrhizal conditions of *Machilus* species.

In order to achieve this, field survey will be carried out. The soil characteristics, mycorrhizal infection rate of the roots and spore density in the rhizospheric soil will be determined.

(2) To characterize the morphology of the AM fungal spores isolated from the rhizospheric soil samples of the *Machilus* species studied.

Most of important taxonomical parameters will be recorded in details and different spore types will be identified to the genus level. The morphological information will be used in constructing a database for the diversity in the AM fungi associated with the *Machilus* species studied.

(3) To study the impact of field-collected AM fungal inoculum on the growth of two types of hosts in pot-culture experiments and to obtain newly-formed AM fungal spores from trap cultures.

Astragalus sinicum (herbal species) and two *Machilus* species (tree species), treated with various levels of fungal inoculum, will be maintained in pot cultures and different growth parameters will be monitored at regular intervals. Meanwhile the trap cultures of spores initiated from the rhizospheric soil collected from *Machilus* species will be set up in pots to produce the healthy AM fungal spores for further study. The effect of phosphorus on the production of AM spores will also be studied.

CHAPTER 2 FIELD SURVEY OF ARBUSCULAR MYCORRHIZAL (AM) FUNGI ASSOCIATED WITH THE THREE STUDIED *MACHILUS* SPECIES IN TAI PO KAO NATURE RESERVE

2.1 Abstract

The characteristics of soil in Tai Po Kao Nature Reserve were examined. The textural, physical and chemical properties of the collected soil samples were analyzed. The mycorrhizae of three *Machilus* species obtained from this forest were examined with respect to the number of AM fungal spores in soil and the infection rate of roots. This field survey provided the foundation for studying the fungal-plant relationship in the local forest.

2.2 Introduction

AM fungi are ubiquitous soil microorganisms that have great potential to enhance plant growth and soil aggregation. Despite major advances in understanding the symbioses between plants and AM fungi, how these fungi function in the field and how they affect the ecology of the communities are still poorly understood (Read, 1991), especially in Hong Kong. Understanding the extent, diversity, and function of these relationships is vital to predict future productivity and resiliency of ecosystems. Ecological field surveys should be conducted in order to obtain basic background information and knowledge of the processes that lead to the successful colonization of roots by the beneficial fungi. Such knowledge will be useful in maximizing the benefits of symbiosis.

2.2.1 Soil properties

The nature of both higher plants and soil microflora are partly determined by the soil property in which they are growing. The soil properties will have important impact on the natural population of AM fungi and they include the soil texture and moisture, water retention capacity, cation exchange capacity, acidity or alkalinity and organic matter.

AM fungi differ in their manner and extent to which they colonize roots. The hyphae, spores and propagules of different species may have different relative importance in colonizing roots. Even for the same species, pattern and intensity of colonization may also vary under different habitats with different environmental conditions (Molina et al., 1992). The relationship between roots colonization and propagule formation, and between propagule distribution/abundance and subsequent mycorrhizal formation, for different fungi in field environments, are not well understood. Without knowledge of these processes, it will be difficult to predict the outcome of inoculating fungi into plant roots.

Techniques used for estimating the importance of AM fungal populations usually depend on enumerating the number of AM spores in the soil and (or) on measurements of infection levels in roots developing in the soil.

2.2.2 AM fungal spores in soil

Most plants are capable of forming mycorrhiza with numerous fungal species. The spectrum of host receptivity, however, can range from narrow to broad. There is no known plant species that form mycorrhizae with only one type of fungi (Molina et al., 1992). In order to study the fungal-plant relationship, it is important to identify the fungi of interest. However, most of the methods commonly used for quantifying mycorrhizal fungi are not particularly suitable for distinguishing among different fungi **within roots**. Therefore, it is important to

expand the quantification procedure to include the propagules of different fungi **within soil**. By improving the quantification procedure, one can identify the key species of fungi within a population and evaluates its ability to enhance the growth and survival of the host. To this end, the spore density of different AM fungi in the rhizospheric soil collected from the three studied *Machilus* species found in a local forest was enumerated in this project.

2.2.3 Infection rate of AM fungi

Under experimental condition using known mycorrhizal fungi, it was found that their beneficial effect on their respective host plant is generally related to the rate and extent of mycorrhizal formation (Abbott et al., 1992). To this end, the infection rate of three studied *Machilus* species from the local forest was measured in this survey. Furthermore, as a preliminary step towards improving the growth properties and survival of nursery seedlings by inoculating indigenous mycorrhizal fungi, this work also investigated the infection rate of the three *Machilus* species obtained from a local nursery.

2.3 Materials and methods

2.3.1 Collection of soil and root samples

Soil and root samples from the rhizospheres of the three *Machilus* species studied were collected from the Tai Po Kau Nature Reserve. The three *Machilus* species were *Machilus thunbergii*, *Machilus oreophila* and *Machilus ichangensis*. The mature trees were sampled, which were higher than 3 meters. Three soil and root samples were collected randomly from within 1 m in diameter in the rhizosphere and the surface soil at 0-20 cm in depth, depending on topography of the site. Three individual trees were sampled for each species, i.e. a total of 3 soil subsamples for each species.

Seedlings of the same three *Machilus* species from Tai Tong nursery were also collected to check for their mycorrhizal infection rate. This was for the purpose of comparison with those collected from the field.

2.3.2 Soil analysis.

The soil analysis was carried out by Soil Analysis Center, Hubei Agricultural Institute. The three soil samples from each *Machilus* species were tested separately for their textural, physical and chemical properties. The soil samples were dried at a moderately low temperature of 40°C for two weeks before analysis. The soil texture was analyzed by determining the particle size distribution with a hydrometer method modified from Bouyoucos method, which inserted a hydrometer in the sample suspension to indicate the stage of settlement (Allen et al, 1974a; Bouyoucos, 1926). The soil pH was determined by using a pH meter and a soil:water ratio of approximately 1:5 by volume (Bates, 1964; Willard et al., 1965). The cation exchange capacity (CEC) was determined by ammonium acetate method (Allen et al, 1974b). Soil moisture was measured by oven drying the samples at 105°C to a constant mass and then calculating the percentage moisture from loss in weight (Allen et al, 1974c). The soil organic matter content was roughly determined by combusting the soil at 550°C for 2 hours, followed by calculating the percentage loss on ignition (Allen et al. 1974d). The organic carbon was tested by acid oxidation method (Allen, et al., 1974e). Total nitrogen was determined by micro-Kjeldahl digestion followed by steam distillation (Allen. et al., 1974f) and total phosphorus was determined by perchloric-sulphuric acid-molybdenum method (Allen et al., 1974g).

2.3.3 Staining of root samples with trypan blue in lactoglycerol

Each of the fine root samples was digested with 10% (w/v) KOH solution in a vial at 90°C for about 2.5 hours. Clearing time and temperature depend on the type of root being evaluated. For example, delicate roots will be cleared in shorter periods or at lower temperatures. Then KOH solution was poured off and the roots were rinsed with tap water for at least 3 times.

Bleaching was necessary only for heavily pigmented root material, which was not cleared adequately in KOH alone. The roots were immersed from 10 min to 1h in alkaline H_2O_2 solution at room temperature until roots are bleached. Alkaline H_2O_2 was prepared by adding 3 ml of liquid ammonia, 30 ml of 10% H_2O_2 to 567 ml of tap water (Kormanik and McGraw, 1982). The roots were rinsed thoroughly in water to remove all H_2O_2.

The roots were then submerged under 1% HCl for 3 minutes. The roots were not washed after this step as an acidic medium was required for subsequent staining. After acidifying, the roots were stained with 0.05% trypan blue in lactoglycerol (1:1:1 lactic acid, glycerol and water) at 90°C for 10-15 min and then immersed in lactoglycerol for destaining. The stained roots were stored in lactoglycerol or poured into a Petri dish for immediate microscopic observation.

2.3.4 Observation of AM features in the roots

The stained fine roots (<2mm diameter) were examined under a dissecting microscope at 10-50 × magnification which was adequate for rapid assessment of root colonization. The root samples were mounted on microscope slides, either temporarily in glycerol or lactophenol, or permanently in PVA (polyvinyl alcohol resin)-lactophenol. They were then observed under a

compound microscope at 100 - 400 × magnifications. Hyphal spread and arbuscule formation within the cortex was recorded and photographs were taken.

The gross morphology of the roots of the three *Machilus* species was observed and recorded.

2.3.5 Evaluation of AM infection

The stained roots were cut into 1cm segments. The infection was determined by the presence of arbuscules and/or vesicles and the infection rate was calculated as the percentage of segments colonized (Giovannetti and Mosse, 1980).

The infection rate of the three *Machilus* species from both forest and nursery were determined by evaluation of 100 root segments from the pooled subsamples of each species.

2.3.6 Spore counting

1 gram of air-dried soil was used for each counting. There were 3 sub-samples for each species and each sub-sample was counted three times (three samples from each sub-sample); hence there were a total of 3×3×1g sample counted for each species. Different types of spores were distinguished and the total numbers of spores were enumerated.

2.4 Results

2.4.1 Soil analysis

Soil samples were collected from the rhizospheres of the three *Machilus* species (**Plate 2a**) in Tai Po Kau Nature Reserve (**Plate 1a-b**). Data for the soil analysis is shown in **Table 3**. It was found that the properties of rhizosphere soil collected from the three *Machilus* species were very similar (**Table 3**). The soil particles had a rather even range size distribution (an

average of 28.3% coarse sand, 25.6% fine sand, 21.5% silt, 24.6% clay). It was classified between loam to silty loam soil according to the international system for particle fraction (Allen et al, 1974a). For the soil physical and chemical properties, it was found that the soil was acidic (average pH = 5.3). The average cation exchange capacity (CEC) of 9.61 cmol/kg was comparable to that of other previous reports in Hong Kong (Chan, 1996). The water content, as indicated by the soil moisture (2.73%) was lower than previously reported (Zhuang and Chan, 1997). The soil organic matter was not particularly high compared to other forest sites in Hong Kong (Zhuang and Chan, 1997), which was reflected in both the physical analysis of "loss on ignition" (average 11.27%) and the chemical analysis of organic carbon (average 4.33%). The total nitrogen of 0.17% (average) suggested the soil was mineral soil in which the concentration ranges generally encountered are from 0.1% to 0.5% (Allen et al, 1974f). The total phosphorus of 0.03% (average) were found to be very low (Zhuang and Chan, 1997).

2.4.2 Observation of AM features in the roots

The morphology of the examined roots was round and thick with heavily pigmented epidermal tissue and almost no root hair was observed (**Plate 3**). When the stained root samples were examined under a microscope, the typical structures of AM infection were found (**Plate 4**). The appressorium (**Plate 4a**) was formed on the root surface, the inter- and intracelluar hyphae (**Plate 4b**), arbuscules (**Plate 4c**), hyphal coils (**Plate 4d**), vesicles (**Plate 4d**) were scatted throughout the roots, the extramatrical hyphae and vesicles (**Plate 4e**) were found outside the roots. The intracellular hyphal coils were extremely extensive and large compared with the arbuscules present in the roots, which were very small, few or not observable.

2.4.3 Density of AM fungal spores in the soil samples

Table 4 showed the results of the total spore count of the three *Machilus*, ranged from approximately 500-800 spores per gram air-dried soil. The detailed spore characteristics were recorded and further analyzed for each *Machilus* species in **Chapter 3**.

2.4.4 Evaluation of AM infection

In order to understand the basic AM colonization status of the *Machilus* species studied, infection rate of the root sample collected from Tai Po Kau Nature Reserve were determined. All the three species were heavily infected by AM fungi and the infection rate was almost 100% (**Table 5**).

The mycorrhizal infection rate of the *Machilus* seedlings grown in the nursery (**Plate 2b)** was also assessed. Although it was not as high compared with that in the natural forest, more than 40% were infected for *M. thunbergii* and *M. oreophila*, and for the *M. ichangensis* it was as high as 91% (**Table 6**).

Table 3: Results of analysis of rhizosphere soil of three *Machilus* species

***Machilus* species**	*M. thunbergii*	*M. ichangensis*	*M. oreophila*
Soil particle size			
> 2 mm	4.05±0.99	4.71±2.19	5.78±1.05
2-0.2 mm (coarse sand)	28.77±0.76	28.23±4.83	27.97±7.61
0.2-0.02 mm (fine sand)	27.13±3.61	24.33±2.54	25.26±0.75
0.02-0.002 mm (silt)	21.30±0.78	21.23±0.95	21.87±1.80
<0.002mm (clay)	22.80±7.08	26.20±6.50	24.90±6.50
Soil physical and chemical properties			
Soil pH	5.30±0.20	5.30±0.20	5.20±0.50
CEC cmol/kg	10.43±0.47	9.58±1.73	8.82±0.73
Percentage moisture %	2.63±0.06	2.83±0.32	2.73±0.06
*Loss on ignition %	10.75±1.42	12.29±1.57	10.77±1.13
Organic carbon %	4.390±0.390	4.610±0.490	3.980±0.610
Total nitrogen %	0.159±0.019	0.196±0.032	0.154±0.026
Total phosphorus %	0.039±0.019	0.033±0.013	0.033±0.008

Data were means of three subsamples from each species tested in duplicates.
* Samples were from 105°C oven-dried soil; other samples were from air-dry soil.

Table 4. **The mean number of spores per gram air-dried soil sample of different *Machilus* species.**

Species	*M. thunbergii*	*M. oreophila*	*M. ichangensis*
*spores/g soil	561±43	596±101	767±15

*Values were the mean of 3 replicates.

Table 5. **Infection rate of AM fungi in the root samples of the three *Machilus* species from Tai Po Kau Nature Reserve**

Machilus Species	Infection rate
M. thunbergii	99%
M. oreophila	97%
M. ichangensis	98%

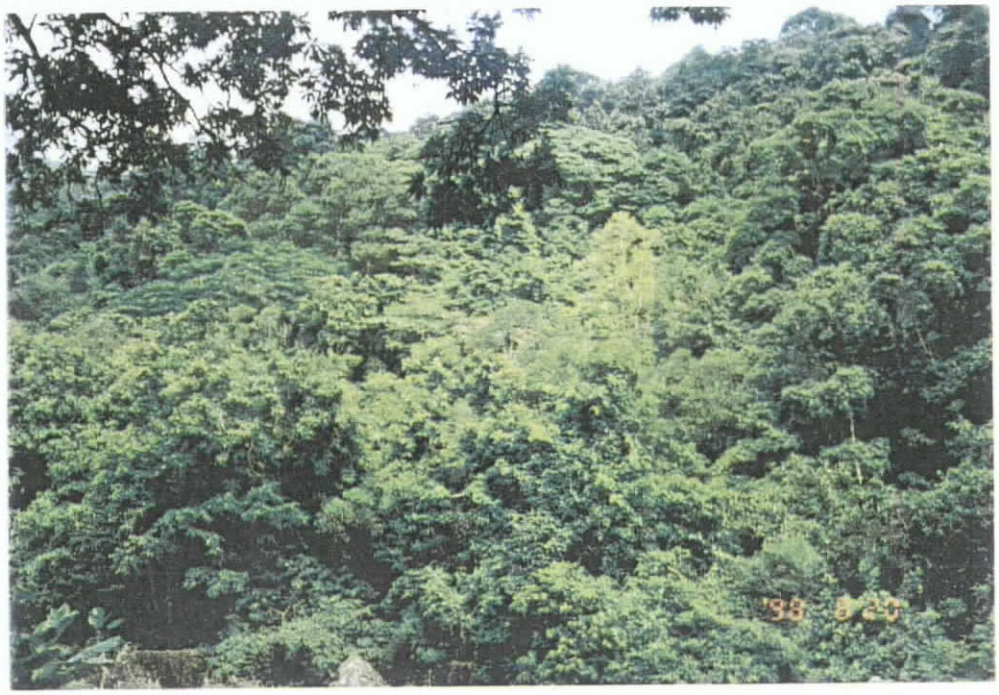
Table 6. **Infection rate of AM fungi in the root samples of the three *Machilus* species from Tai Tong Nursery**

Machilus Species	infection rate
M. thunbergii	43%
M. oreophila	43%
M. ichangensis	91%

Plates 1(a-b) Tai Po Kao Nature Reserve where the soil and root samples were collected

Plate 1(a) Entrance of Tai Po Kau Nature Trail

Plate 1(b) Part profile of Tai Po Kao Nature Reserve



Plates 2(a-b) *Machilus* species growing in the forest and nursery

Plate 2(a) *Machilus* species growing in the forest

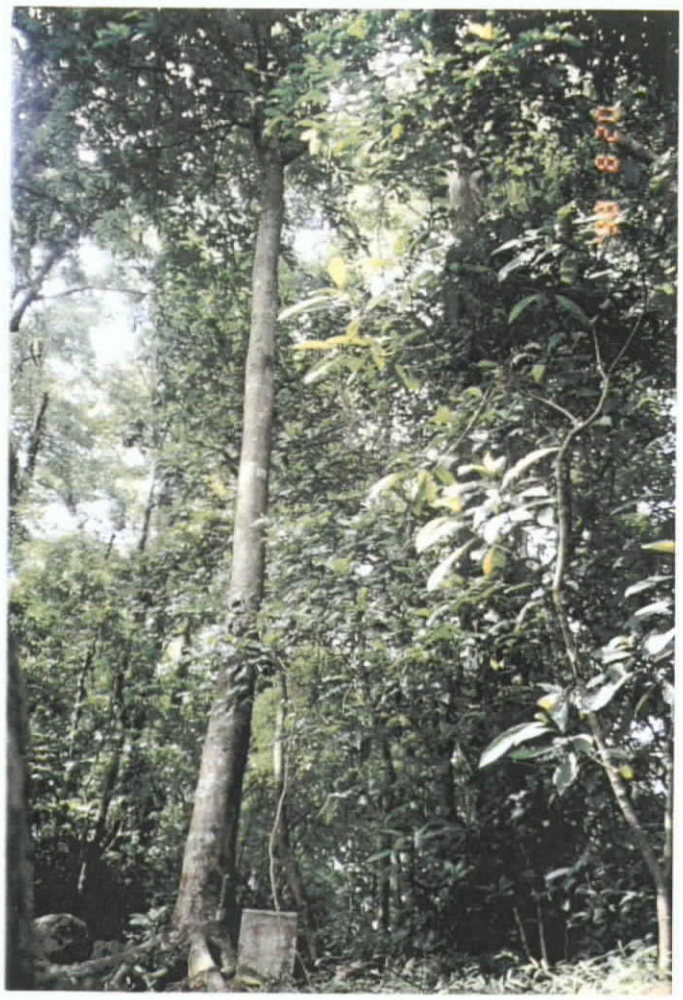
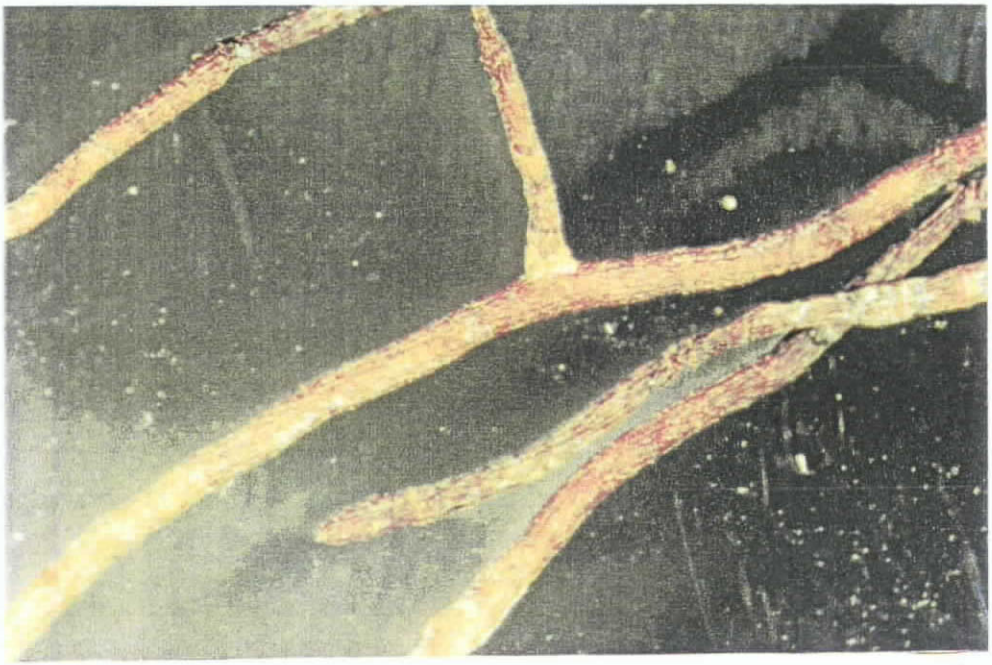


Plate 2(b) *Machilus* species growing in the nursery

Plates 3(a-b) Root morphology of a *Machilus* species

Plate 3(a) The heavily pigmented roots of a *Machilus* species

Plate 3(b) Roots of a *Machilus* species with almost no root hairs observed; × 6.3



Plates 4(a-g) Typical structures of AM infection observed in the *Machilus* roots: appresorium (A), hyphal coils (HC), vesicles (V), arbuscules (AR), inter-& intracellular hypha (IH), and extramatrical hyphae (EH)

Plate 4(a) Hyphal coils; × 200

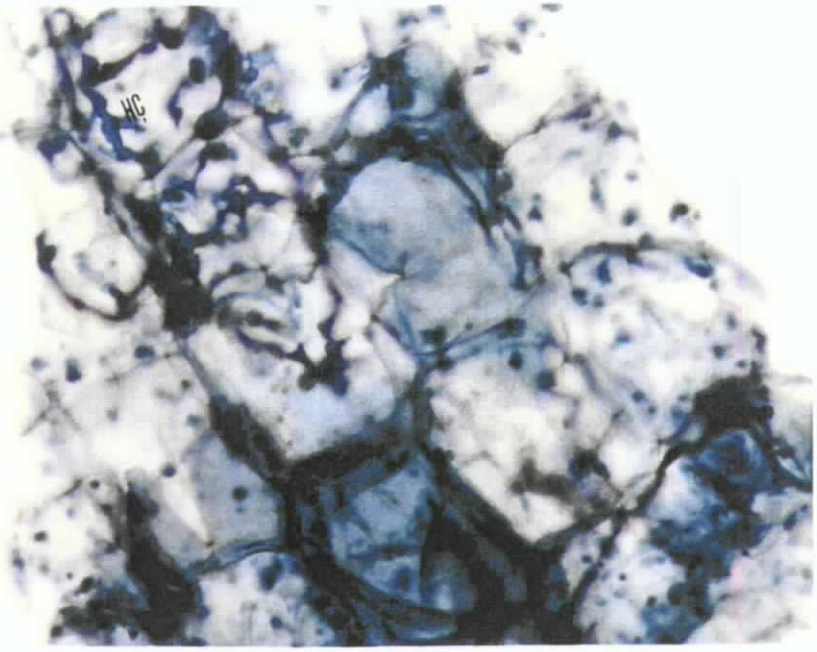


Plate 4(b) Hyphal coils and vesicles; × 200

Plate 4(c) Hyphal coils with a terminal vesicles; × 400

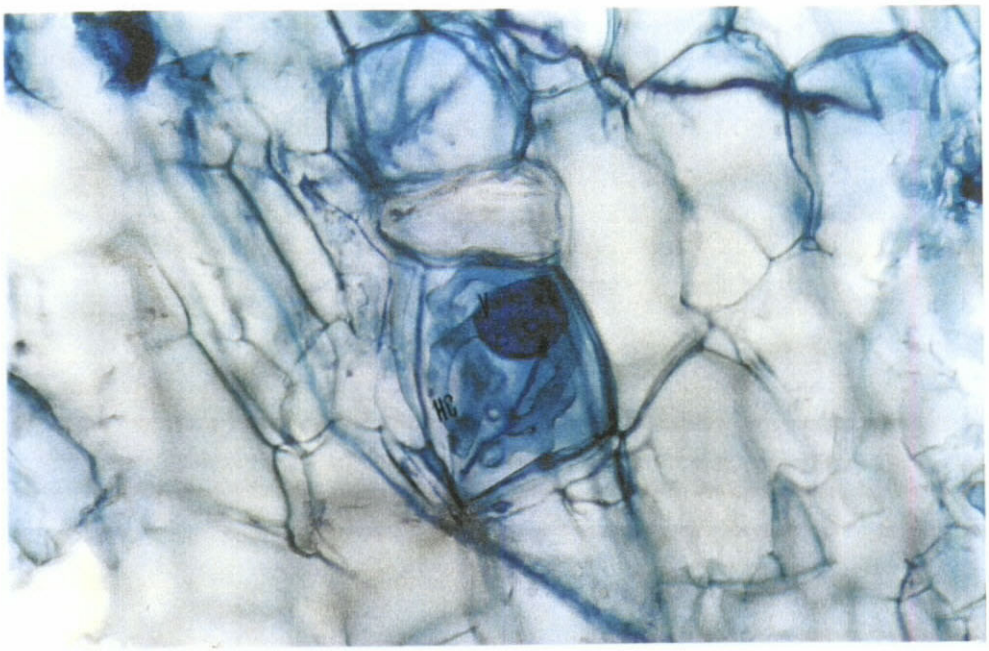


Plate 4(d) Inter-and intracellular hypha and fine arbuscules; × 400

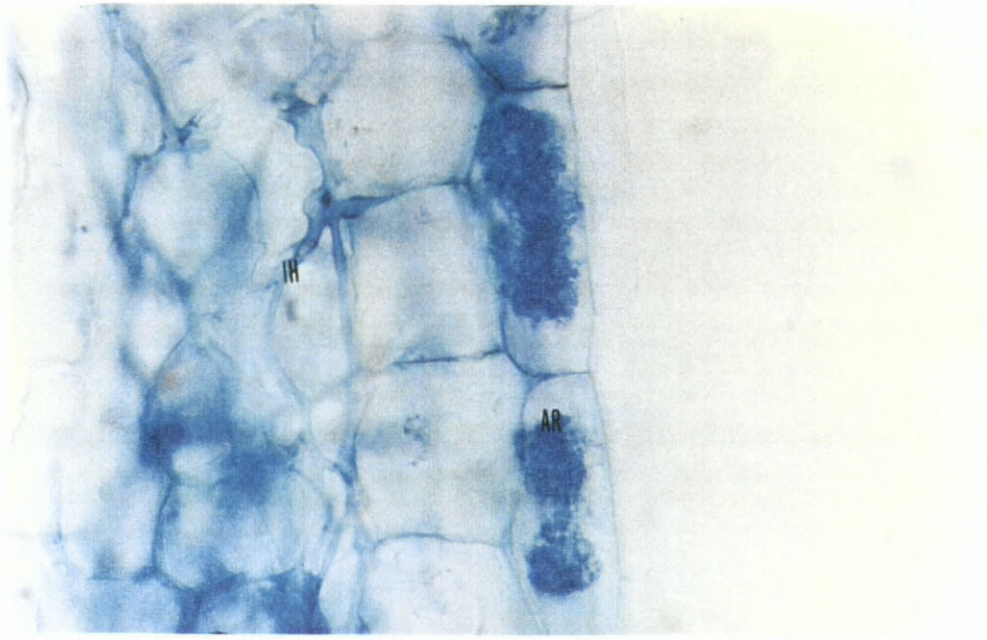


Plate 4(e) Vesicles scattered throughout the root; × 100

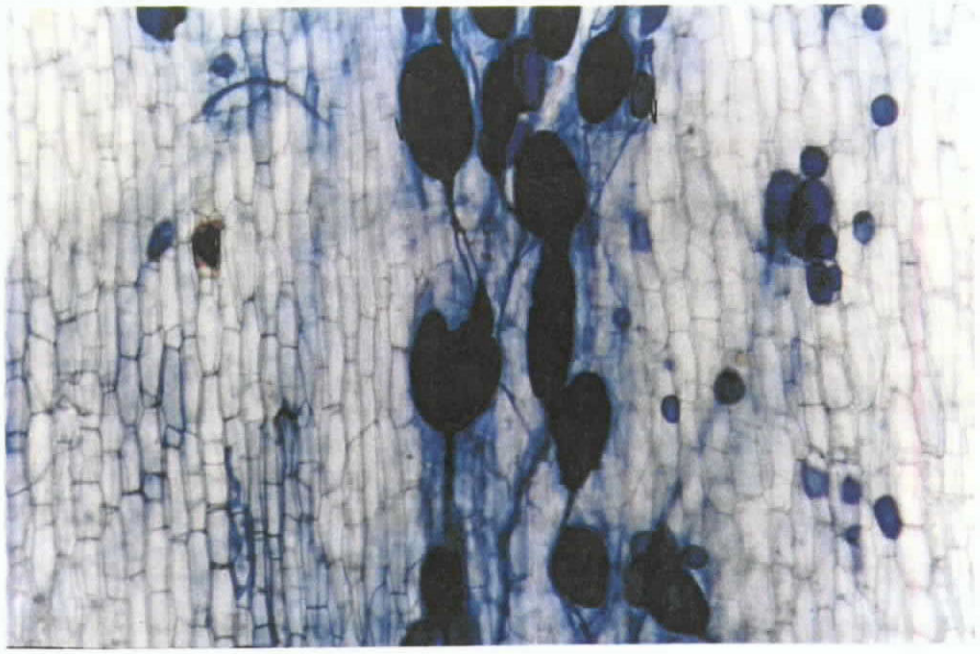


Plate 4(f) Extramatrical hyphae outside the root; × 100

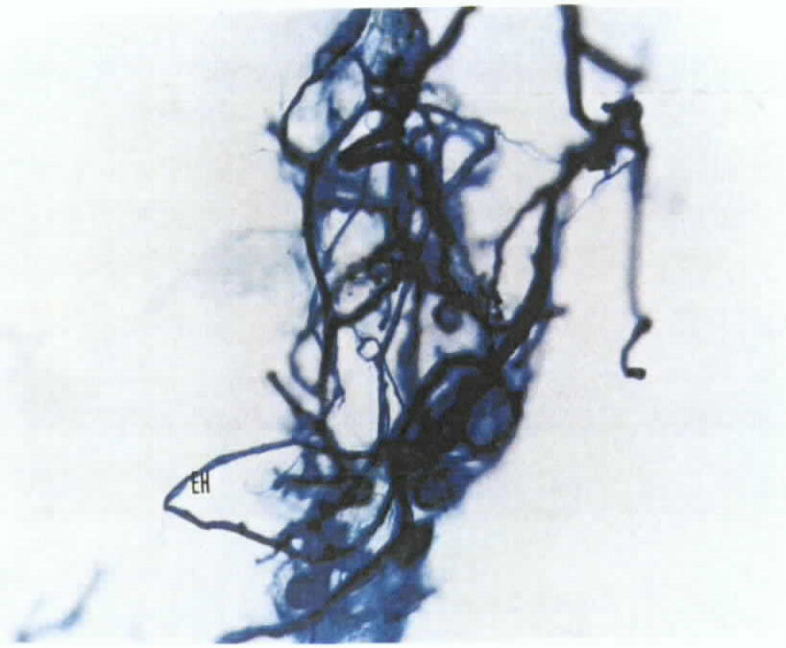


Plate 4(g) Appresorium formed on the root surface; × 100

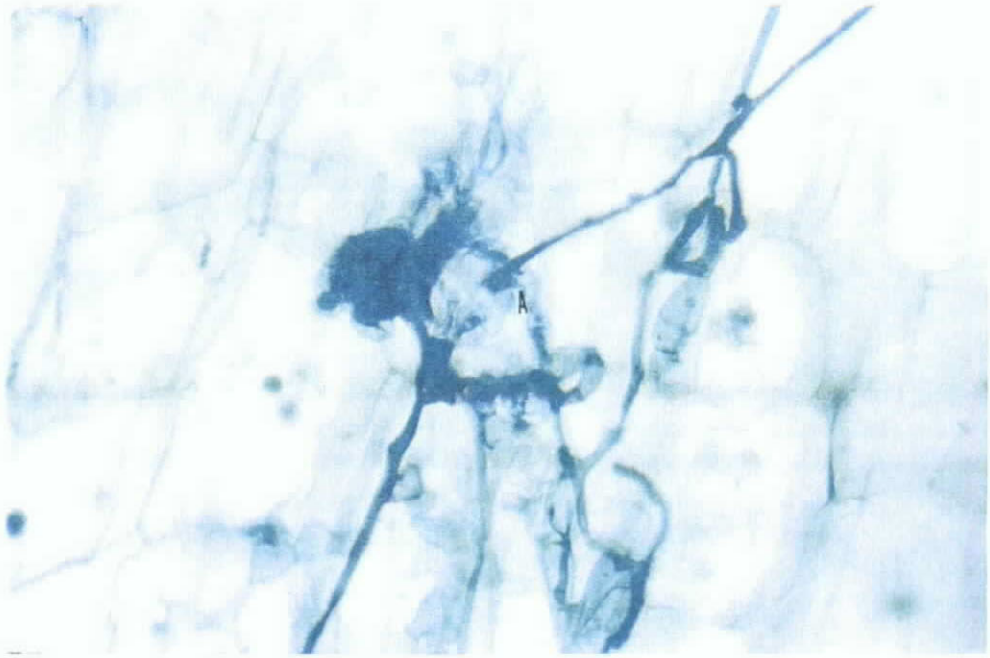


Plate 5 The *Paris*-type hyphal coils (HC) formed in the *Machilus* in pot culture; × 400

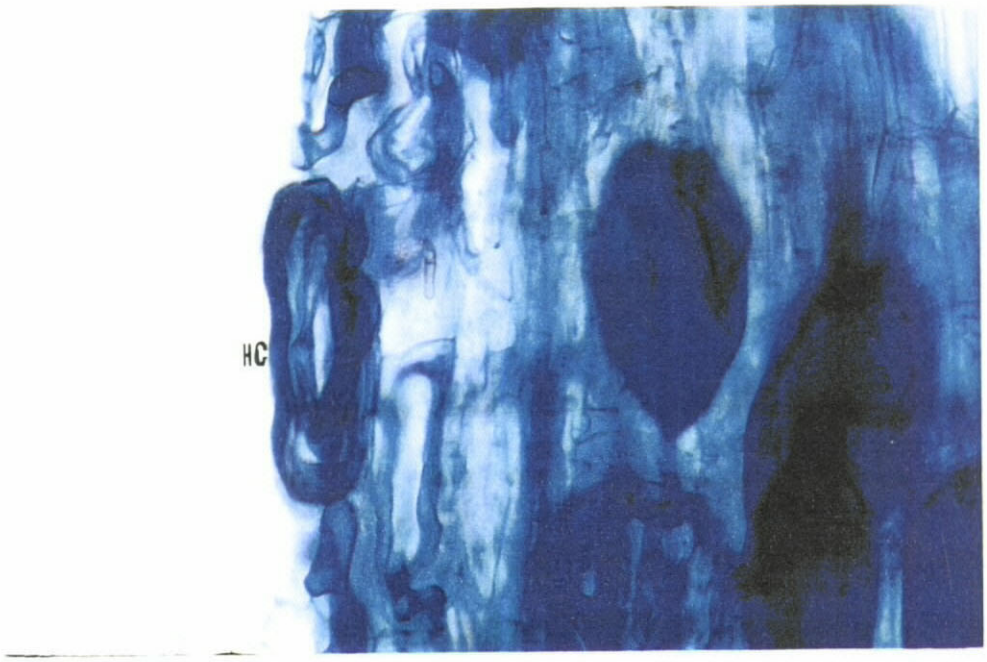
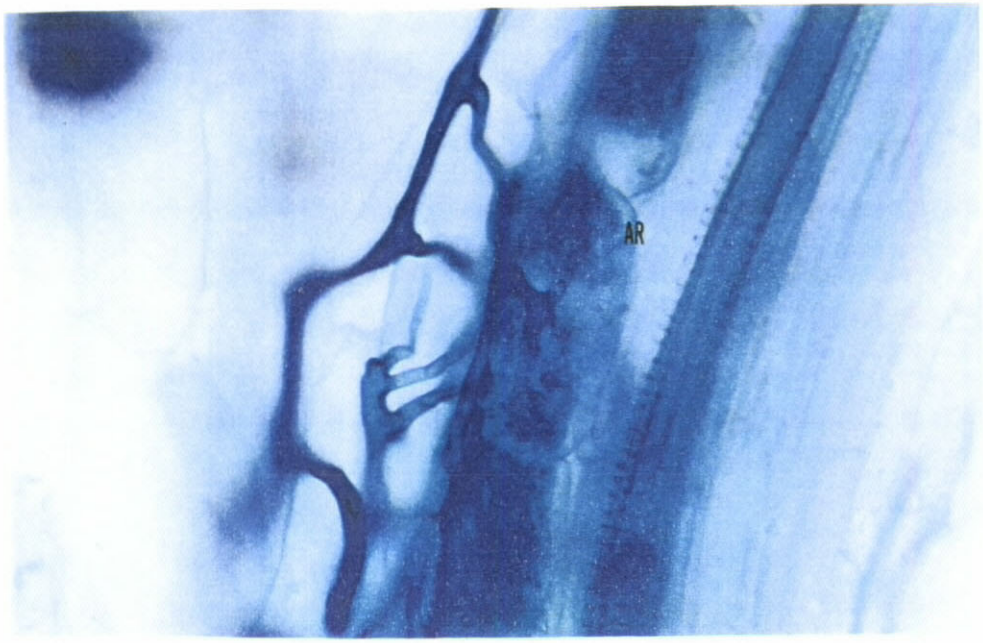


Plate 6 The *Arum*-type fine arbuscules (AR) formed in the *Astragalus sinicum* in pot culture; × 400



2.5 Discussion

Soils in the forests of Hong Kong are typically acidic, deficient in phosphorus (Chan, 1996; Zhuang and Chan 1997). The results of our soil analysis were consistent with these reports. In our investigation, the spore density in the rhizospheric soil (500-800 spores/g soil) and the mycorrhizal infection of *Machilus* (infection rate almost 100%) were rather high compared with other reports (Picone, 1996; Cuenca and de Andrade, 1996; Gould et al., 1996). The high spore density is in sharp contrast with the relatively few (only six) morphological species found (see **Table 7** in **Chapter 3**).

In this investigation the average organic matter in the soil was 11.3%. It was relatively lower compared with that in another two forests in Hong Kong, which was 27.7% and 57.6% respectively (Zhuang and Chan, 1997). It is obviously that more organic matter can hold more water. Therefore the water moisture in the soil of this two other site (14% and 17%) were higher than that in our study (2.73%). It has been speculated that organic matter could enhance mycorrhizal development (Hayman, 1987). There was only a few reports supporting this speculation except that AM fungi may be able to grow saprophytically in soil or organic matter (Warner and Mosse, 1980; Hepper and Warner, 1983; St. John et al., 1983; Warner, 1984). Our observation of the high mycorrhizal infection and high spore density in the relatively low organic soil seems to be inconsistent with this speculation. In contrast it was notable that the spore density in the other two high organic soil were much lower with only 10-17 spores per gram soil (Chan, unpublished).

The effects of soil pH on natural populations of AM fungi have not been studied systematically and there was also little information on the occurrence of species in relation to pH (Mosse et al., 1981). The high mycorrhizal infection rate in our investigation suggests that the six types of AM fungi present in the soil samples were very tolerant to acidic soil.

though they may differ in their infectivity. Although both the spore density and the mycorrhizal infection rate were very high, there was no evidence that infectivity has direct relationship with spore density. What more, *Machilus* was not the only type of plant present in the site. There is not always a clear relationship between numbers of spores of a particular fungus and the extent of root colonization in the field (Ebbers et al., 1987). But In field soils, spore numbers appear to reach a maximum in conditions where the phosphate level are less than those required for maximum shoot growth (Hayman et al., 1975; Porter et al., 1978). The stressful soil conditions in this study, as well as the fact that *Machilus* are long-lived species, may explain the high spore density and high mycorrhizal infection level.

The mycorrhizal infection rate of the *Machilus* in the nursery was also assessed. The infection rate was high, especially in *M. ichangensis* (91%); but it is still lower than that of natural forest. The difference of infection rate between the nursery grown seedlings and forest grown trees may be due to the fact they were grown under very different environmental and soil conditions. Meanwhile there are very few AM spores observed in the soils that are used to support the *Machilus* in the nursery. This relatively high mycorrhizal infection rate in the soils with very few AM fungal spores may suggest that mycorrhizal formation is a natural trait of the hosts with respect to the special root morphology observed in this study. However, this has to be interpreted carefully because the physiological response of the three studied *Machilus* species to the different studied AM fungi is still unknown and the exact spore density in the nursery soil should be counted in the future which is an omission in this study.

Unfortunately, we were unable to establish the relationship between the types of spore found in the rhizospheres and the mycorrhizae formed in the *Machilus* roots by simply using the

morphological characteristics of the spores. Molecular characterization of the spores and the mycorrhizal roots will be investigated later.

The findings that the arbuscules are relatively few in numbers, small, or absent altogether compared with the extremely extensive intracellular hyphal coils in the *Machilus* roots, suggested that the AM infection in *Machilus* belonged to the *Paris*-type instead of the *Arum*-type according to the definition first described by Gallaud (1905). The *Arum*-type is defined on the basis of an extensive intercellular phase of hyphal growth in the root cortex and development of terminal arbuscules on intercellular hyphal branches. The *Paris*-type is defined by the absence of the intercellular phase and presence of extensive intracellular hyphal coils. The *Paris*-type mycorrhizal association were also found in many other plants in the natural ecosystems (e.g. Brundrett and Kendrick, 1990; Whitbread et al., 1996; Widden, 1996).

Furthermore, another interesting phenomenon found in the pot culture experiments (**4.3.1** & **4.3.2**) was that AM inoculation produced a *Paris*-type (**Plate 5**) AM in *Machilus* (tree species) and an *Arum*-type (**Plate 6**) in *Astragalus sinicum* (herbal species). The results may indicate that the host, not the endophyte, determines the host-fungus relationships. This result has also been observed by others (Barrett, 1958; Gerdemann, 1965; Smith and Smith, 1997).

In summary, by this survey the basic mycorrhizal conditions of the *Machilus* in a local forest have been investigated. It was found that the soil in this forest were very acidic, deficient in phosphorus. Three species of *Machilus* have been found in this area. All their roots were heavily colonized by AM fungi with the infection rate almost 100%. Besides that, the spore density was also found very high with 500-800 spores/g air-dried soil. Although all the typical AM structures could be observed in the roots, it was notable that the mycorrhizal roots

of the *Machilus* in this forest belonged to the Paris-type, which was reflected in their extremely extensive intracellular hyphal coils compared with the relatively few, small or absent arbuscules. This field survey provided the foundation for studying the fungal-plant relationships in local forests.

CHAPTER 3 MORPHOLOGICAL CHARACTERIZATION OF THE FIELD COLLECTED SPORES

3.1 Abstract

Six types of AM fungal spores (Type 1 – 6) have been isolated from the rhizospheres of the three *Machilus* species in Tai Po Kau Nature Researve. The morphological characteristics of each type were described and recorded. Three of these types (spore Type 1, 3 and 4) belong to the genus *Glomus* and one (spore Type 5) belongs to the genus *Sclerocystis*. Two types (spore Type 2 and 6) remain unidentified but could possibly be in the genus *Acaulospora*. Within the soil from the rhizospheres of the three *Machilus* species collected from Tai Po Kau Nature Reserve, spore Type 2 was the most dominant; spore Type 3 and 6 were of the lowest abundance.

3.2 Introduction

AM fungi are obligate biotrophs (Lewis, 1973). Pure cultures can only be maintained in pot-cultures with host plants. Most of them form relatively large, asexual spores in soil. For this reason, AM fungal identification has traditionally been determined almost entirely by their spore morphology (Gerdemann and Trappe, 1974; Morton and Benny, 1990). Diversity in AM fungi can be manifested at the molecular, morphological, and ecological levels. Characters of any of these levels can be grouped into hierarchical patterns defining taxonomic groups if they are conserved enough to be heritable through geologic time in all descendants of a common ancestor. At present, only morphological characters associated

with the mode of spore formation and in subcellular structure of spores are sufficiently stable and diverse enough to recognise at least 150 species (Morton and Bentivenga, 1994).

Although the stable spore morphologies can be useful to group different geographical isolates into cohesive species, one should remember that this characteristic is only derived from one cell lineage of the whole organism and it might or might not have any correlation with its underlying genetic, biochemical, behavioral or physiological characteristics (Morton et al., 1992). Despite this, spore morphology is commonly used to study mycorrhizal processes in natural or managed plant communities, in different environments or geographical locations, or at the beginning and the end of a particular experiment. If spore morphology were not recorded, large collections would be impossible to manage.

There is a long history of the development of the AM fungal taxonomy as reviewed in **Chapter 1**. Until 1990, AM fungi were placed in their own order Glomales consisting of two suborders, the Glomineae (Families: Glomaceae, Acaulosporaceae) and the Gigasporineae (Family: Gigasporaceae), with six genera, Glomaceae (*Glomus* and *Sclerocystis*). Acaulosporaceae (*Acaulospora* and *Entrophospora*) and Gigasporaceae (*Gigaspora* and *Scutellospora*). Taxonomic keys for the above classification have been established based on the morphological criteria (Morton and Benny, 1990). Spores from different families and genera of the Glomales are different. Spores in *Glomus* are formed on cylindrical to flared sporogenous hyphae. The sporogenous hyphae may form individually, in loose to tight aggregates with little or no organisation, or in highly organised aggregation that results in compact sporocarps (Morton, 1988). The genus *Sclerocystis*, having only one species *S. coremioides* Berk and Broome, has a more advanced step in the sporocarp specialization series compared with *Glomus*. In *Acaulospora* and *Entrophospora*, a saccule forms terminally on a sporogenous hypha, after which spores are initiated laterally from or within

this hypha. Spores of *Gigaspora* and *Scutellospora* species expand from a bulbous sporogenous cell and can grow much larger than those of other genera - up to 700 μm in *S. scutata* Walker and Diederichs. Owing to the equivalent hyphal attachments and mode of spore formation, spores in each genus cannot be distinguished easily under a stereomicroscope.

The main morphological variables for identifying AM fungal spores were grouped in different categories as described (Giovannetti and Gianinazzi-Pearson, 1994):

(1) sporocarp occurrence, shape, colour and size;
(2) peridium occurrence and characteristics;
(3) spore colour, size and shape;
(4) spore wall number, colour, thickness and ornamentation and
(5) hyphal attachment, shape and type of occlusions.

Sporocarp phenotype is highly variable and all the different possibilities may occur within the same genus. Spore colour varies from white to yellow, red, brown and black, with all the intermediate shades, subjected to personal interpretation. Spore colours, therefore, should be checked against universal colour charts as references (Walker, 1992). Spore size is also very variable, even within the same species, ranging from less than 50 μm up to 800 μm between different genera. In contrast, spore shape varies little from globose to sub-globose, elliptical or pear-like; but peculiar shapes can sometimes be observed as the result of physical constraints during spore development, especially in natural environments. The shape of subtending hypha is also a character that is quite stable within each species, while the mode of occlusion may vary, probably depending on the age of spores. Number, size, structure and ornamentation of spore walls can vary considerably between species of the same genus. The Melzer's reagent can be used to mount spores to look for diagnostic iodine staining reactions

(hydrophobic regions of structures). However, the staining may fade or even disappear in lightly-stained structures in prepared slides after a year or longer storage. These wall features have assumed a major role in recent descriptions of new species, and this importance has been widely recognised (Walker, 1983; Morton, 1988). Because of the stability of wall structures in spores and the extent of diversity between species, their components have constituted the main characters for assessing evolutionary relationships within the Glomales. Briefly, two main branches have been hypothesised to have evolved from a common ancestor: the Gigasporaceae on the one hand and the Glomaceae and the Acaulosporaceae on the other (Morton, 1990).

The morphological characterisation of AM fungi associated with *Machilus* species in Hong Kong was one of the main focuses of this study. Most of the important taxonomical parameters mentioned above were recorded in details by examination under light microscope and scanning electron microscope. Different spore types were identified to the genus level. Herbarium mounts of spores were prepared. The results from this work will be useful to establish a database for future study.

3.3 Materials and methods

3.3.1 Spore isolation

AM fungal spores were isolated by wet sieving and decanting (Gerdemann and Nicolson, 1963) and subsequent sucrose centrifugation (Daniels and Skipper, 1982). Soil at the site of collection was visually inspected for epigeous sporocarps. The top 0-20 cm soil samples was collected as described in **2.3.1**. It was blended with water in a Waring blender for 30 seconds. After that, a water spray was used to pass the blender contents through nested

sieves with 500, 250, and 38 (or 45) μm openings. Material from the 500 and 250 μm sieves were placed in separate large petri dishes and examined for smaller sporocarps and large spores. Material on 38 or 45 μm sieve were transfer gently into centrifuge tubes (preferably those with 50 cm^3 volume) containing a sucrose gradient of 20% and 60%. A swinging bucket rotor was best for optimal separation of soil particulate from spores. The supernatant in each tube can be poured into a 38-45 μm sieve and washed for 1-2 min, rinsed in distilled water, and placed in a petri dish for storage.

3.3.2 Voucher preservation

The whole or broken spores were mounted on slides in PVLG (Polyvinyl-Lacto-Glycerol) or PVLG + Melzer's for permanent preservation. The PVA (polyvinyl alcohol) should have the following properties: 50-75% hydrolyside, and a viscosity of 20-25 centipoise in 4% aqueous solution at 20°C. For PVLG preparation, all the ingredients were mixed in a lightproof bottle before adding PVA. The PVA were added as powder and the mixed ingredients were then placed in a hot water bath to dissolve (70-80°C) for 4-6 hours. And the PVLG was prepared. For the permanent storage of stained spores with Melzer's reagent, the Melzer's reagent was mixed in equal proportions with 1:1 dilution.

The contents of PVLG are shown as follows:

Ingredient	**Quantity**
Distilled water	100 ml
Lactic acid	100 ml
Glycerol	10 ml
Polyvinyl alcohol (PVA)	16.6 g

The contents of the Melzer's reagent are shown as follows:

Ingredient	Quantity
Chloral hydrate	100 g
Distilled water	100 ml
Iodine	1.5 g
Potassium iodide	5.0 g

3.3.3 Herbarium mounts of spores in PVLG-based mountants

Two small drops (0.5-0.75cm in width) were added to two slides, one of PVLG and the other of PVLG + Melzer's reagent. The same type of spore was mounted on separate slide with PVLG and PVLG + Melzer's. Spore then was added with minimum water if using a pipette. The slides were allowed to stand for at least 5 minutes to let the surface of drop dry slightly (increasing viscosity of edges, reducing flow when coverslip is added). Then a coverslip was placed gently on each slide. During microscopic examination, the spore can be crushed to varying degrees (thus exposing hard-to-see thin flexible inner layer if present) with the blunt tip of a pencil, forceps or needle. More mountant could be added to the edge of the coverslip if needed. After 5 days at room temperature or two days at 60°C, the mountants were hardened and the slides were prepared.

3.3.4 Morphological characterization of spores

The characteristics of whole spores (colour, shape, size distribution, field collection spore formation), subcellular structure of spores (spore walls, ornamentation of spore wall, reaction of spore wall in Melzer's reagent), structure of subtending hypha (shape, width, occlusion) were recorded. The sieved spores were examined under a Leica Wild MZ8 Stereo microscope for gross morphology and a Leica DMRB light microscope for the structural details. Scanning electron microscope (SEM) (model: Leica Stereoscan 440 systems) was used to observe the different ornamentations on spore walls. Spore sizes were measured with

a Leica Q500MC Image Analysis System. The spore colour was described by matching with the "INVAM colour chart for spores" (Morton et al., 1996).

Photographs of both the whole and the broken specimens and SEM micrographs of whole spores were taken for record.

3.4 Results

3.4.1 Analysis of spore morphology

At least 6 types of spores were found in the rhizospheric soil of all the three *Machilus* species studied. The detailed morphological characteristics of the spores were summarized in **Table 7** and the images from both light and electron microscopy analyses were in **Plates 7 - 12.**

The identification of the spores was based on the keys as described (Hall, 1984; Morton and Benny, 1990). Type 1, 3 and 4 have features of a subtending hypha typical of the genus *Glomus*. Chlamydospores of *Glomus* species usually have only one subtending hypha and the spores are formed on the end of a hypha that can be constricted at the point of attachment to the spore. They have parallel sidewalls, or became markedly wider at the point of attachment to the spore (Hall, 1984).

Type 5 was presumably of *Sclerocystis* species. The bundle of the spores was detached from a tight sporocarp with the spores arranged in a single layer around a central plexus of glebal hyphae. According to Morton and Benny (1990), the key to taxa in *Sclerocystis* is: fruiting body of a sporocarp composed of spores with lateral walls adherent to one another: chlamydospores in a single layer except at the base; base composed of sterile hyphae.

Type 2 and 6 remain unidentified but possibly be *Acaulospora* species in which most species form sessile spores and lack a subtending hypha (Hall,1984; Morton and Benny, 1990).

Although there were six types of spores found in the soil samples, their abundance distribution was different. **Fig. 1** showed the spore density of different spore types found in the rhizosphere soil of three *Machilus* species. It was notable that the spore Type 2 accounted for a considerable proportion of the total spore number and was more than 50% of the total spore count. The spore Type3 and 6 were of the lowest abundance.

Table 7. Summary of the characteristics of the six types of spores found in the rhizospheres of the three *Machilus* species

	Spore Type 1	Spore Type 2	Spore Type 3
Whole spores			
Colour	Red brown (0-60-50-10) to dark red-brown (40-80-80-0) (according to the standard colour chart)	Black	Deep black red (60-80-70-10)
Shape	Globose or sub-globose	Globose, sub-globose or ellipsoid	Globose to sub-globose
Spore formation	Singly or compact clusters	Singly	Singly or compact clusters
Size distribution	60-170 μm×80-190 μm	60-350 μm ×60-370 μm	80-160 μm × 90-190 μm The singly formed spores all existed in the 125 μm sieve; the compact clusters of spores were in the 250 μm sieve
Subcellular structure of spores			
Spore walls	One layer with laminations	The spore wall consisted of at least 2 layers: an outer dark layer and a red brown colour layer which appeared inside the dark layer when the spore was crushed	It consisted of a series of laminations, detailed information was unknown
Ornamentation of spore wall	Under a light microscope, the spore wall surface was smooth but deep fissures on the spore wall appeared when crushing the spore; SEM showed ornamentation on the spore wall	Under a dissection microscope, the spore surface appeared reticulated; due to the dark colour, detailed structure can not be clearly observed; SEM showed ornamentation on the spore wall	Under a light microscope, the spore wall surface was smooth but deep fissures on the spore wall appeared when the spore was crushed; SEM showed a smooth spore wall surface
Spore wall in Melzer's	No change in colour	No change in colour	No change in colour
Structure of subtending hypha			
Shape	Flared, straight or occasionally curved	The spores are sessile after wet-sieving; detailed information was unknown.	Straight subtending hypha; the outer layer of spore wall continue along the subtending hyphae for a short distance
Width	11-29 μm		15-45 μm
Colour	Subtending hypha often paler in colour than the spore		Subtending hypha similar in colour as the spore
Occlusion	By thickening of inner spore wall, without a septum at spore hypha conjunction		Without occlusion (septa) by thickening of inner spore wall

Table 7. (cont'd) Summary of the characteristics of the six types of spores found in the rhizospheres of the three *Machilus* species

	Spore Type 4	Spore Type 5	Spore Type 6
Whole spores			
Colour	Pale yellow (0-0-20-0) to pale yellow brown (0-40-80-0) It was transparent and some oil-like content was observed inside the spore. It came out when the spore was crushed.	Pale yellow (0-0-20-0) to pale yellow brown (0-10-60-0); spore wall is transparent; the oil like content was observed inside the wall and came out when crushing the spore	Reddish orange (0-60-60-0) to red brown (20-80-100-0); transparent, oil-like material was seen inside the spore
Shape	Globose or sub-globose	Ellipsoid to sub-ellipsoid or globose to sub-globose	Globose to ellipsoid
Spore formation	Singly	Spores were formed in sporocarps, which are composed of spores with their lateral walls adherent to one another. Connecting hyphae embedded in a central hyphal plexus	Singly
Size distribution	70-140 μm × 80-170 μm. Mainly found in the 63 μm sieve, with a few in the 45 μm sieve and the least in the 125 μm sieve	40-70 μm ×50-130 μm	60-90 μm×90-110 μm
Subcellular structure of spores			
Spore walls	One layer with a series of laminations	One layer with laminations	One layer with laminations
Ornamentation of spore wall	Under a light microscope, the spore surface was smooth but deep fissures appeared when crushing it; SEM showed ornamentation on the spore wall	Under a light microscope, the spore surface was smooth but deep fissures appeared when crushing it; SEM showed a smooth spore wall surface	Under a light microscope, the wall surface was like reticulate; deep fissures appeared when crushing it; SEM showed ornamentation on the spore wall
Spore wall in Melzer's	No change in colour	No change in colour	No change in colour
Structure of subtending hypha			
Shape	Flared, straight or slightly constricted	Connecting hyphae embedded in a central hyphal plexus	The field-collected spores were sessile after wet-sieving; detailed information was unknown
Width	6-23 μm	5-17 μm	
Colour	Often little paler than the spore	Often little paler than the spore	
Occlusion	Hypha occluded by the thickening of the inner spore wall, no septa	Without occlusion (septa)	

Plates 7(a-d) Morphology of spore Type 1

Plate 7(a) Spore clusters; × 50

Plate 7(b) Globose or sub-globose spores with black brown to red brown colour; × 50

Plate 7(c) Spore with subtending hypha (S): × 400

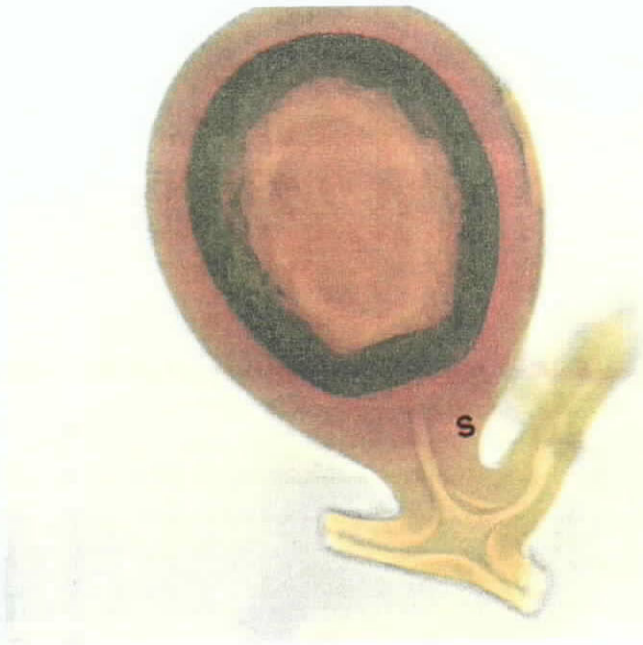
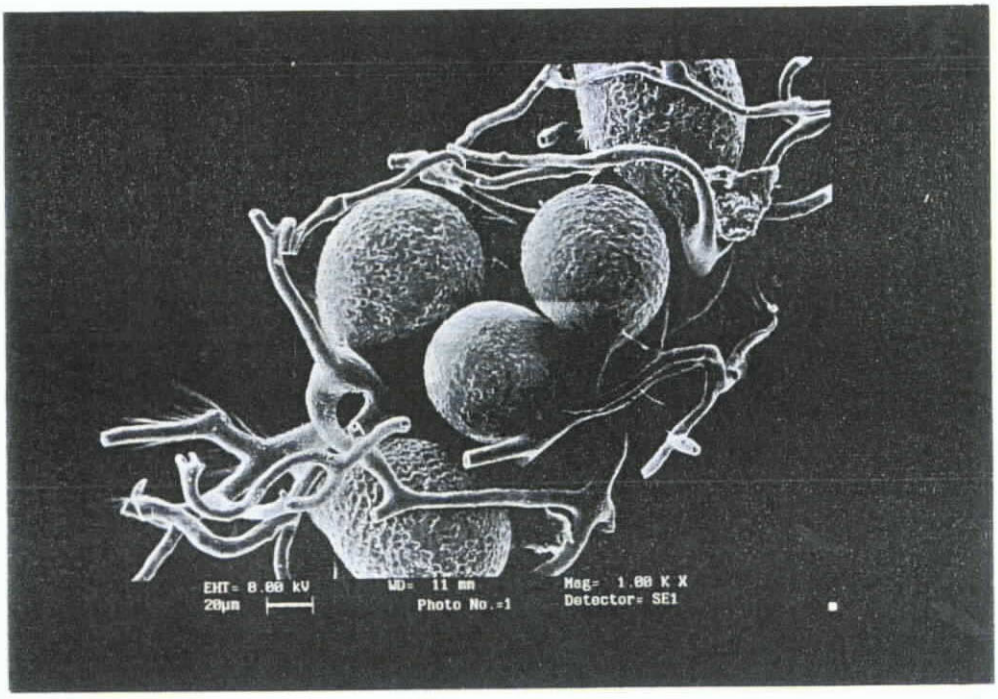


Plate 7(d) Scanning electromicrograph of spores with subtending hypha and ornamentation on the spore walls.



Plates 8(a-c) Morphology of spore Type 2

Plate 8(a) Globose and sub-globose black spores without subtending hypha; × 50

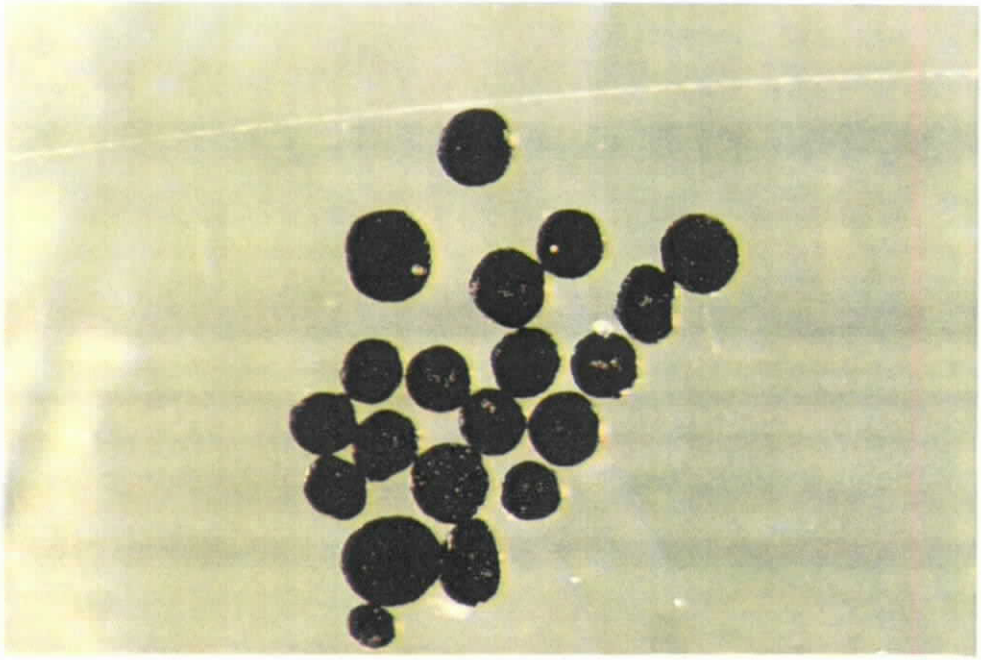


Plate 8(b) Spore wall with two layers (L); × 200

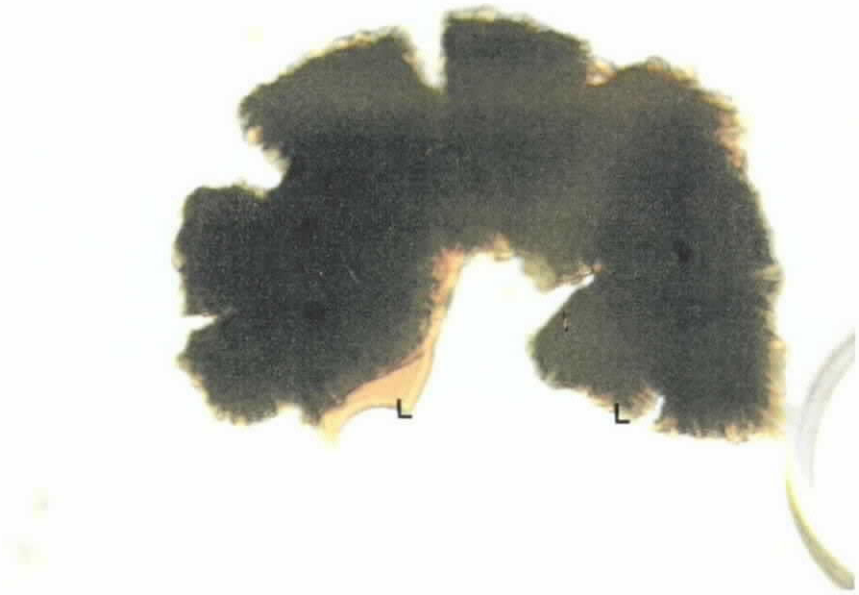
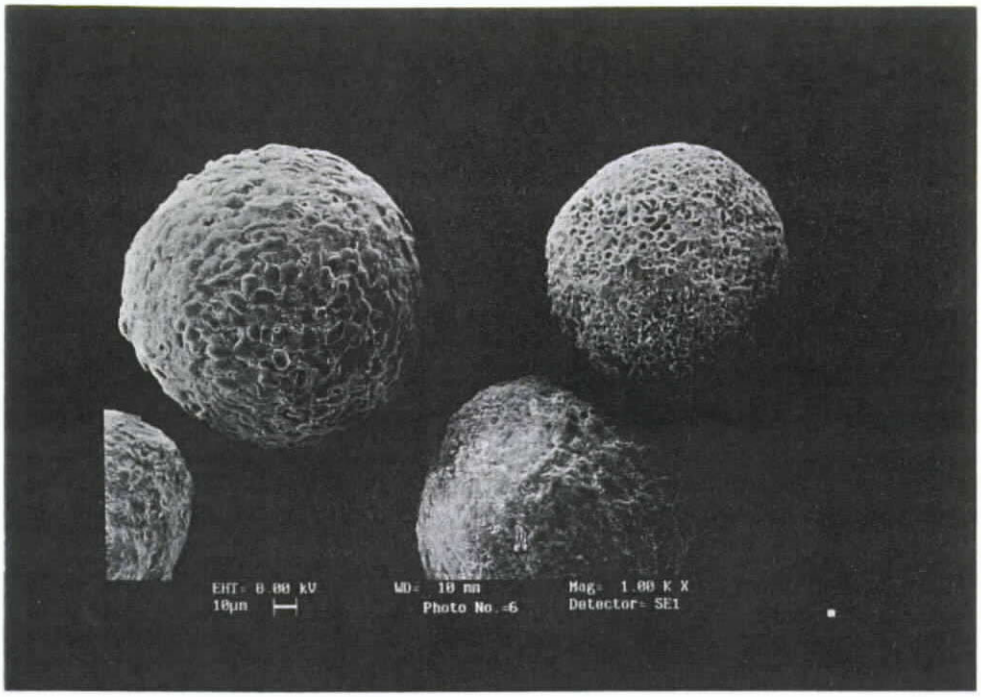


Plate 8(c) Scanning electromicrograph of spores with ornamentation on the spore walls.



Plates 9(a-d) Morphology of spore Type 3

Plate 9(a) A compact spore cluster; × 50

Plate 9(b) The deep black red spores with subtending hyphae; × 200

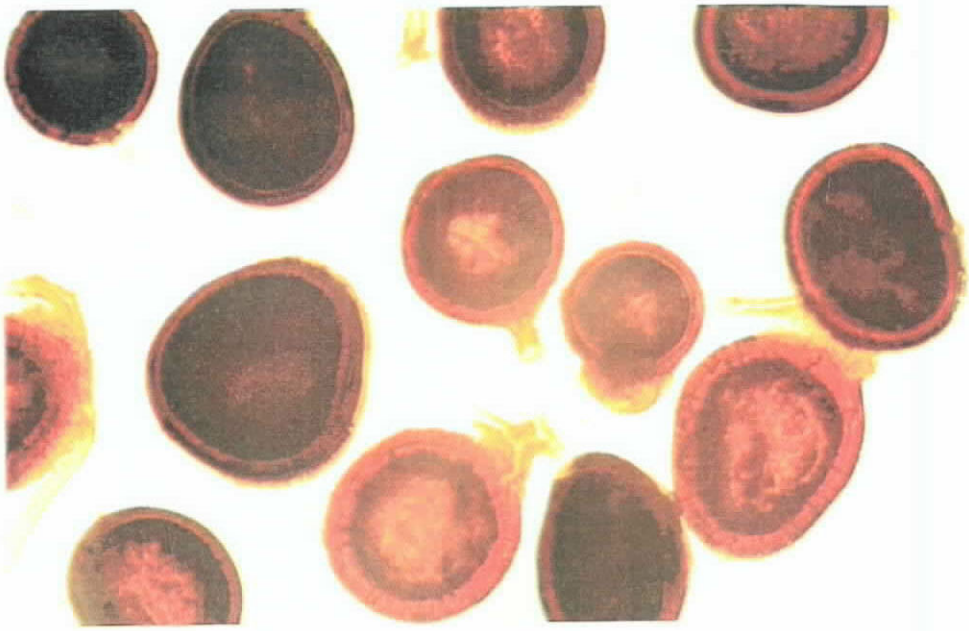


Plate 9(c) Spore with straight subtending hypha; × 400

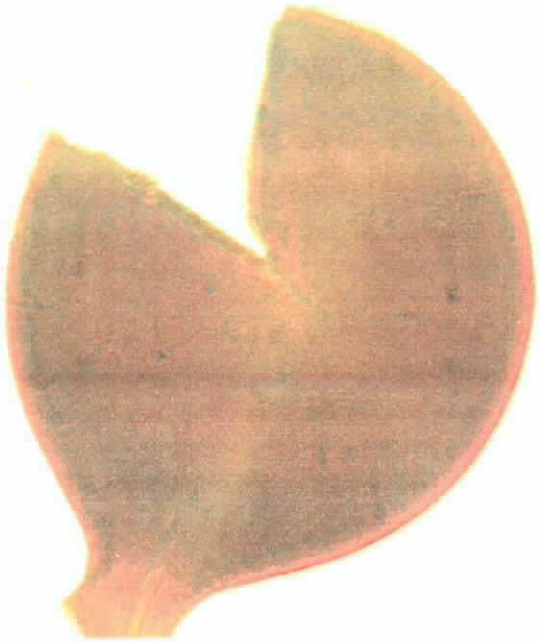
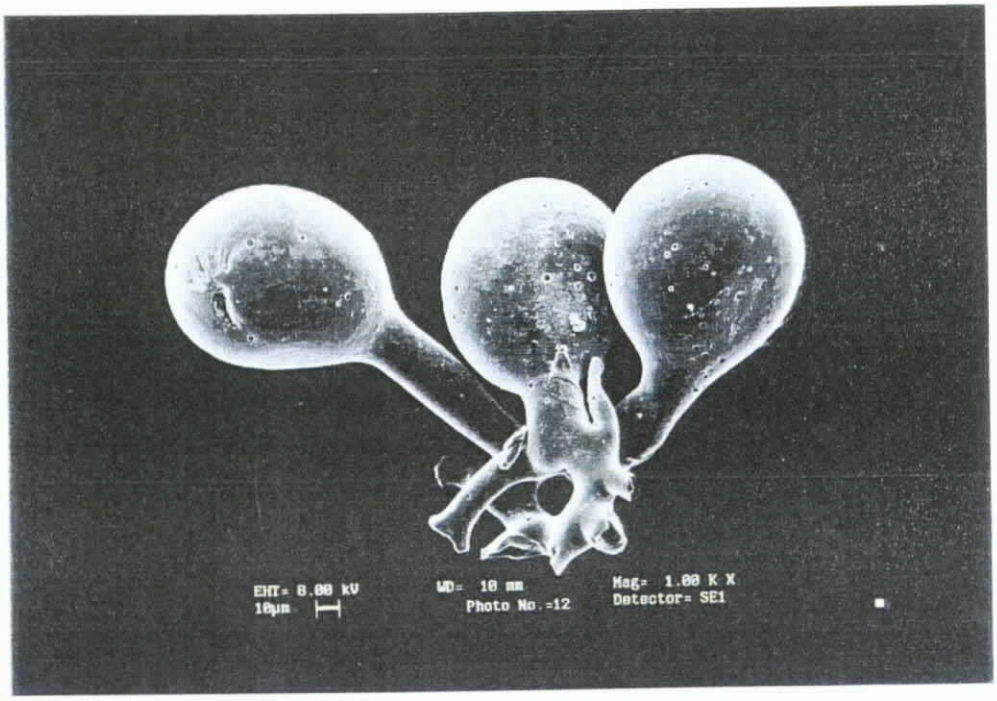


Plate 9(d) Scanning electromicrograph of spores with subtending hypha and smooth spore wall surface



Plates 10(a-c) Morphology of spore Type 4

Plate 10(a) Pale yellow or pale yellow brown spores with subtending hyphae; × 50

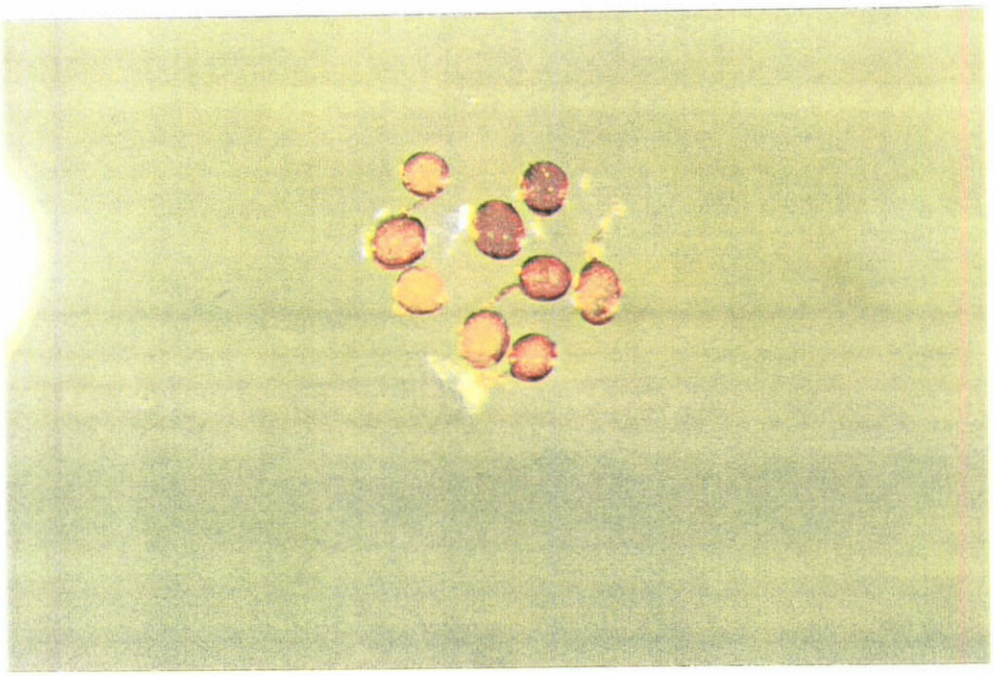


Plate 10(b) Oil-like content (O) inside the spore; × 400

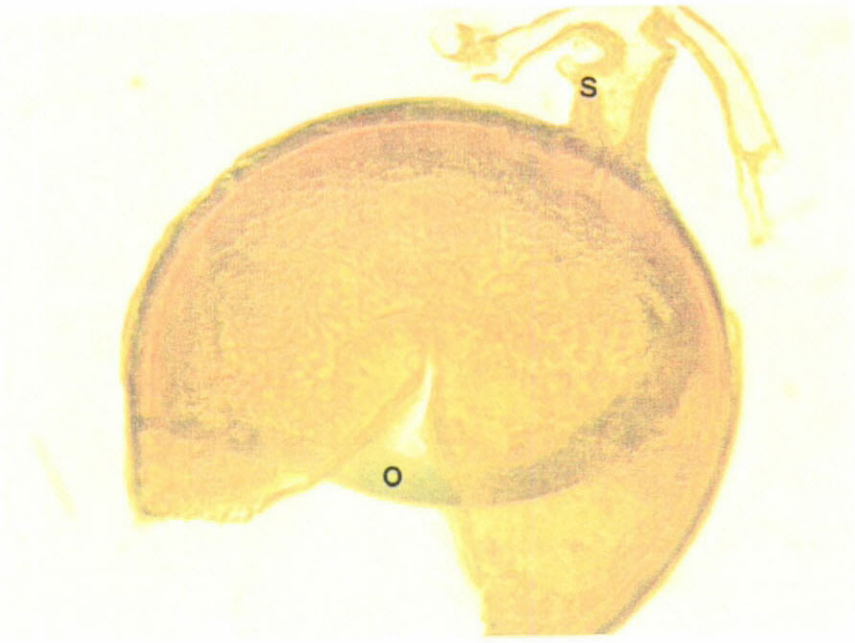
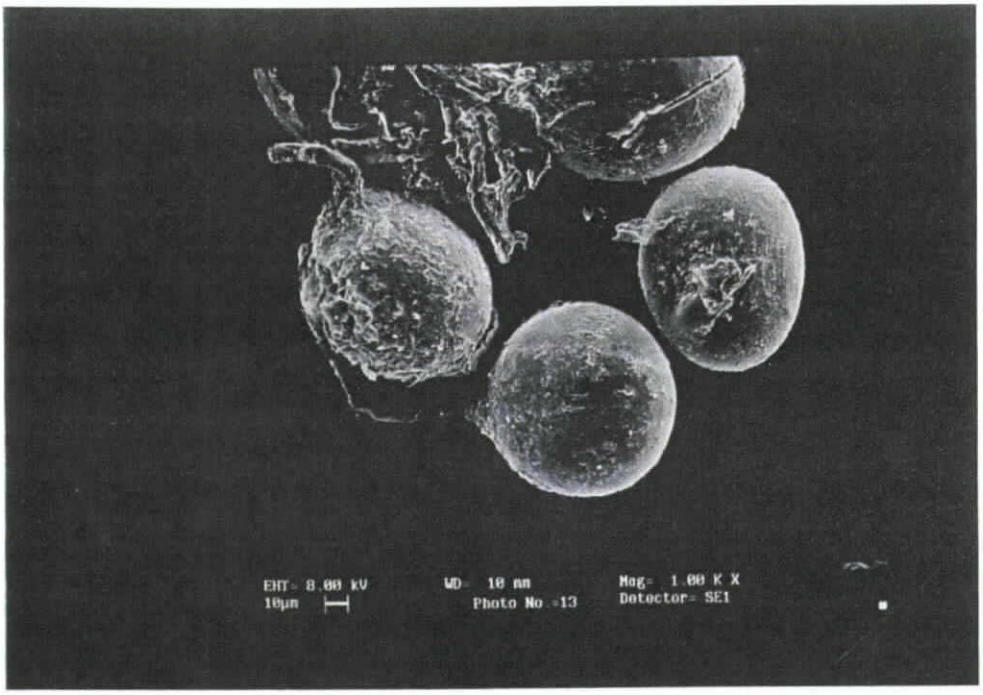


Plate 10(c) Scanning electromicrograph of spores with subtending hyphae and ornamentation on the spore wall



Plates 11(a-c) Morphology of spore Type 5

Plate 11(a) Clusters of pale yellow or pale yellow brown spores; × 50

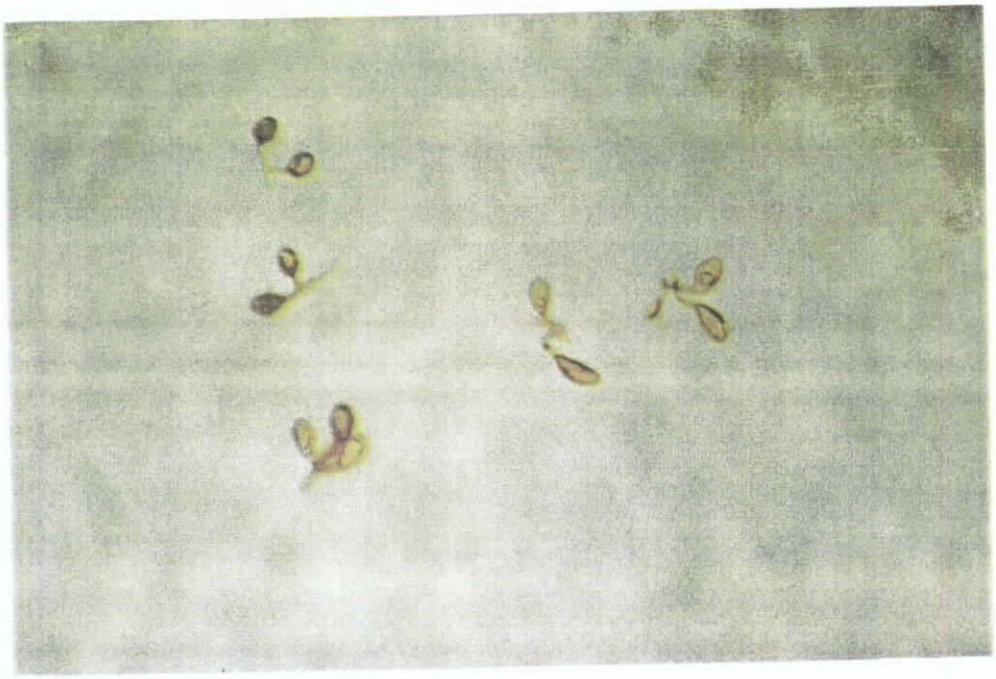


Plate 11(b) Spores with remains of hyphal plexus; × 50

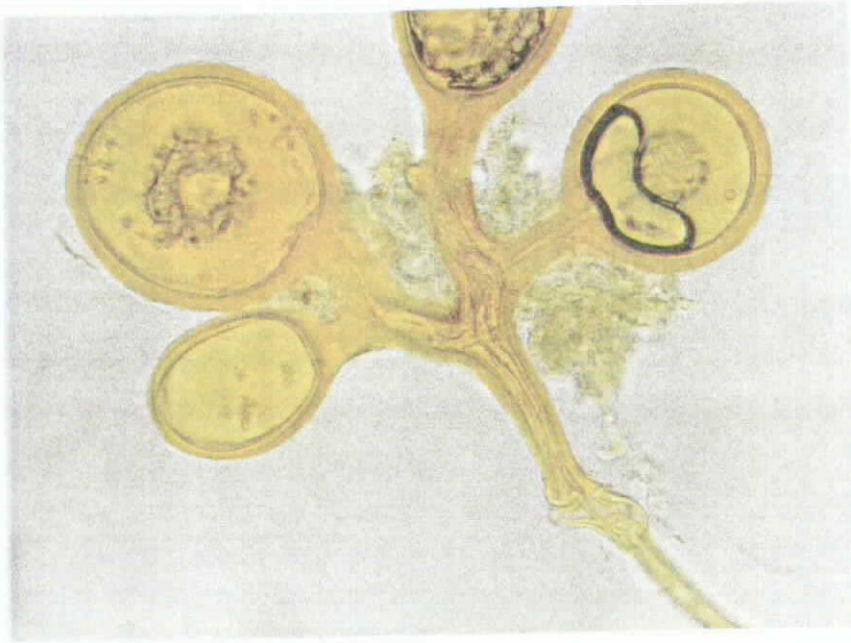
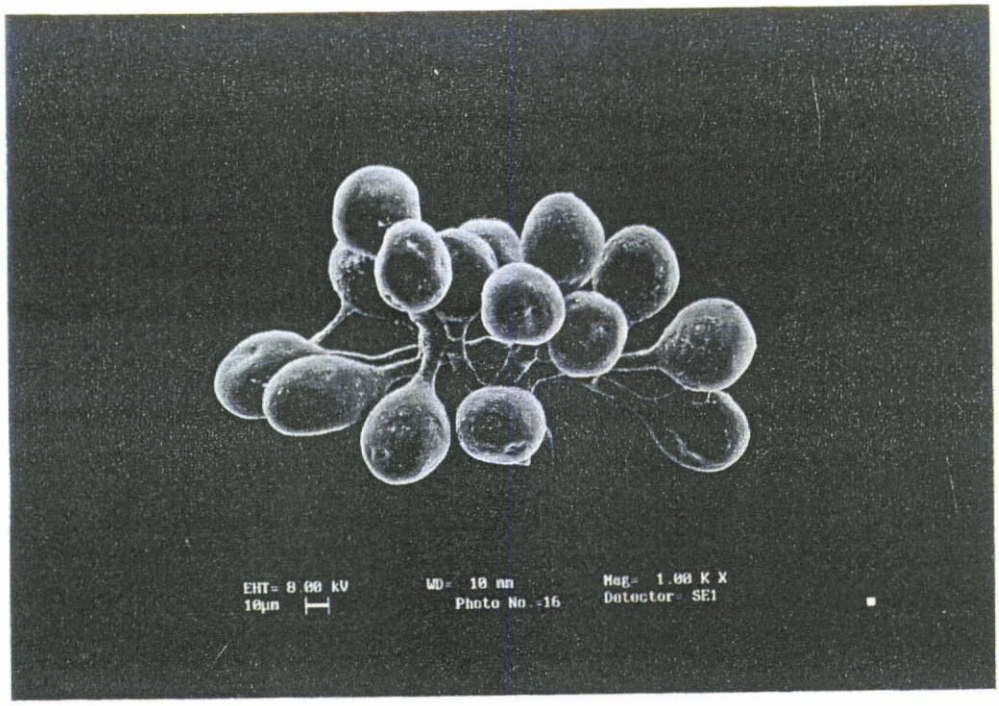


Plate 11(c) Scanning electromicrograph of spores with hyphal plexus and smooth spore wall



Plates 12(a-c) Morphology of spore Type 6

Plate 12(a) Reddish orange colour spores with oil content seen inside the spore wall; × 50

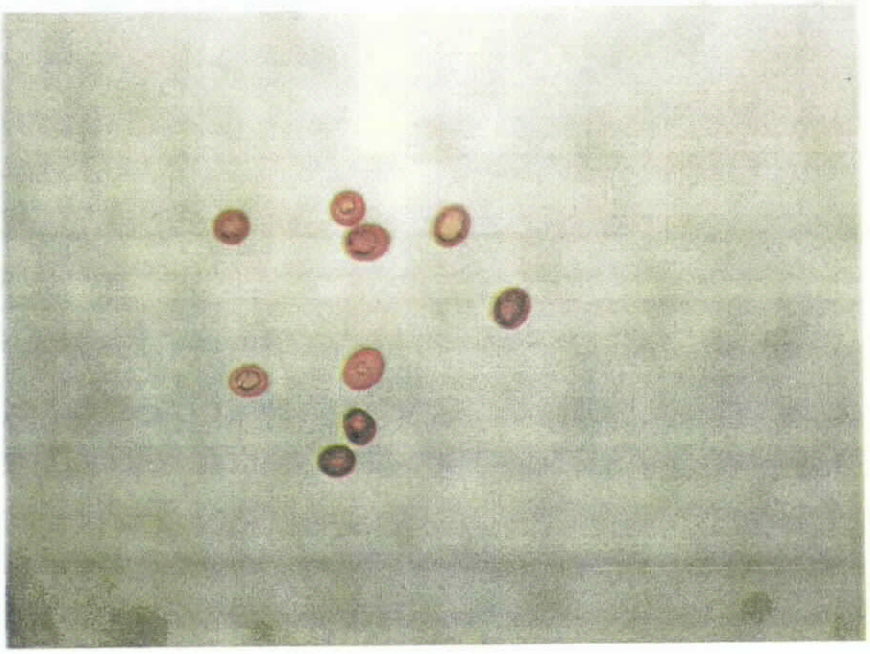


Plate 12(b) Spore without subtending hypha; × 400

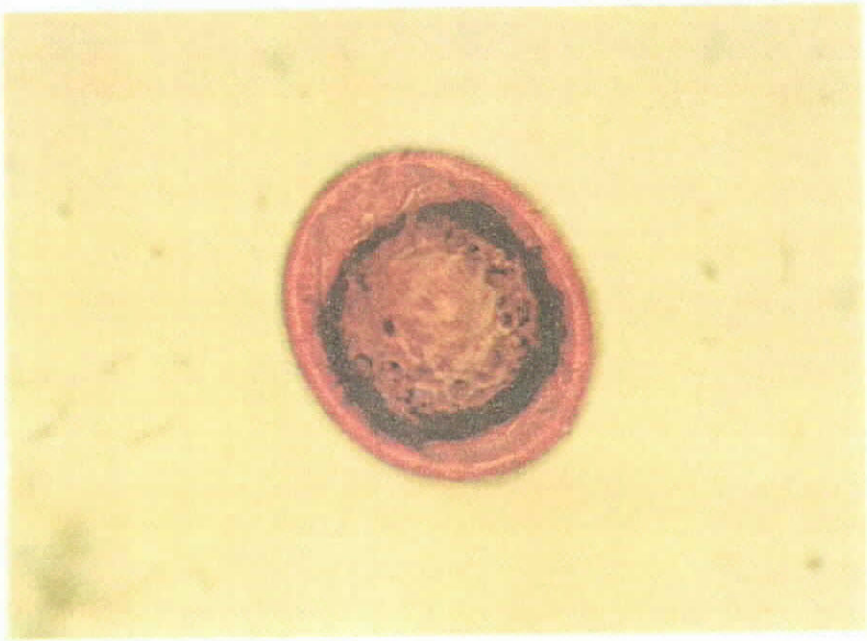


Plate 12(c) Scanning electromicrograph of spore without subtending hypha and with ornamentation on the spore wall

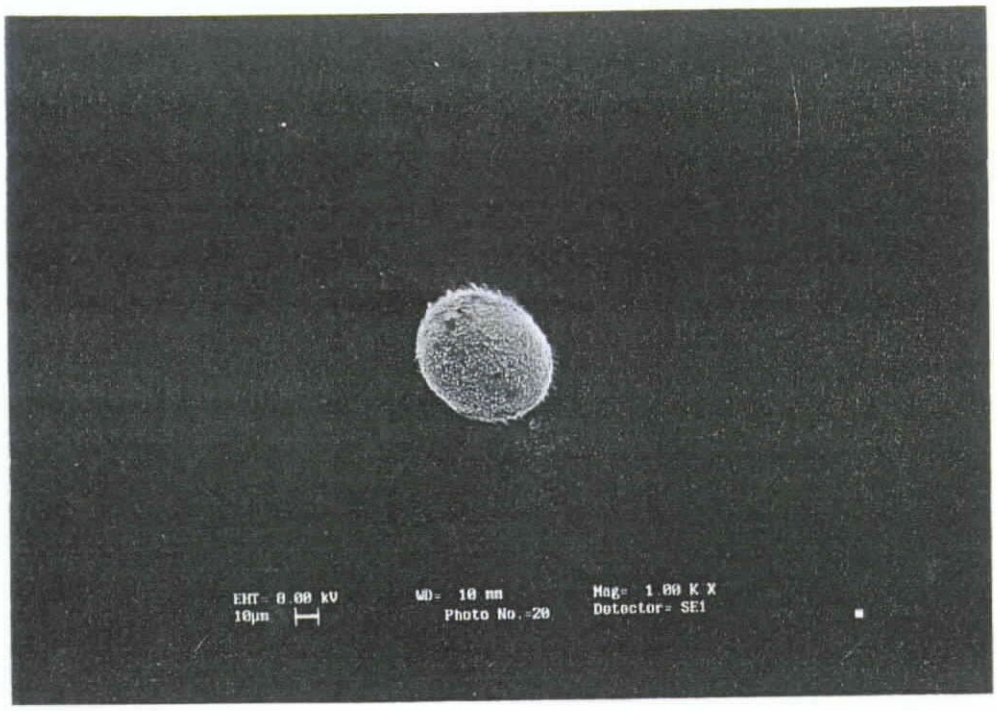
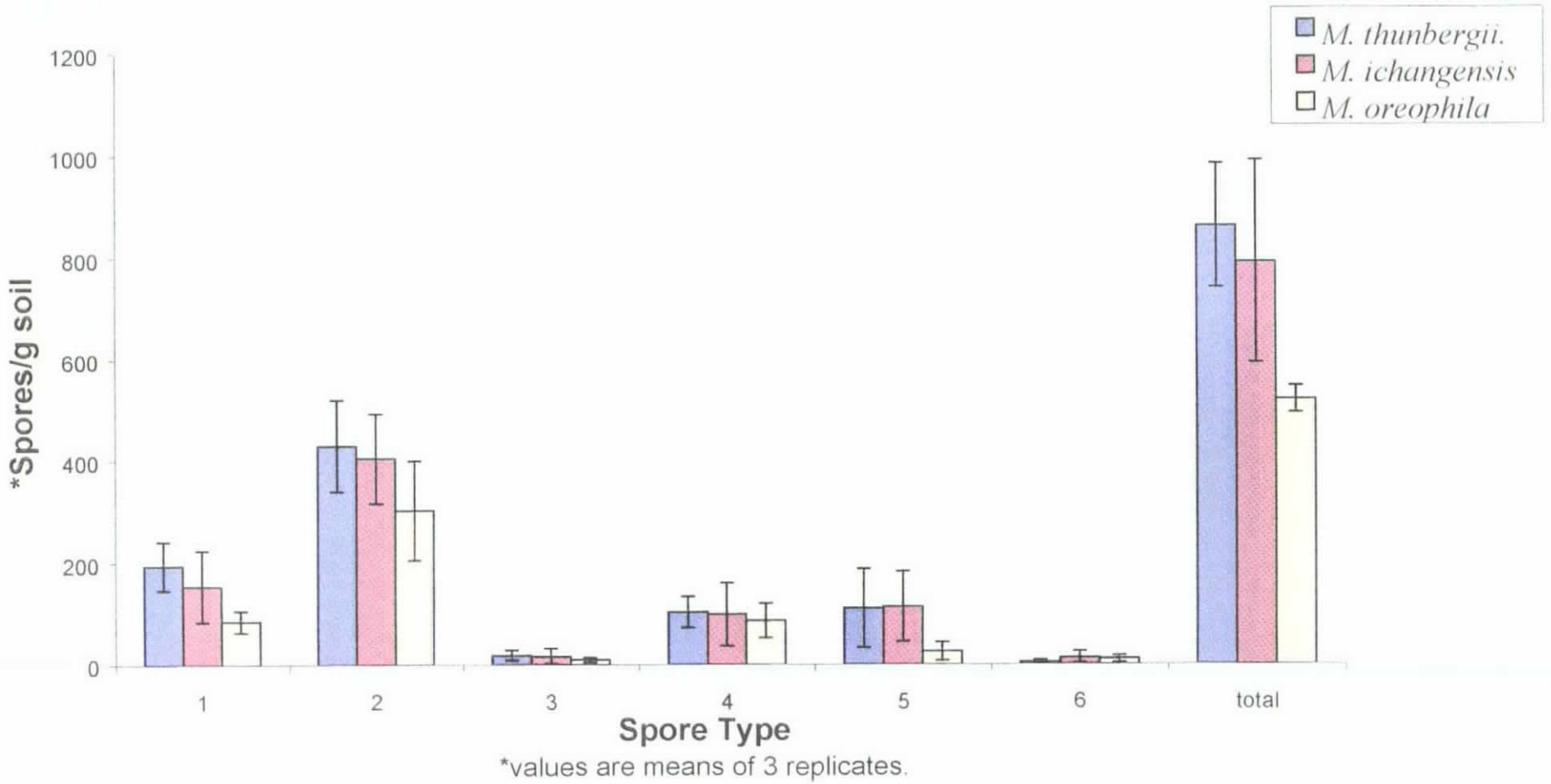


Fig. 1 The number of different types of spores per gram air-dried soil collected from the rhizospheres of different *Machilus* species.



*values are means of 3 replicates.

3.5 Discussion

In this investigation, six types of AM fungal spores were isolated from rhizospheres of three *Machilus* species in a natural secondary forest in Hong Kong. The morphological characters of each type were described and recorded in details based on the observations at both light microscopy and SEM level. Three of these types were believed to be the genus *Glomus* and one type belonged to the genus *Sclerocystis*; two types remained unidentified but possibly be *Acaulospora* species. The spores of Type 5 (*Sclerocystis* species) have been described previously in the rhizospheres of *Acacia confusa* Merr. and *Melaleuca quinquenervia* Cav. growing in a forest plantation in Hong Kong (Chan and Griffiths, 1992). The remaining five types were described for the first time in Hong Kong. The study of spore morphology is important not only for the taxonomic classification but also for understanding the dynamics of root colonisation by different AM fungi in natural ecosystems (Abbott and Gazey, 1994; Morton and Bentivenga, 1994).

None of the six types of spore showed colour change in Melzer's reaction. This may indicate that the spores we had examined were in a late maturation stage, as Melzer's reaction work best when spores are tested at earlier stages of maturation when the hyaline wall is present (Dodd et al., 1996).

It was notable that the proportion of the spore abundance is not even in the six spore types. the Type 2 spore accounted for more than half of the total number of spores. Whether the Type 2 spores play the most important role in this microecosystem and what makes this natural selection of species suited to this very soil conditions merit further study.

Up till now, taxonomic information is inadequate for even the most experienced persons to make accurate identification on all the isolates they encounter (Morton et al., 1992). Exact

identification of spores was not always necessary but careful characterisation is important for ensuring the type of AM fungi used in other experiments. In addition, vouchers of spores have been prepared and deposited so that identification may be determined or verified as required.

The morphological characterisation of AM fungal spores from the natural site can only serve as a reference for further studies because most glomlean fungi cannot be identified accurately from field-collected spores (Morton et al., 1992). These spores rarely are identical to those in culture, the latter being newly formed and with all morphological features intact. The solution of more accurate descriptions is to obtain cultured spores of indigenous species derived from trap and monospecific culture, compare their morphology to those in the field and determine their differences and similarities.

CHAPTER 4 POT-CULTURE EXPERIMENTS AND PROPAGATION OF THE STUDIED AM FUNGI IN TRAP CULTURES

4.1 Abstract

The indigenous AM fungi associated with *Machilus* species collected from the field were used as inoculum to assess their impact on the plant growth response. Growth parameters under controlled conditions were monitored using three different host plants: *Machilus. ichangensis*, *Machilus. oreophila* and *Astragalus. sinicum*. It was found that the inoculation had positive effects on each type of plants, although the effects on the *Astrugalus sinicum* were much greater than on the two *Machilus* species. There were also obvious differences among different inoculum on the *Machilus* species. The trap cultures of the AM fungi were set up and a few types of spores had been successfully propagated. Meanwhile the effects of different P-fertilisation on AM fungal infection and spore production were also assessed. It was found that the effect of medium level phosphorus applied were most appropriate for both the survival of the hosts and the production of AM fungal spores in the trap culture.

4.2 Introduction

It is well known that mycorrhizal fungi and plants can form a mutually beneficial, symbiotic relationship. The fungal partner, by associating with plant roots, receive a supply of energy in the form of carbohydrates from the host. The plant partner, on the other hand, absorb higher level of mineral nutrients and result in an enhanced plant growth. Though there are other benefits to both fungus and plant, most of the demonstrated benefits of mycorrhizae for

forest trees are improved nutrition for phosphorus and nitrogen (Gianinazzi-Pearson and Gianinazzi, 1986).

This nutritional benefit to plants is of interest to plant growers, who would like to understand whether the choice of a particular fungal species as a partner for a given tree species on a given reforestation site can significantly affect the survival, growth, and nutrition of the tree. One of the most important factors that determines whether the plant can benefit from inoculation in a specific site is the inoculum potential of indigenous mycorrhizal fungi present in that soil. In addition, to assess whether introduction of AM fungi into a given ecosystem will be beneficial, it is necessary to evaluate the importance of the indigenous mycorrhizal populations.

Machilus species are dominant in some local secondary forest. The spore density in rhizosphere soil of *Machilus* was found to be very high, and infection rate in roots was almost 100%. This may indicate that the *Machilus* species studied are highly mycorrhiza-dependent or the indigenous AM fungal population is playing an important role in the ecosystem. However, it is essential to understand more about the physiology of fungal species and their ability to form efficient associations with the host plants.

Plants in natural ecosystems are known to have varying degrees of dependence on mycorrhizal associations, which have resulted in the designation of obligate and facultative associations. These categories are the result of inherent properties of the plants themselves, as well as the availability of nutrients in the soils in which they naturally occur. According to Trappe (Trappe, 1987), if about 12% of the examined angiosperm species are facultatively mycorrhizal and 18% are typically nomycorrhizal, then about 70% will be obligate mycotrophic plants. The potential benefits resulting from the inoculation of plants with

mycorrhizal fungi will depend on the properties of the host plant, the identity of mycorrhizal fungi and the characteristics of the soil where they are grown.

The root morphology of the *Machilus* species is very particular: the roots are round and thick and almost no root hairs were observed (see **2.4.2 & Plate 3**). Baylis (1970, 1972) suggested that the lack of root hairs is a good predictor of the extent to which a plant will respond to mycorrhizal infection. The phosphorus uptake is likely to be negatively correlated to the root parameters such as root length and surface area (Baylis, 1975). This hypothesis is consistent with observation in many cases. It may be due to the fact that the plant phosphorus inflow is partly influenced by both the degree of root hairiness and the degree of rhizosphere modification. Inconsistency, however, may sometimes occur because the prediction of response to infection based on the traits of non-mycorrhizal plants may still be difficult. The latter is due to the fact that mycorrhizal infection may alter the inherent traits of plants and phosphorus availability has an effect on the degree of infection.

This work aims to (1) assess the benefits of inoculating indigenous AM fungi collected from the *Machilus* rhizosphere to two *Machilus* species and *Astragalus sinicum*, (2) collect newly formed spores from trap culture for identification purposes, and (3) evaluate the different levels of P-fertilization to AM infection and spore production in the trap cultures.

4.3 Materials and methods

4.3.1 Experiment 1: growth responses of two *Machilus* species to three different levels of AM fungal inoculation collected from the rhizosphere of *Machilus* species

4.3.1.1 AM fungal inoculum preparation

Inoculum consisting of a mixture of soil, spores and infected roots were collected from the rhizospheric soil of *M. ichangensis* and *M. oreophila* in the field. The AM fungal propagule densities of the inoculum were 760 per gram soil and 600 per gram soil for *M. ichangensis* and *M. oreophila*, respectively. Air-dried soil was passed through a 2-mm sieve. Roots that cannot pass through the sieve were blended and then mixed thoroughly with the sieved soil.

4.3.1.2 Soil preparation

The soil used was a mixture of sandy soil and horse manure (in a ratio of 6:1 v/v) collected from Tai Tong nursery. The soil had a pH of 6.2. Air-dried soil was passed through a 2-mm sieve. The soil was autoclaved for 1.5 h on three successive occasions prior to packing into 12.5-cm diameter pots.

4.3.1.3 Seed preparation

The seeds of *M. ichangensis* and *M. oreophila* were surface-sterilized in a 70% alcohol solution for 5 minutes and then 30% H_2O_2 solutions for 15 minutes. After washing 5 times with sterile water, the seeds were soaked in sterile water at 28 °C for 3 days and then incubated in sands at 28 °C for 3 weeks.

4.3.1.4 Experimental set up

Four treatments including 3 mycorrhizal treatment and 1 control were assessed for each of the two *Machilus* species. The two different *Machilus* species received the inoculum collected from their respective species. The inoculum for different treatments (**Table 8**) was placed on the surface of the sterile soil as a band (the control treatment received the sterilised inoculum instead, by autoclaved at 121°C, 15 p.s.i. for 60 min). The total amount of potting material added up to 120 gram and was allowed to stand for 7 days at 28 °C in a growth chamber. Twelve replicate pots per treatment were arranged in a randomised block in a growth chamber under controlled environmental conditions. 4 pots per treatment were harvested 52, 84 and 125 days after planting.

The pots were watered every two days to field capacity with distilled water over a 125-day period in a Conviron growth chamber under a controlled environmental conditions: day/night temperature 30/20°C, 16 h photoperiod, relative humidity 70-80%, irradiance from 140 $\mu E.m^{-2}.s^{-1}$ at the beginning and at the end of the light period, up to 800 $\mu E.m^{-2}.s^{-1}$ in the middle of the day (maintained for 7 hours).

Table 8 Levels of AM fungal inoculum in pot culture experiment 1

Treatment	Control	Low inoculum	Medium inoculum	High inoculum
Soil per pot (g)	75	105	90	75
Inoculum per pot (g)	45 *	15	30	45

* sterilized by autoclave for 60 minutes

4.3.1.5 Data treatments and analyses

Different parameters of plant growth, including shoot height, leaf area, leaf dry matter, stem dry matter, shoot dry matter, root dry matter, root/shoot ratio, shoot phosphorus (P) and nitrogen (N) concentration, root colonization, were recorded at each harvest. Dry weight of shoot and root were determined by drying at 70 °C for 72 h. Root subsamples were examined microscopically to confirm the presence of arbuscules, vesicles, hyphal coils or appressoria in AM fungal infected roots and to ensure that there was no infection. Non-mycorrhizal roots were also examined. Method for clearing and staining roots to determine the degree of AM fungal infection were performed as described in **2.3.3**. Statistical analysis of data were performed using nonparametric Kruskal-Wallis H tests in SPSS software.

4.3.2 Experiment 2: growth responses of *Astragalus sinicum* to AM fungal inoculation collected from the rhizosphere of *Machilus* species

4.3.2.1 AMF inoculum preparation

The inoculum consisting of a mixture of soil, spores and infected roots were collected from the rhizospheric soil of *Machilus* in the field. The AM fungal propagule densities of the inoculum were about 700 spores per gram of soil. Air-dried soil was passed through a 2-mm sieve. The roots that cannot pass through the sieve were blended and then mixed thoroughly with the sieved soil.

4.3.2.2 Soil preparation

A sandy soil obtained from Tai Tong nursery of the Fishery & Agriculture Department was air-dried and passed through a 2-mm sieve. The soil pH is 6.2 and was sterilized (121°C, 15 p.s.i. for 1.5h) on three successive occasions prior to packing into 6-cm diameter pots.

4.3.2.3 Seed preparation

Seeds of *Astragalus sinicum* were surface-sterilized in 3.5% calcium hypochlorite solution for 10 min. After washing 5 times with sterile water, the seeds were left to germinate in a Petri dish at 26 °C in dark.

4.3.2.4 Experimental set up

Two treatments including one with mycorrhizal inoculation and one control were set up. For the inoculated treatment, 10 gram of the inoculum prepared from 4.3.2.1 were placed on the top of 100 gram of sterile soil in a plastic pot. The pots were allowed to stand for 7 days at 28 °C in a growth chamber. Then six uniform, germinated seeds prepared from 4.3.2.3 were sown in each pot. Control pots received 10 gram of similar but sterilized (121°C, 15 p.s.i. for 60 min) inoculum soil. 4 replicate pots and 4 control pots were arranged in a randomized block in a growth chamber.

The pots were watered every two days to field capacity with distilled water over 91-day period in a Conviron growth cabinet under a controlled environmental conditions: day/night temperature 24/18°C, 16h photoperiod, relative humidity 70%-80%, irradiance varied from 140 $\mu E.m^{-2}.s^{-1}$ at the beginning and at the end of the light period, up to 800 $\mu E.m^{-2}.s^{-1}$ in the middle of the day (maintained for 7 hours). Each pot was fed weekly 20ml nutrient solution (modified from Hoagland solution, described below at **Page 75**) when the nutrient deficiency symptom was observed in the growing plants 35 days after planting.

One week after seedling emergence, number of seedlings in each pot was reduced to only four. All four plants from each pot per treatment were harvested at 21, 35, 63 and 91 days after sowing. 91 days was chosen for the last harvest because mycorrhizal plants tended to become pot-bound after this period.

4.3.2.5 Data treatments and analyses

Fresh weights of shoots and roots, dry weights of shoots are recorded at each harvest. Dry weight of shoot and root were determined by drying at 70 °C for 72h. Root subsamples were examined microscopically to confirm the presence of arbuscules, vesicles, hyphal coils or appressoria in AM fungal infected roots and to ensure that there was no infection in non-mycorrhizal roots were also examined. Mycorrhizal infection rate (hyphal, arbuscules or vesicles) is expressed as M%, while the arbuscule and vesicle development (arbuscules or vesicles) as A%. The amount of mycorrhizal root (mycorrhizal root mass) was calculated by multiplying M% by total root fresh mass. Due to the small size and lightweight of the individual *A. sinicum* plants, four plants per pot were balanced together for their fresh and dry weights. Therefore, data represent the means of 4 replicate plants and the statistical analysis was not done in this experiment due to the above reasons.

4.3.3 Experiment 3: propagation of the studied AM fungi in trap cultures

Commonly, trap cultures are those produced in pots using whole inoculum (roots, infectious propagules, and surrounding material {soil or growth medium} from a field sample or a previous culture). They are set up to meet one or more of the following goals: to trap and propagate fungi indigenous to a particular field site; to increase density and infectivity of all types of infective propagules; to increase volume of inoculum of a monospecific culture for internal use or for release. Different types of AM fungi in the inoculum may have different potential to infect the host plants, thus the hosts could be infected by different fungi at different time. Therefore, AM fungi infection order could be determined by transplanting the inoculated hosts at different time point to a "clean" pot medium (without AM fungi) for propagating the studied fungi. Meanwhile the trap cultures of different types of AM fungi could be obtained, and the monospecific culture or fewer-type-spore culture may be obtained

at the earlier transplanting time point. In this experiment the trap culture of the studied AM fungi were set up by this way. The autoclaved sand was used as culture medium in this experiment for their easier separation of spore production at harvest. Nutrient solution was used to maintain normal plant growth. Further more the effects of different P-fertilization on AM fungal infection and spore production were assessed by applying different P-level nutrient to the transplanted plants.

The inoculated pot cultures of *Astragalus sinicum* were set up as described in Experiment 2 (**4.3.2.4**). At 3, 5 and 13 weeks after sowing, plants from inoculated pots were transplanted into pots with sterilized sands and each pot contained 2 transplanted seedlings. Different levels of P-fertilizers were applied to the transplantation plants in four treatments (P_0, P_L, P_M, P_H) (**Table 9**) with each treatment two pot replicates. Each pot was fed weekly 20 ml nutrient solution.

Table 9. Different phosphorus levels in the nutrient solution applied to *A. sinucum* in pot experiment 3

Phosphorus concentration	μM	mg/L
P_0	0	0
P_L	40	1.24
P_M	400	12.4
P_H	1200	37.2

The cultures were harvested after the total growth period of four months. Then the roots of plants in each pot were assessed for their mycorrhizal infection rate by the same method described in **2.3.5**. The pot mediums were checked for the AM spores produced. Different spore types found at different transplantation time were recorded.

A master nutrient solution was prepared as described below (modified after Hoagland and Arnon, 1938). The nutrient solution containing different P levels applied in this experiment were prepared by changing the P concentration of the master nutrient according to **Table 9**.

A master nutrient solution contains:

KNO_3	50.0 g litre^{-1}
$Ca(NO_3).4H_2O$	11.6 g litre^{-1}
KH_2PO_4	12.4 g litre^{-1}
$MgSO_4.7H_2O$	49.3 g litre^{-1}
Fe-citrate/Fe EDTA	8.0 g litre^{-1}
$MnSO_4.H_2O$	0.15 g litre^{-1}
$ZnSO_4.7H_2O$	0.02 g litre^{-1}
$CuSO_4.5H_2O$	0.08 g litre^{-1}
$Na_2B_4O_7.10H_2O$	0.2 g litre^{-1}
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.2 g litre^{-1}
$CoNO_3.6H_2O$	0.25 g litre^{-1}

The pH of the nutrient solution is adjusted to 5.5 with HCl.

4.4 Results

4.4.1 Experiment 1: growth responses of two *Machilus* species to three different levels of AM fungal inoculation collected from the rhizosphere of *Machilus* species

The results for this experiment were presented in **Table 10**. Due to the small sample sizes, nonuniform plant seeds and short growth period, the differences between each treatment were not statistically significant (**Table 11**). However, in the reality I think there were difference existing between the treatments.

4.4.1.1 Mycorrhizal development

No obviously difference was observed in terms of mycorrhizal infection pattern between the two *Machilus* species except some slight differences in the values of infection rate (**Fig. 2a** and **Fig. 3a**). The roots were found colonized by the AM fungus at the first harvest (52 days

after planting) with infection rate not more than 20%. Abundance of the mycorrhizal fungi within roots steadily increased with development of the root systems by time.

For *M. ichangensis*, the infection rate corresponded with the levels of inoculum in the treatment except that at the first harvest (52 days after planting) the medium inoculum resulted in the highest infection rate of 19%, slightly higher than the infection rate of 15% caused by the high inoculum (**Fig. 2a**).

For *M. oreophila*, only at the first harvest (52 days after planting), the infection rate corresponded with the levels of inoculum in the treatment. At the second harvest (84 days after planting), the low inoculum resulted in the lowest infection rate, the medium and high inoculum gave rise to almost the same infection rate (62% and 61% respectively). At the third harvest (125 days after planting), the low, medium and high inoculum brought about very similar infection rate (77%, 77%, 82% respectively), though that of the high inoculum were slightly higher.

No mycorrhizal colonization was observed in the control plants of two *Machilus* species.

4.4.1.2 Biomass response

For the *M. ichangensis*, the shoot height and stem dry matter of the inoculated plants was greater than the control plants at all the three harvests. The leaf area (**Fig. 2c**), leaf dry matter (**Fig. 2d**), shoot dry matter (**Fig. 2f**) and total dry matter (**Fig. 2h**) of the inoculated plants was greater than the control plants at the first (52 days after planting) and the third harvests (125 days after planting). The root/shoot ratio of the control plants was highest throughout the three harvests (**Fig. 2i**).

At the second harvest, the plant responses to the low mycorrhizal inoculation were better than the other treatments in the following aspects: shoot height (**Fig. 2b**), leaf area (**Fig. 2c**), leaf

dry matter (**Fig. 2d**), stem dry matter (**Fig. 2e**), shoot dry matter (**Fig. 2f**) and total dry matter (**Fig. 2h**). Also, plants with low-level mycorrhizal inoculation had best shoot development at the second harvest as they have the lowest root/shoot ratio (**Fig. 2i**) with the highest shoot dry matter (**Fig. 2f**) and relatively lower root dry mass (**Fig. 2g**).

At the third harvest, the shoot height (**Fig. 2b**), stem dry matter (**Fig. 2e**), shoot dry matter (**Fig. 2f**), root dry matter (**Fig. 2g**) and total dry matter (**Fig. 2h**) of the plants with high level mycorrhizal inoculation was the highest.

At the second harvest of *M. oreophila*, the yields of leaf dry matter (**Fig. 3d**), stem dry matter (**Fig. 3e**), shoot dry matter (**Fig. 3f**) and total dry matter (**Fig. 3h**) of the plants with low level mycorrhizal inoculation was the highest. Also, the plants with medium level of mycorrhizal inoculation yielded the lowest value of the leaf area, leaf dry matter, shoot dry matter, root dry matter, total dry matter and the root/shoot ratio.

At the third harvest of *M. oreophila*, the yields of stem dry matter (**Fig. 3e**), shoot dry matter (**Fig. 3f**) and the total dry matter (**Fig. 3h**) of the inoculated plants were higher than that of control plants.

Another trend that merits attention was that the biomass responses to low level mycorrhizal inoculation were greater than that of control plants throughout the three harvests of the two *Machilus* species except the root dry matter of *M. oreophila* at the third harvest (**Fig. 3g**). Meanwhile the root/shoot ratio of low level inoculated plants were less that that of the control plants may suggest that the inoculated plants had better shoot development compared with the control plants. All these indicated that the plants with low-level inoculation had better growth than the control ones.

The ultimate effects of the inoculation on the growth of *Machilus* species were assessed by averaging the effect of different levels of inoculation to the plant growth. At the last harvest (125 days after planting) it was found that the inoculated plants had average increases in *M. ichangensis* shoot height, leaf area, leaf dry matter, stem dry matter, shoot dry matter, root dry matter and total dry matter of 28.9%, 10.9%, 12.1%, 47.1%, 20.6%, 2.9% and 14.7% respectively, compared with the control plants. For the *M. oreophila*, the according increases were 3.5%, -1.2%, 0.8%, 20.5%, 5.6%, -2.5% and 3.2% respectively.

4.4.1.3 Foliar phosphorus and nitrogen responses

No convincing results were obtained from the foliar analysis because the dry matter of the leaves was too small for any accurate analysis.

4.4.2 Experiment 2: growth responses of *Astragalus sinicum* to AM fungal inoculation collected from the rhizosphere of *Machilus* species

4.4.2.1 Mycorrhizal development

Roots of *A. sinicum* plants were intensely colonized (42% of the root cortex) by the AM fungus 21 days after inoculation and the infection rate (M%) has reached 68% 63 days after planting and then decreased (**Fig. 5**). The frequency of arbuscule and vesicle (A%) gave a similar pattern to the infection rate (M%) (**Fig. 5**). However, mycorrhizal root mass steadily increased with development of the root systems (**Fig. 6**). No mycorrhizal formation was detected in the control pots throughout the experiment.

4.4.2.2 Plant growth

Mycorrhizal *A. sinicum* plants showed significant growth stimulation 21 days after inoculation compared with the control. At the third harvest (63 days after planting), the

inoculated plants already had average increases in plant shoot dry mass of 34% and up to 680% (**Fig. 4c**). The growth parameters (**Fig. 4**) measured in this study including shoot fresh matter (**Fig. 4a**), root fresh matter (**Fig. 4b**) and shoot dry matter (**Fig. 4c**) followed a similar growth pattern with each other. For the inoculated *A. sinicum* plants, all the three parameters had decreased growth at the 21-35 days period after planting and gave a steady increase in growth from 35 days after planting and became pot bound after 91 days of growth. The growth of control plants was exactly opposite of the inoculated plants with steady decrease in growth except the only increase at the 35-63 days period. 63 days after planting, the control plants tended to stop growing and were dying at the last harvest at 91 days after planting.

4.4.3 Experiment 3: propagation of the studied AM fungi in trap cultures

Table 10 summarised the results of the trap culture experiment. The trap culture had been set up and a few types of newly formed spores were found in the rhizospheres of the transplanted plants (**Table 10**). The main purpose of this experiment was to establish the trap culture of the studied AM fungi. Besides that, more insights of the effect of different levels of P-fertilization on AM infection in roots and spore production in soil were obtained qualitatively. One type of *Glomus* spores (**Plate 13a**) was found in the rhizospheric medium of plants transplanted at 3 weeks, one type of *Acaulospora* spores (**Plate 13b**) at 5 weeks, Type 1, Type 2 (same as described in **Chapter 3**) and the same *Acaulospora* spores (**Plate 13c**) at 13 weeks.

The plants applied by P_0-fertilizer (no phosphorus in the fertilizer) after transplantation appeared declined with the shoot withered and yellow gradually and did not survive.

Although some of the plants in the P_L-fertilizer treatment (low phosphorus level in the fertilizer) were dead after transplantation, the remaining plants were intensively infected (infection rate > 70%) in all the three different transplantation.

The plants applied by P_M-fertilizer (middle phosphorus level in the fertilizer) after transplantation all grew well with intensive mycorrhizal infection (infection rate > 70%) in the plants of the three different transplantation time.

The plants applied by P_H-fertilizer (high phosphorus level in the fertilizer) after transplantation all grew well, and the mycorrhizal infection was not high (infection rate < 30%), except those of transplantation time at 13 weeks (infection rate > 70%).

Table 10. Effects of different levels of AM fungal inoculation on the growth and development of two *Machilus* species

	Root colonization			Shoot height (cm)			Leaf area (cm^2)		
Days after planting	52	84	125	52	84	125	52	84	125
Inoculum									
M. kobangensis control	0%	0%	0%	8.80 ± 1.72	8.82 ± 0.96	8.84 ± 0.57	15.88 ± 3.10	36.40 ± 3.85	39.82 ± 3.03
low	13%	27%	65%	9.13 ± 1.31	12.20 ± 1.67	11.28 ± 1.29	28.96 ± 9.31	47.67 ± 7.52	40.64 ± 8.48
mid	19%	40%	78%	10.23 ± 1.02	10.98 ± 0.96	10.48 ± 1.22	29.11 ± 3.50	34.65 ± 8.37	48.15 ± 5.66
high	15%	59%	84%	10.30 ± 0.53	11.00 ± 0.74	12.42 ± 0.90	30.36 ± 8.61	32.92 ± 4.16	43.76 ± 5.94
Days after planting	52	84	125	52	84	125	52	84	125
Inoculum									
M. oreophila control	0%	0%	0%	9.07 ± 0.37	9.10 ± 0.35	9.43 ± 0.81	13.54 ± 1.29	31.96 ± 2.40	33.74 ± 5.39
low	7%	36%	77%	9.53 ± 1.16	8.97 ± 1.23	11.57 ± 0.85	22.73 ± 2.97	29.09 ± 7.86	37.67 ± 1.65
mid	14%	62%	77%	8.23 ± 0.63	9.98 ± 0.73	8.58 ± 0.31	19.12 ± 3.94	22.08 ± 1.97	30.67 ± 4.76
high	19%	61%	82%	8.00 ± 0.64	10.28 ± 0.29	9.14 ± 0.42	15.81 ± 3.77	28.63 ± 3.86	31.65 ± 2.94

	Leaf dry matter (g)			Stem dry matter (g)			Shoot dry matter (g)		
Days after planting	52	84	125	52	84	125	52	84	125
Inoculum									
M. kobangensis control	0.07 ± 0.01	0.20 ± 0.03	0.23 ± 0.02	0.028 ± 0.007	0.055 ± 0.010	0.073 ± 0.010	0.094 ± 0.013	0.25 ± 0.04	0.30 ± 0.02
low	0.11 ± 0.03	0.24 ± 0.05	0.23 ± 0.05	0.033 ± 0.008	0.083 ± 0.023	0.107 ± 0.026	0.146 ± 0.036	0.32 ± 0.08	0.33 ± 0.07
mid	0.12 ± 0.01	0.16 ± 0.05	0.27 ± 0.04	0.042 ± 0.007	0.056 ± 0.012	0.102 ± 0.015	0.157 ± 0.018	0.21 ± 0.05	0.37 ± 0.05
high	0.10 ± 0.03	0.17 ± 0.03	0.26 ± 0.03	0.038 ± 0.005	0.060 ± 0.011	0.111 ± 0.013	0.143 ± 0.033	0.23 ± 0.04	0.38 ± 0.04
Days after planting	52	84	125	52	84	125	52	84	125
Inoculum									
M. oreophila control	0.05 ± 0.01	0.13 ± 0.02	0.18 ± 0.04	0.029 ± 0.004	0.040 ± 0.004	0.060 ± 0.010	0.083 ± 0.010	0.17 ± 0.02	0.24 ± 0.05
low	0.07 ± 0.01	0.14 ± 0.04	0.20 ± 0.02	0.028 ± 0.003	0.051 ± 0.017	0.066 ± 0.006	0.099 ± 0.011	0.19 ± 0.06	0.26 ± 0.02
mid	0.07 ± 0.01	0.10 ± 0.01	0.19 ± 0.04	0.026 ± 0.001	0.043 ± 0.008	0.082 ± 0.013	0.095 ± 0.011	0.15 ± 0.02	0.27 ± 0.05
high	0.06 ± 0.02	0.14 ± 0.02	0.18 ± 0.02	0.028 ± 0.007	0.049 ± 0.005	0.070 ± 0.011	0.088 ± 0.032	0.19 ± 0.03	0.25 ± 0.03

	Shoot and root dry matter (g)			Root dry matter (g)			Root/shoot ratio		
Days after planting	52	84	125	52	84	125	52	84	125
Inoculum									
M. kobangensis control	0.12 ± 0.02	0.32 ± 0.05	0.45 ± 0.05	0.027 ± 0.006	0.071 ± 0.008	0.150 ± 0.028	0.30 ± 0.06	0.28 ± 0.03	0.49 ± 0.07
low	0.18 ± 0.04	0.38 ± 0.09	0.48 ± 0.11	0.029 ± 0.006	0.064 ± 0.016	0.150 ± 0.038	0.23 ± 0.04	0.19 ± 0.02	0.44 ± 0.02
mid	0.19 ± 0.02	0.26 ± 0.06	0.52 ± 0.06	0.033 ± 0.005	0.047 ± 0.009	0.148 ± 0.010	0.21 ± 0.02	0.24 ± 0.03	0.41 ± 0.04
high	0.17 ± 0.04	0.28 ± 0.05	0.54 ± 0.07	0.029 ± 0.008	0.051 ± 0.008	0.166 ± 0.032	0.20 ± 0.01	0.23 ± 0.01	0.44 ± 0.07
Days after planting	52	84	125	52	84	125	52	84	125
Inoculum									
M. oreophila control	0.10 ± 0.02	0.21 ± 0.02	0.35 ± 0.06	0.019 ± 0.005	0.044 ± 0.002	0.104 ± 0.017	0.23 ± 0.04	0.27 ± 0.04	0.43 ± 0.04
low	0.12 ± 0.02	0.24 ± 0.08	0.35 ± 0.04	0.019 ± 0.009	0.053 ± 0.021	0.088 ± 0.017	0.17 ± 0.08	0.27 ± 0.03	0.34 ± 0.06
mid	0.12 ± 0.02	0.18 ± 0.03	0.38 ± 0.07	0.022 ± 0.005	0.036 ± 0.005	0.113 ± 0.018	0.22 ± 0.04	0.25 ± 0.03	0.43 ± 0.03
high	0.11 ± 0.04	0.24 ± 0.04	0.35 ± 0.05	0.019 ± 0.007	0.058 ± 0.010	0.103 ± 0.016	0.22 ± 0.04	0.33 ± 0.06	0.42 ± 0.04

Table 11. Statistical analysis (P value) of the effect of AM inoculum on the growth of two species of *Machilus*

	Shoot height			Leaf area			Leaf dry matter			Stem dry matter		
Days after planting	52	84	125	52	84	125	52	84	125	52	84	125
M. ichangensis	0.89	0.33	0.14	0.25	0.47	0.67	0.26	0.57	0.58	0.60	0.86	0.23
M. oreophila	0.65	0.41	0.09	0.23	0.27	0.64	0.64	0.66	0.96	0.81	0.87	0.69
	Shoot dry matter			**Shoot and root dry matter**			**Root dry matter**			**Root/shoot ratio**		
Days after planting	52	84	125	52	84	125	52	84	125	52	84	125
M. ichangensis	0.25	0.68	0.40	0.43	0.63	0.62	0.64	0.23	0.88	0.34	0.06	0.60
M. oreophila	0.71	0.79	0.94	0.73	0.63	0.98	0.89	0.23	0.62	0.97	0.71	0.48

Four levels of AM inoculum, including negative control, are applied to the two species of *Machilus*. Each treatment consists of 4 to 6 plants. They are harvested on 52, 84 and 125 days after inoculation and analysed for their growth parameters. The effects of AM inoculum on these growth parameters were analysed using the Kruskal-Wallis H tests in SPSS software.

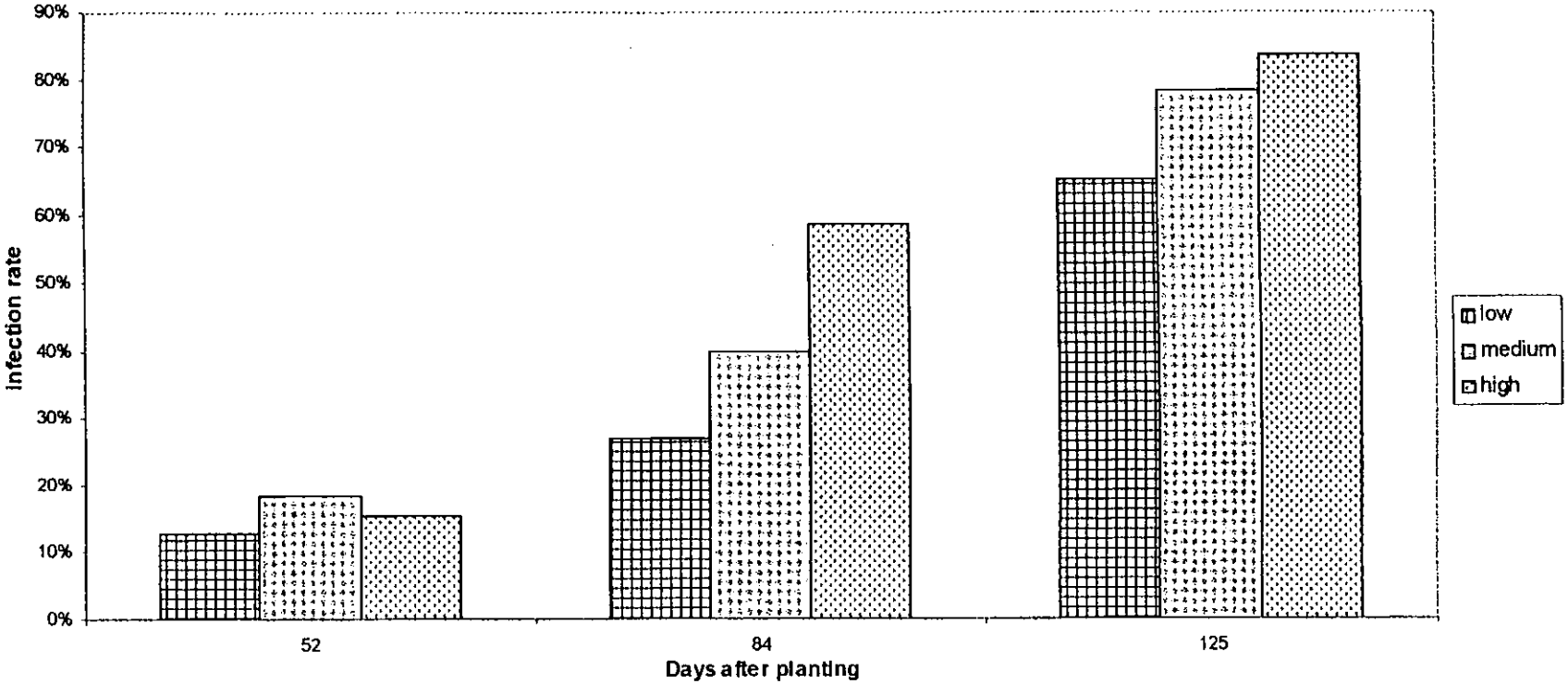
Table 12. Results of trap culture experiment

Weeks of transplantation	P level of the nutrition to plants	Root infection rate	Spore types found
3	P_0	NS	
	P_L	NS/+++	*Glomus* spores
	P_M	+++	
	P_H	+	
5	P_0	NS	
	P_L	NS	
	P_M	+++	*Acaulospora* spores
	P_H	+	
13	P_0	NS	
	P_L	+++	Type 1 spores
	P_M	+++	Type 2 & *Acaulospora* spores
	P_H	+++	Type 1 spores

+++ represents more than 70% root infection; ++ represents 30%-70% root infection; + represents less than 30% root infection; NS represents the transplanted plants can not survive; NS/+++ represents some of the plants in the pots were dead and those that were survived had more than 70% root infection.

Fig. 2(a-i) Growth responses of *M. ichangensis* to three different AM fungal inoculation levels

Fig. 2(a) Infection rate of inoculated *M. ichangensis*



The infection rate was determined by evaluation of 100 root segments from 4 pooled subsamples in each treatment. Therefore no error bars are present.

Fig. 2(b) Shoot height of *M. ichangensis* to three different AM fungal inoculation levels

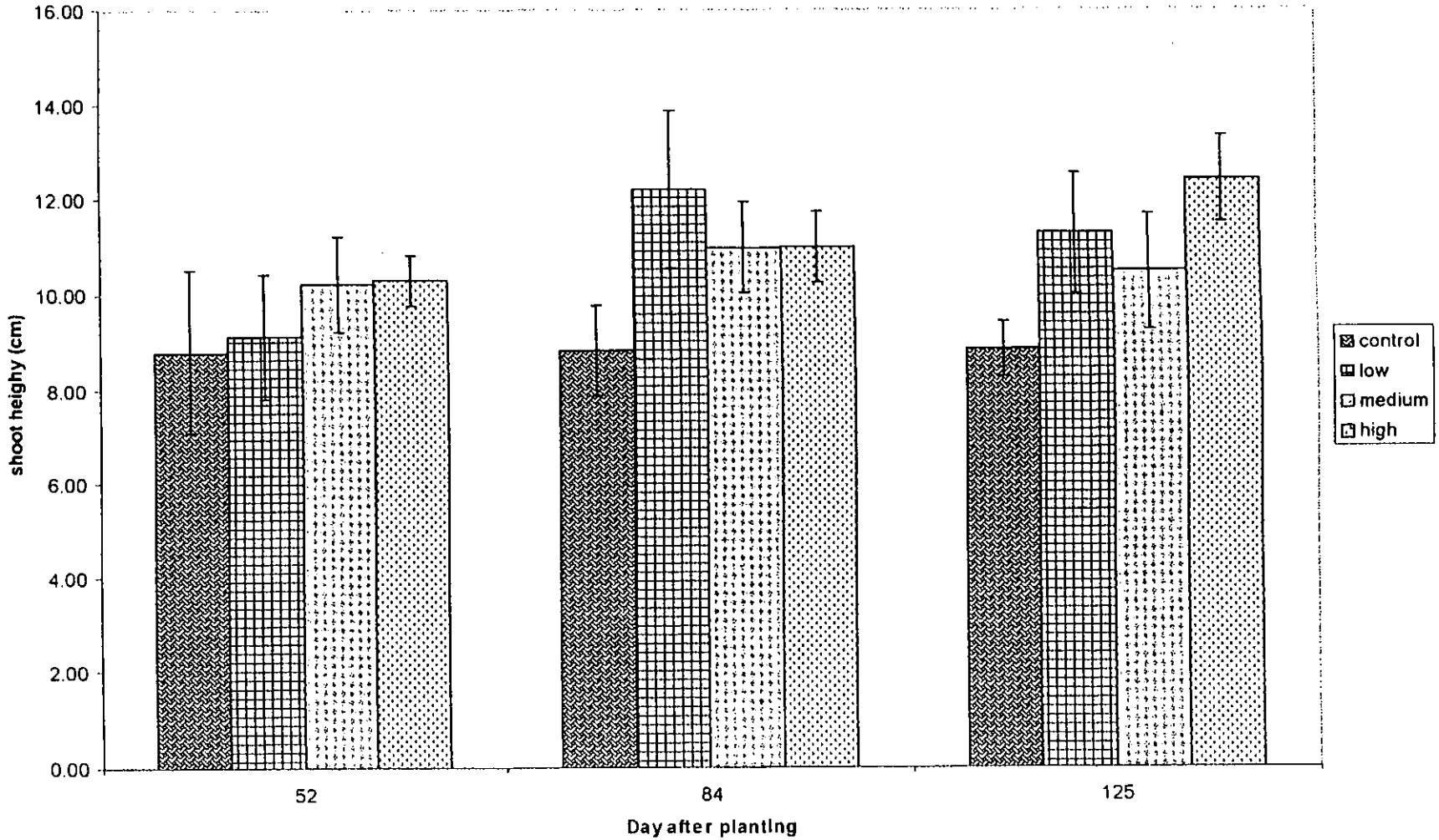


Fig. 2(c) Leaf area of *M. ichangensis* to three different AM inoculation levels

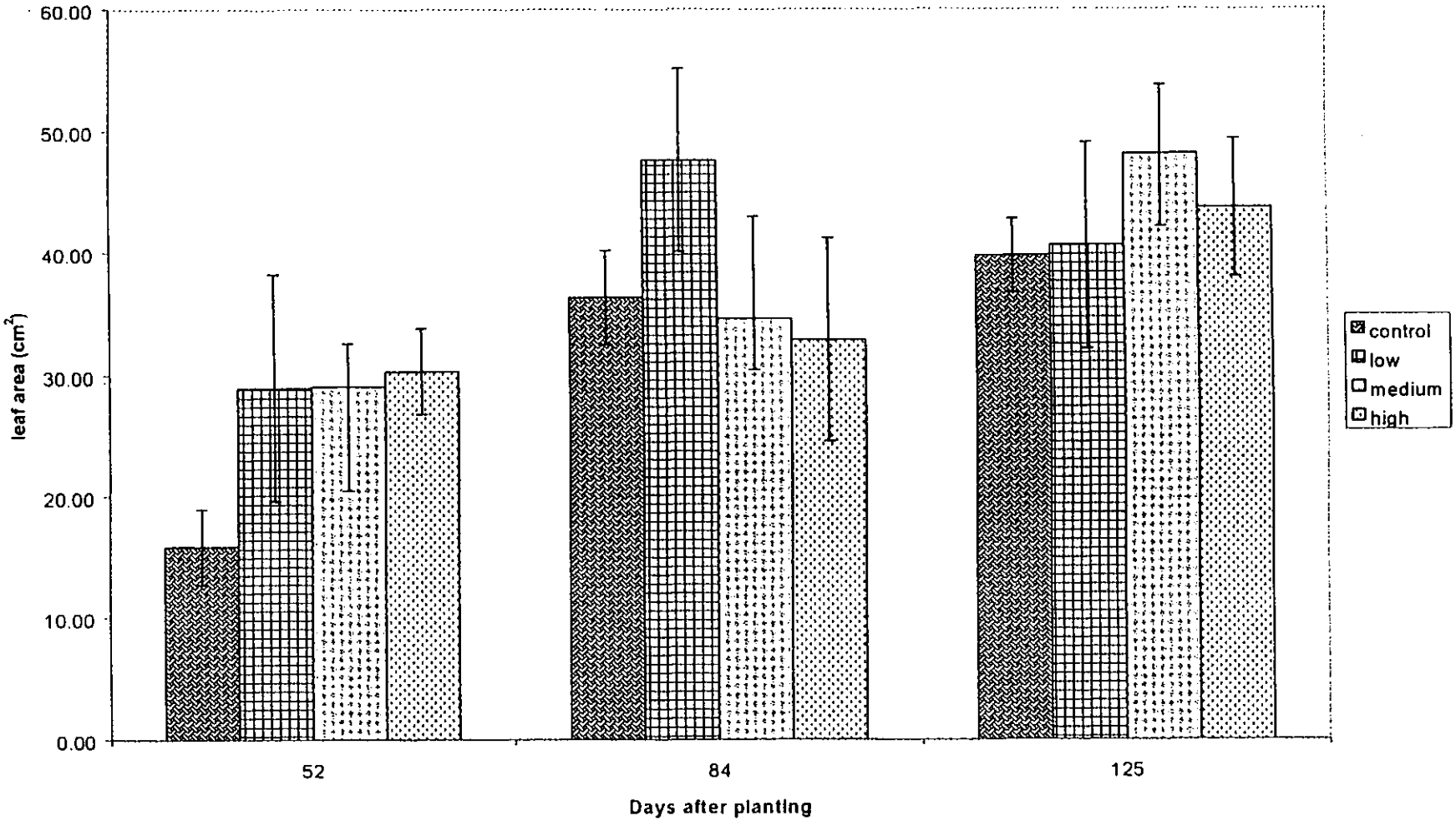
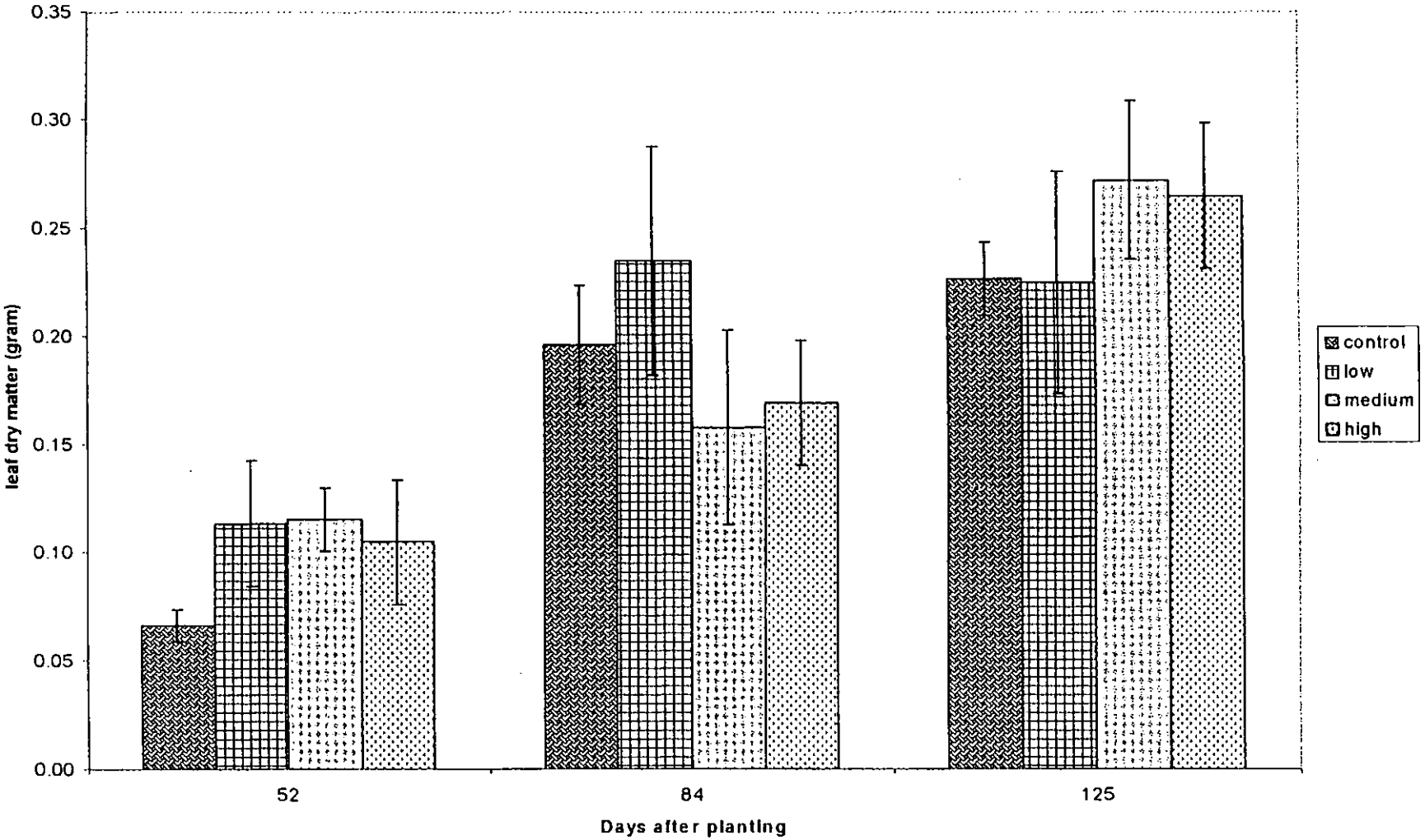


Fig. 2(d) Leaf dry matter of *M. ichangensis* to three different AM fungal inoculation levels



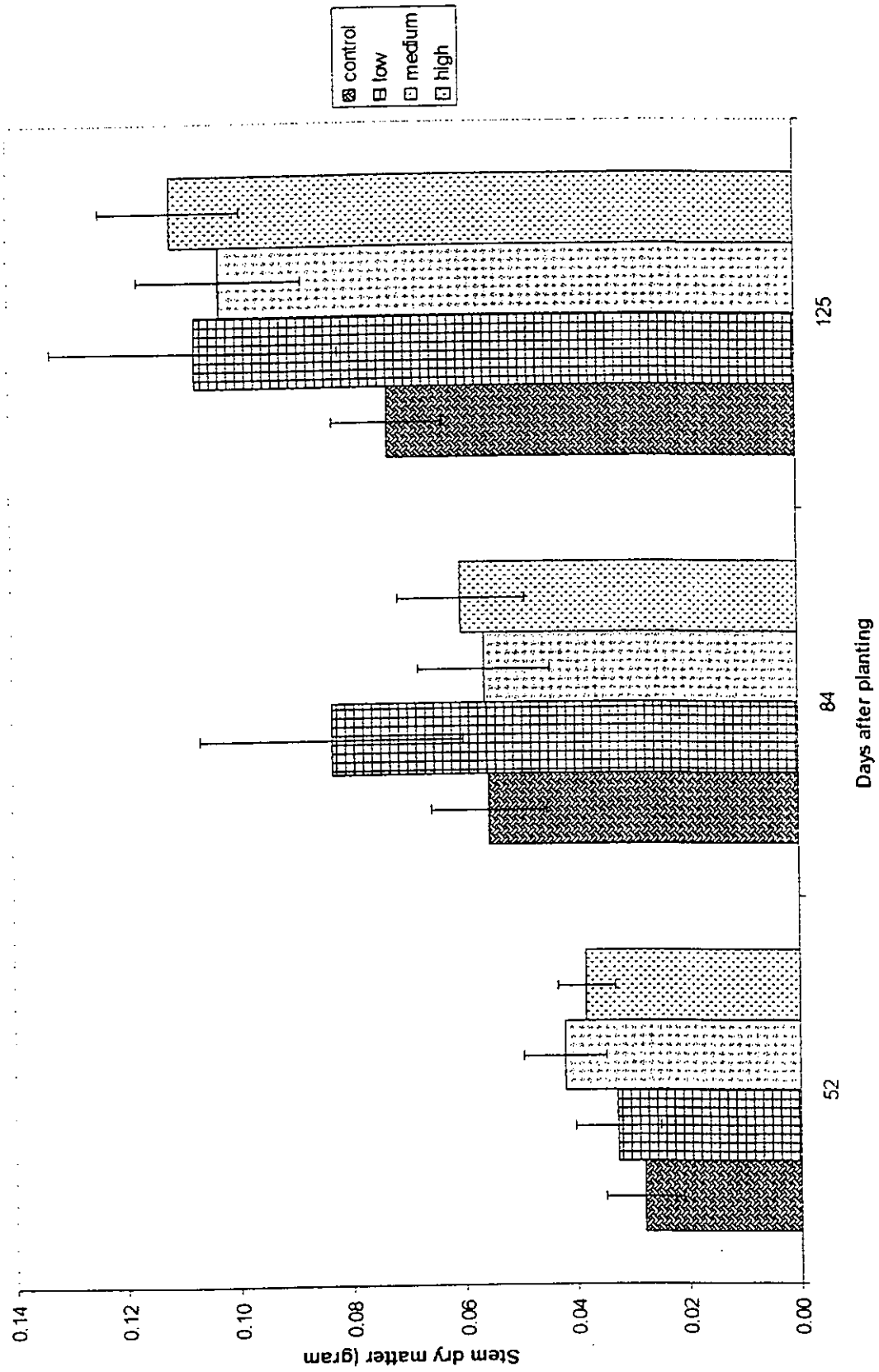


Fig. 2(e) Stem dry matter of *M. ichangensis* to three different AM fungal inoculation levels

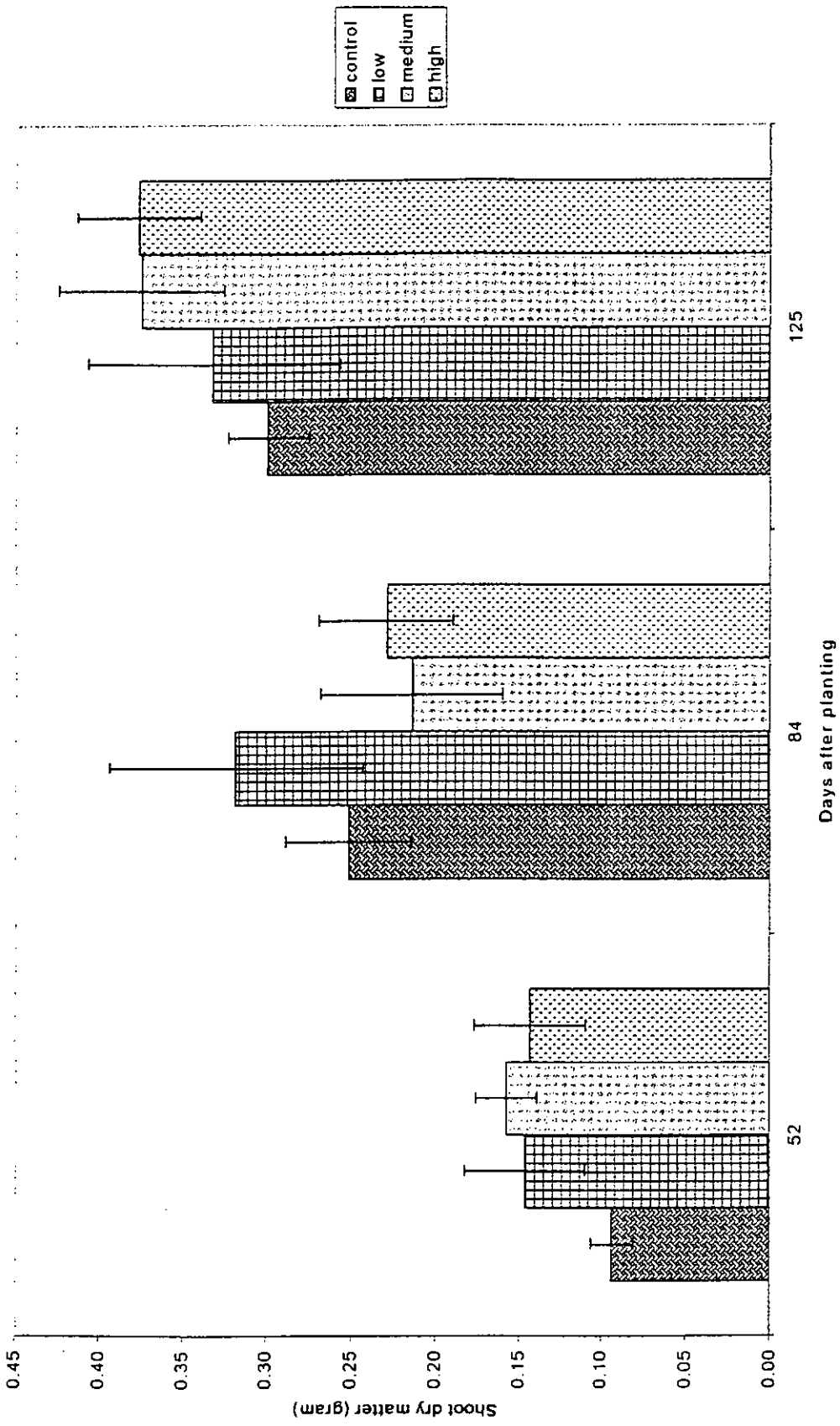


Fig. 2(f) Shoot dry matter of *M. ichangensis* to three different AM fungal inoculation levels

Fig. 2(g) Root dry matter of *M. ichangensis* to three different AM fungal inoculation levels

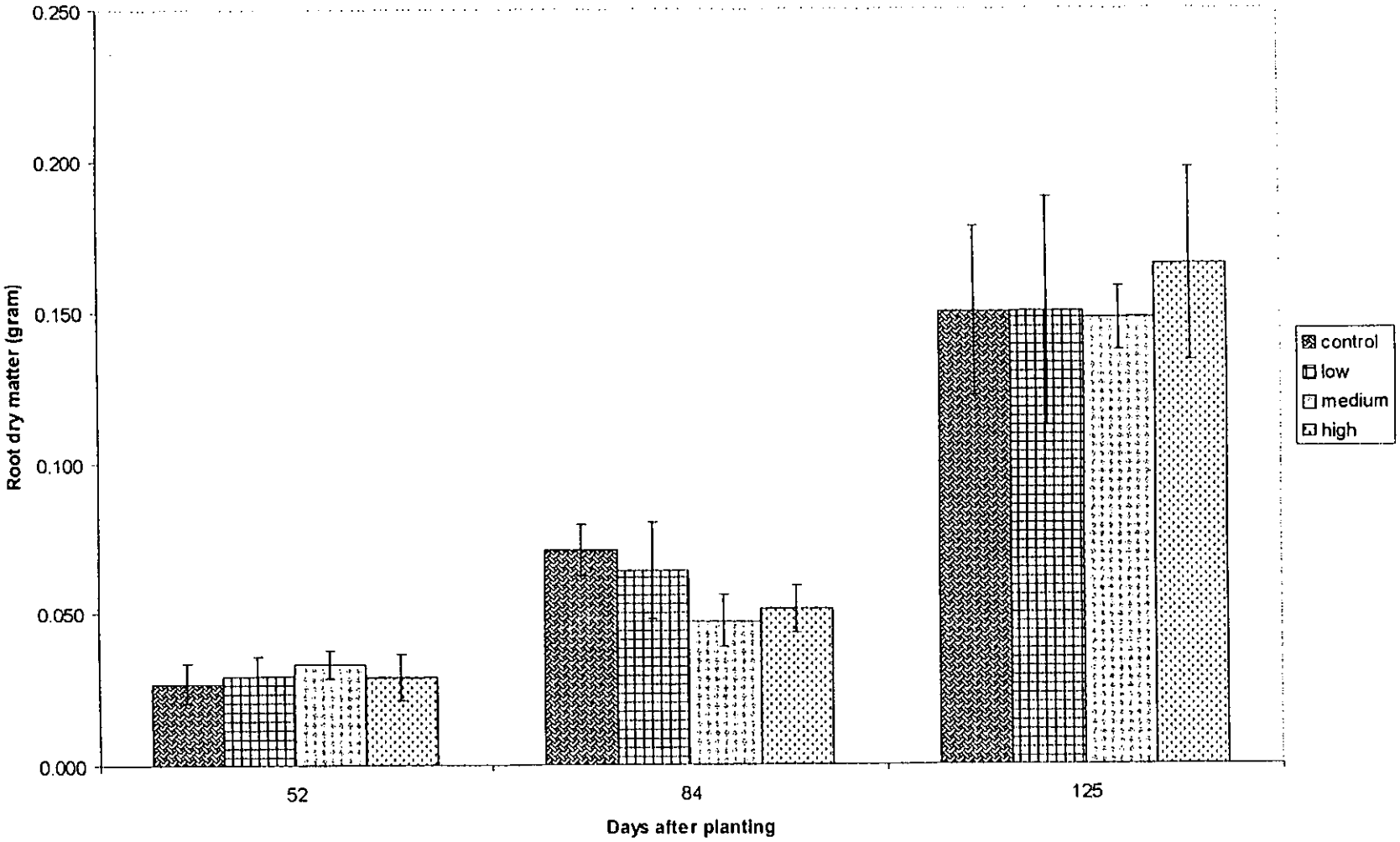
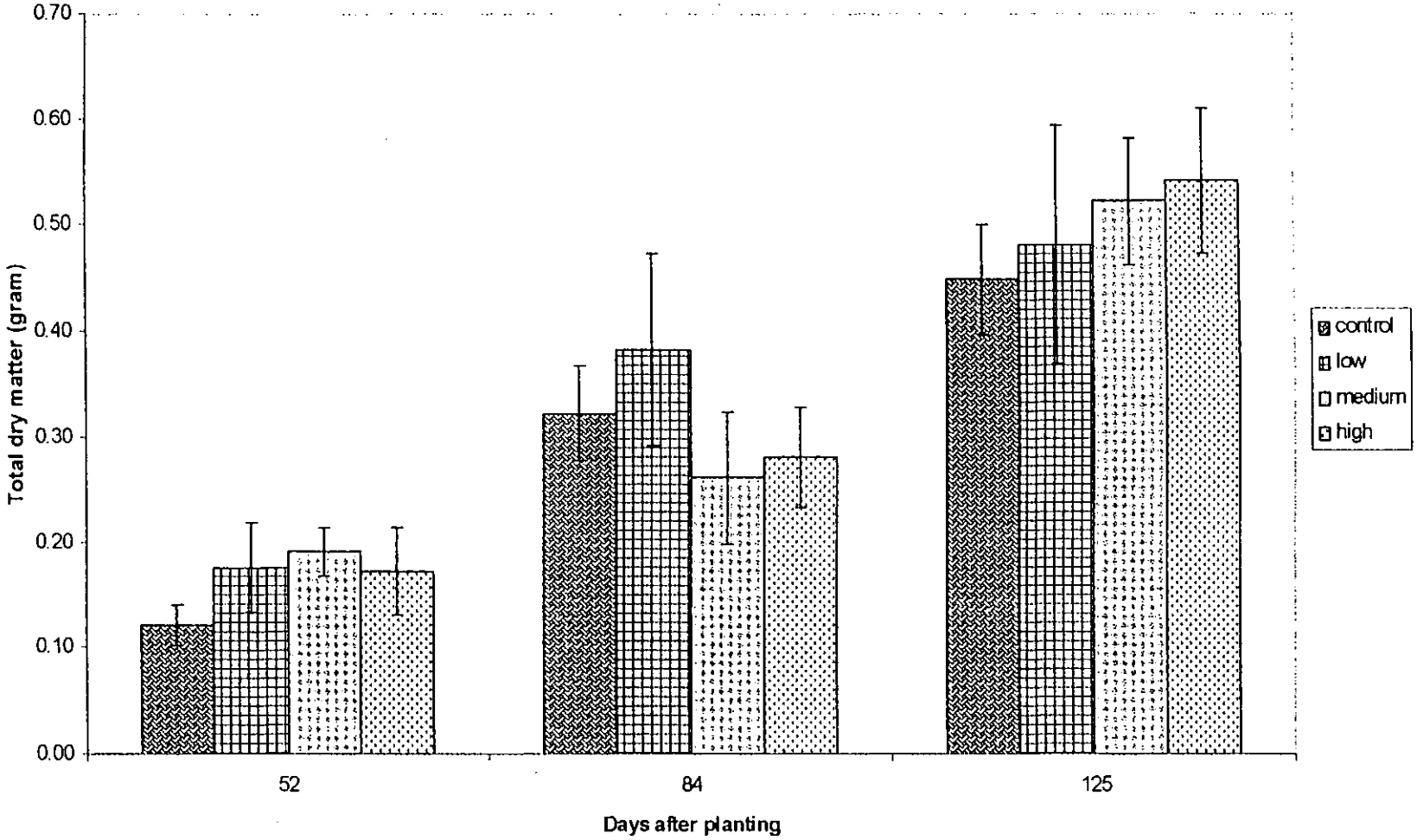


Fig. 2(h) Total dry matter (shoot plus root) of *M. ichangensis* to three different AM fungal inoculation levels



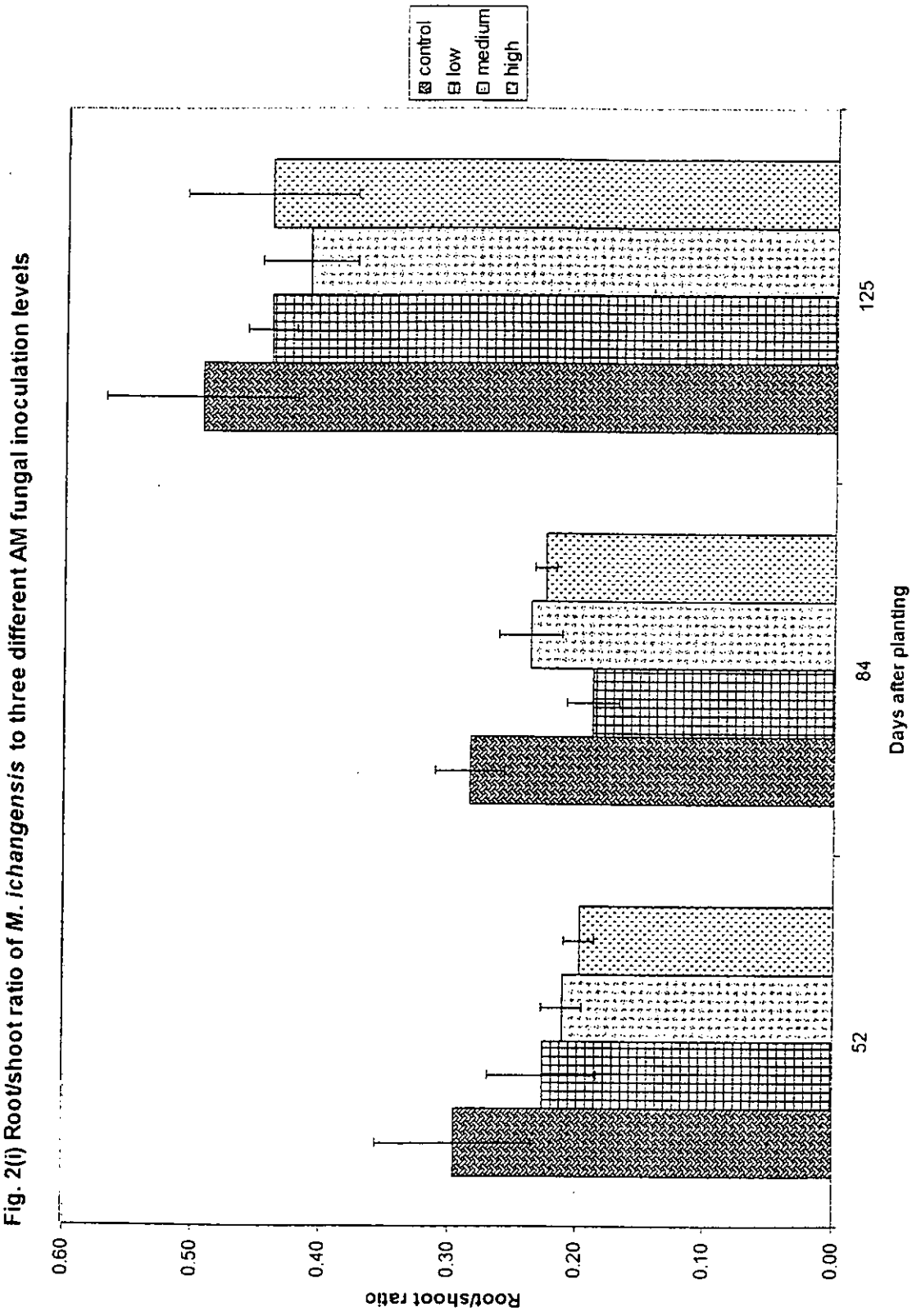
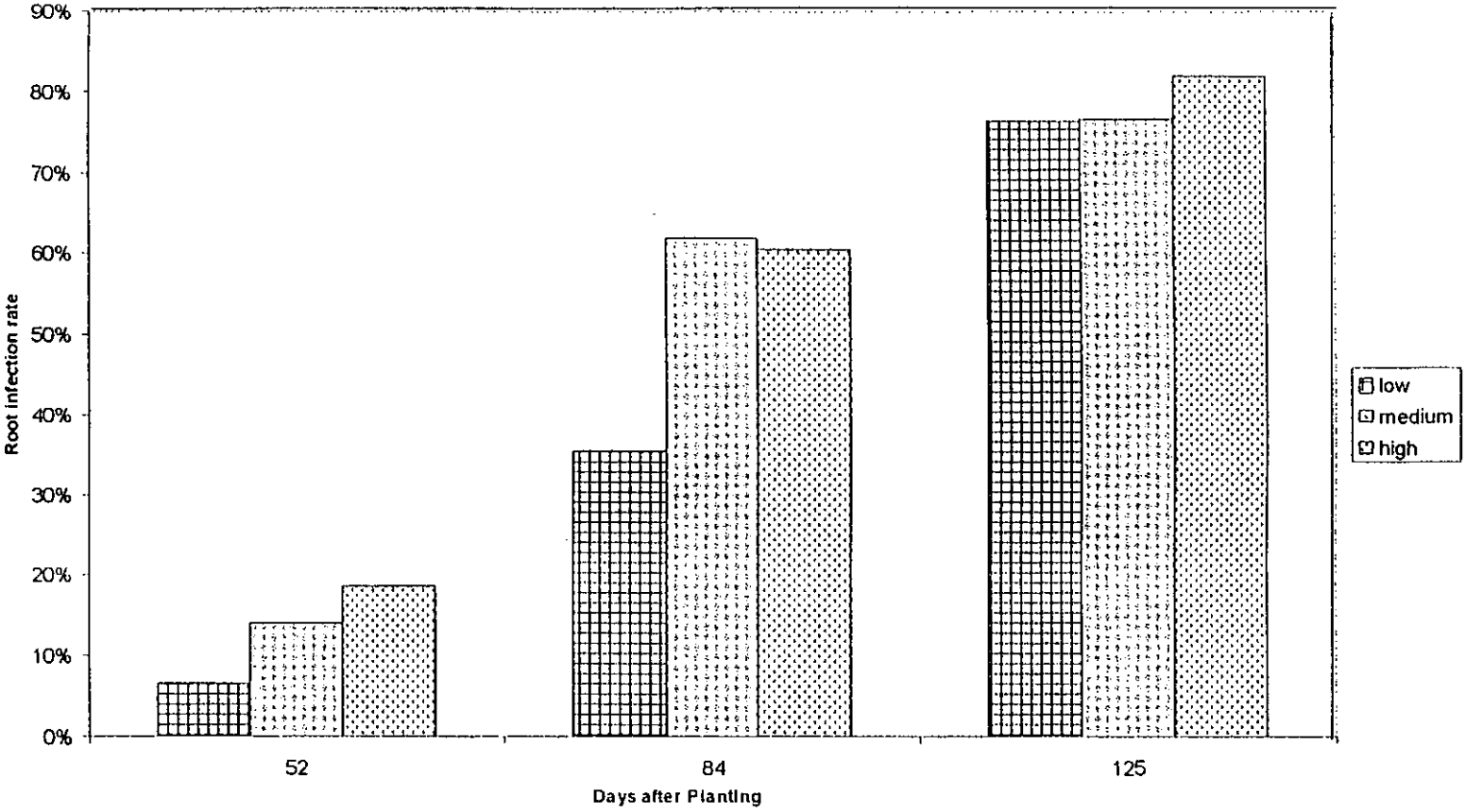


Fig. 2(i) Root/shoot ratio of *M. ichangensis* to three different AM fungal inoculation levels

Fig. 3(a-i) Growth responses of *M. oreophila* to three different AM fungal inoculation levels

Fig. 3(a) Root infection rate of inoculated *M. oreophila*



The infection rate was determined by evaluation of 100 root segments from 4 pooled subsamples in each treatment. Therefore no error bars are present.

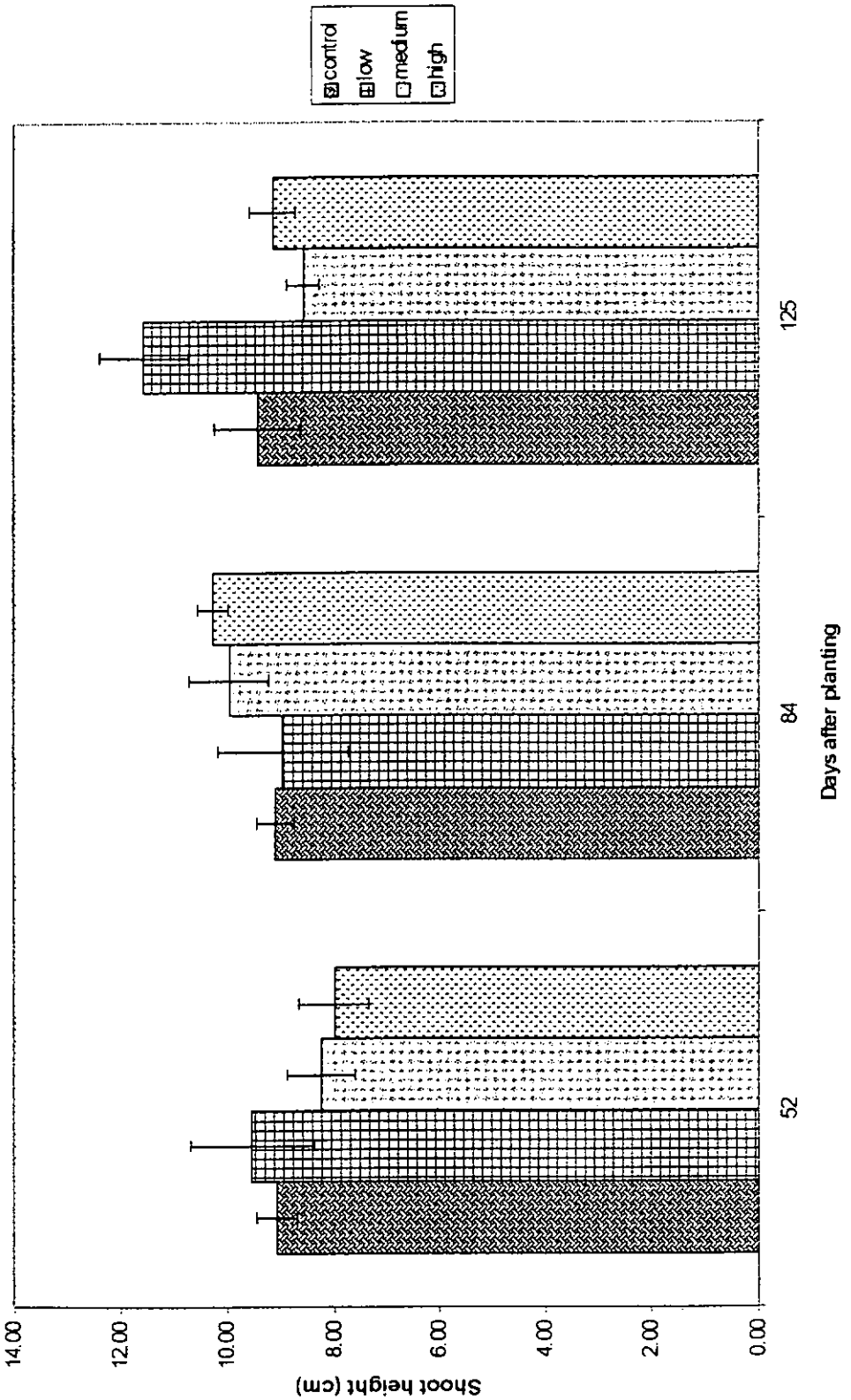


Fig. 3(b) Shoot height of *M. oreophila* to three different AM fungal inoculation levels

Fig. 3(c) Leaf area of *M. oreophila* to three different AM fungal inoculation levels

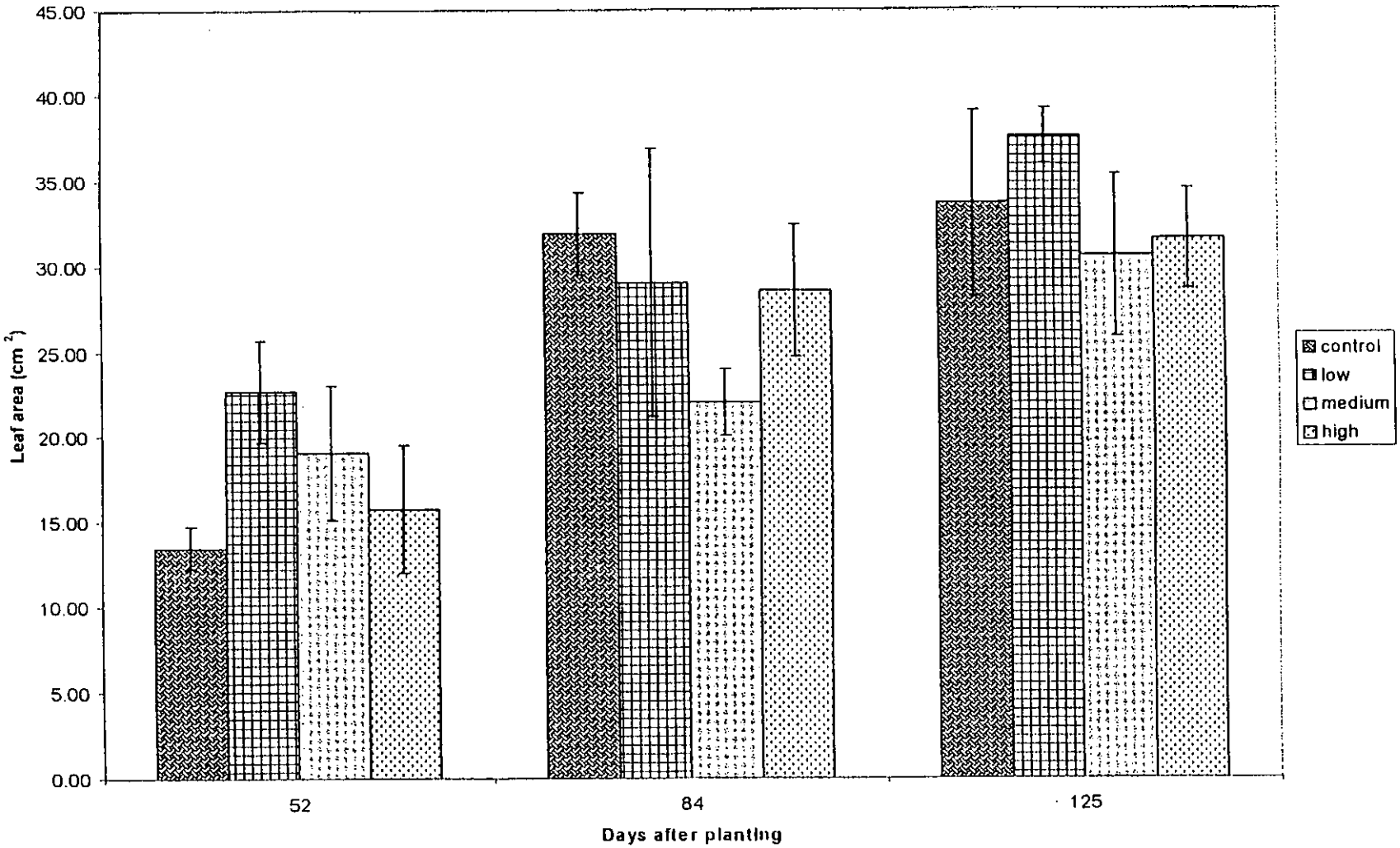


Fig. 3(d) Leaf dry matter of *M. oreophila* to three different AM inoculation levels

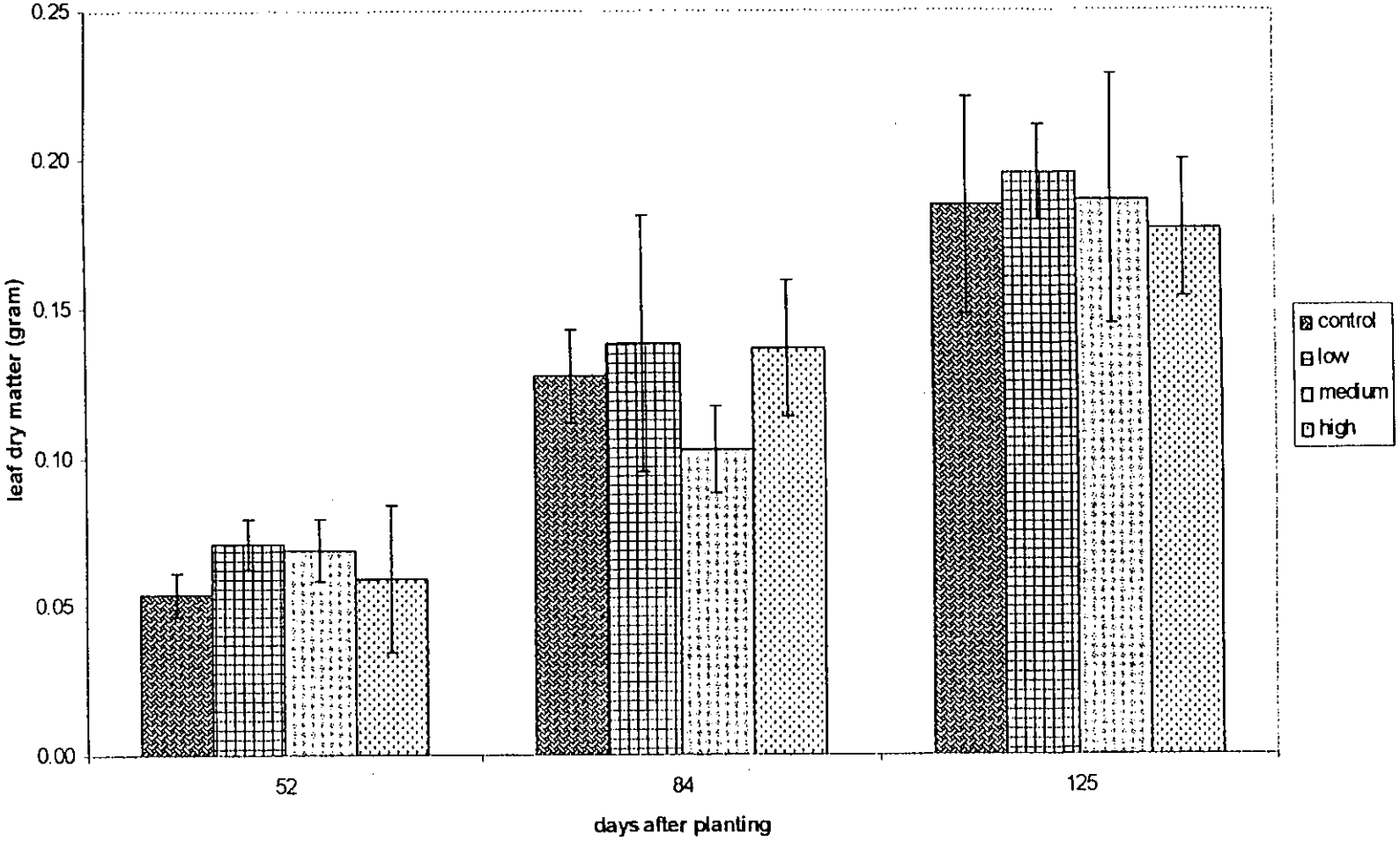


Fig. 3(e) Stem dry matter of *M. oreophila* to three different AM fungal inoculation levels

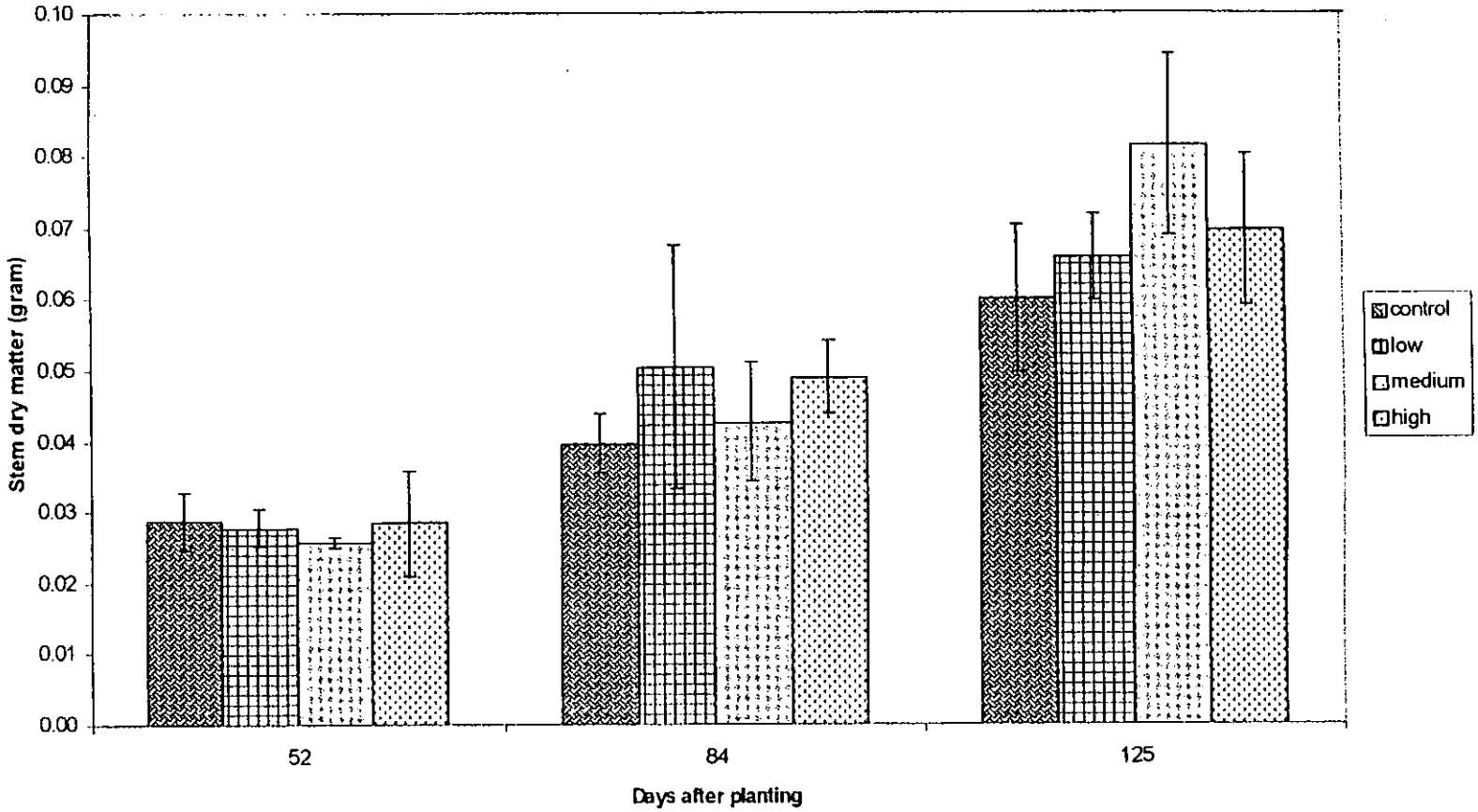
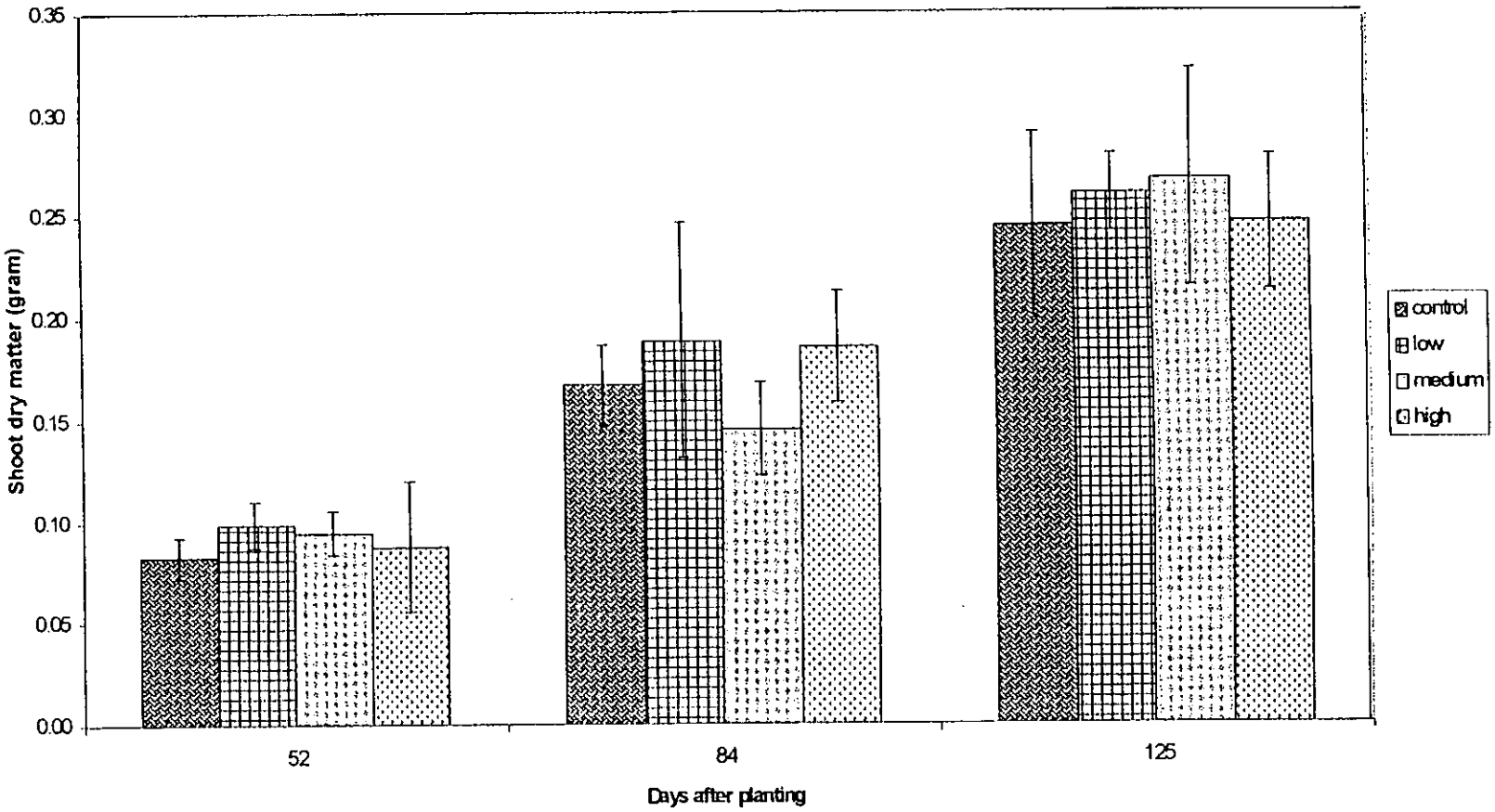


Fig. 3(f) Shoot dry matter of *M. oreophila* to three different AM fungal inoculation levels



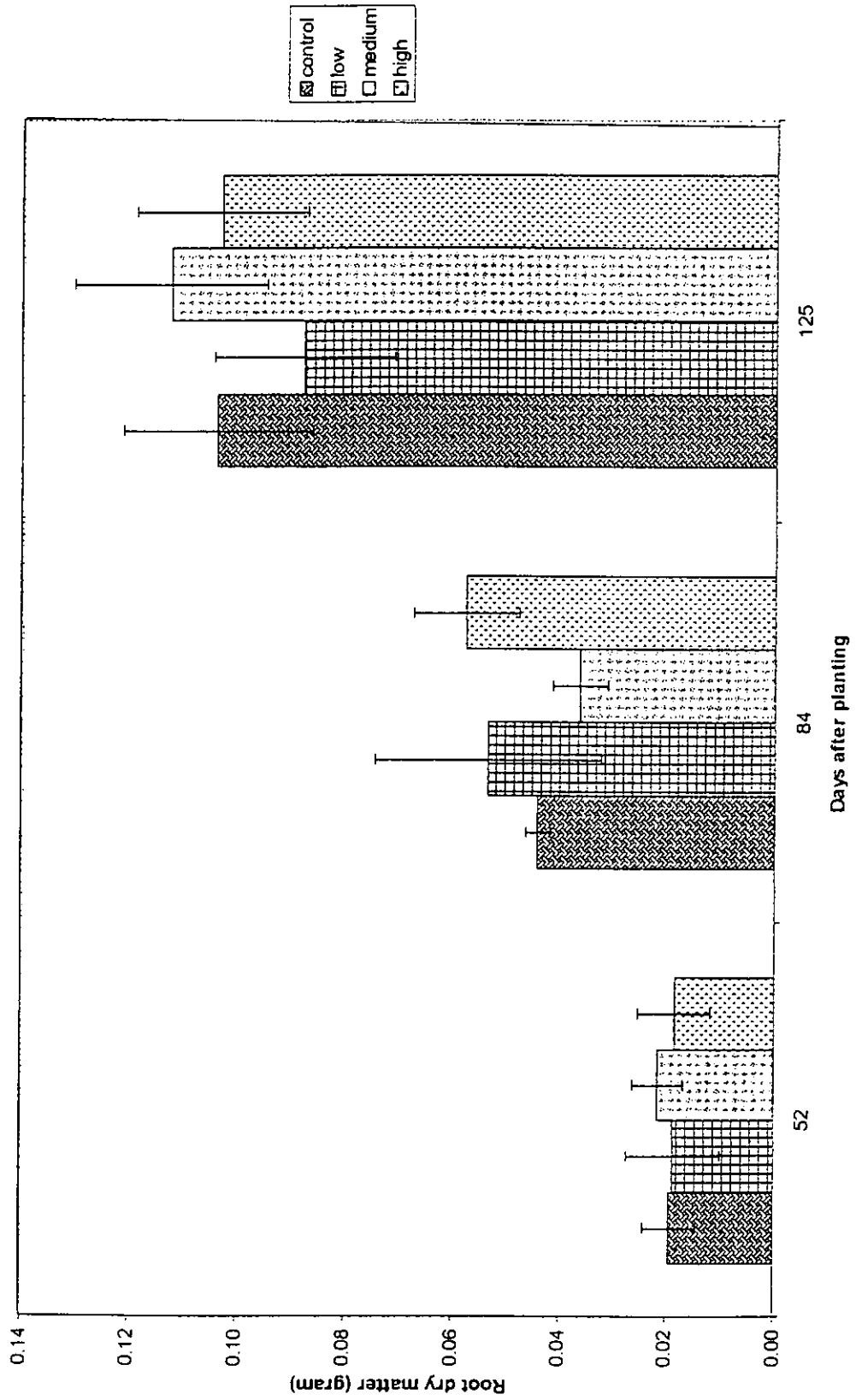
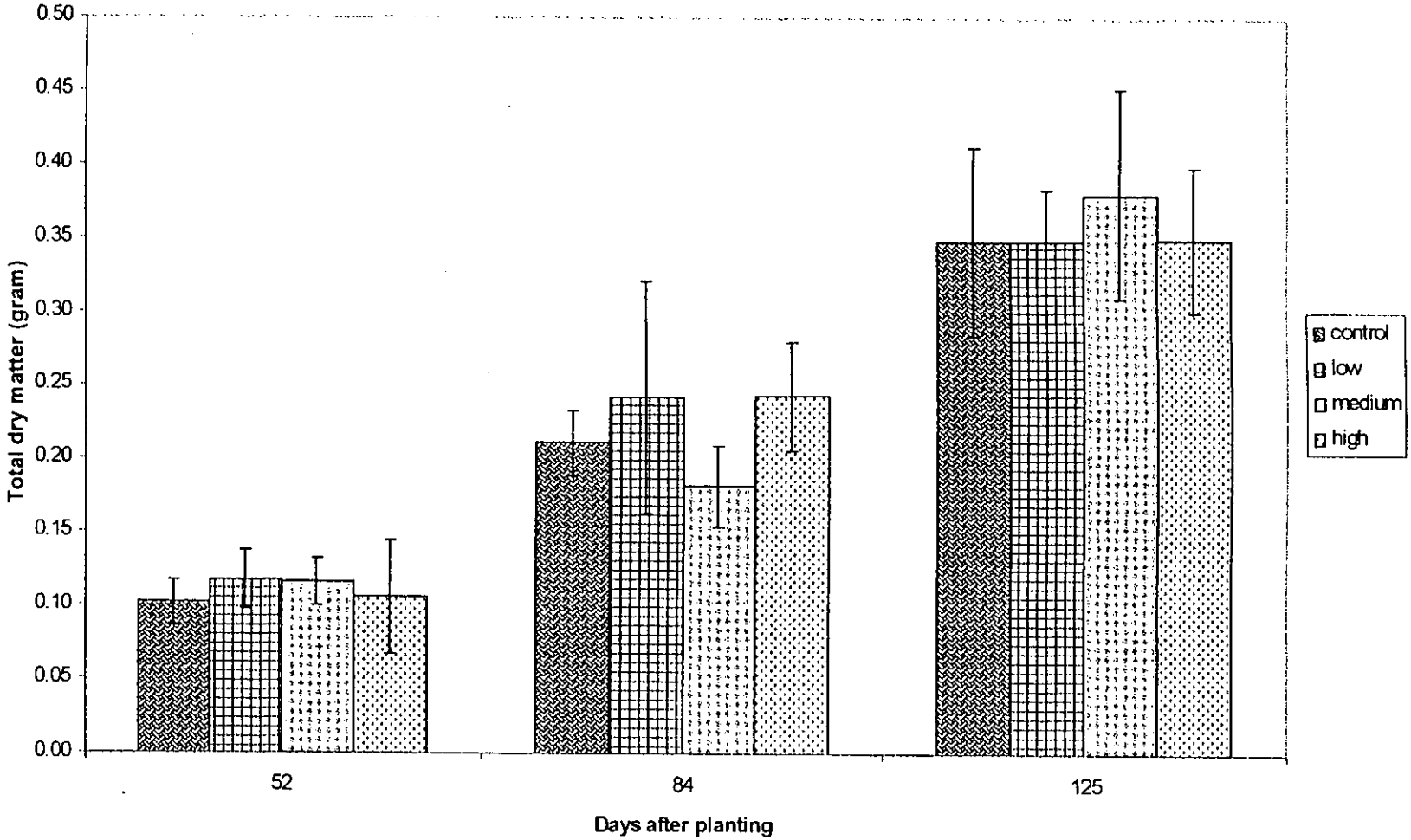


Fig. 3(g) Root dry matter of *M. oreophila* to three different AM inoculation levels

Fig. 3(h) Total dry matter (shoot plus root) of *M. oreophila* to three different AM fungal inoculation levels



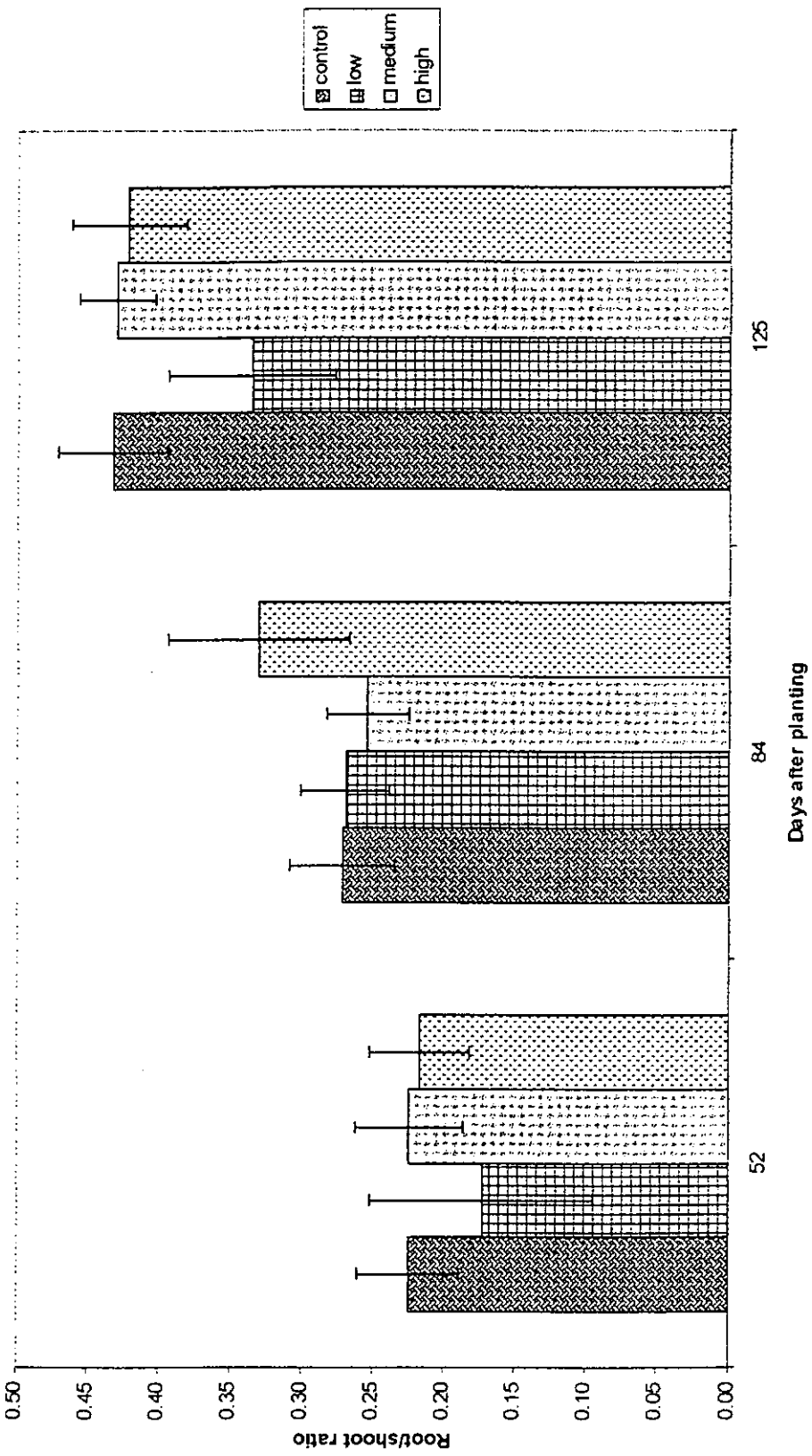
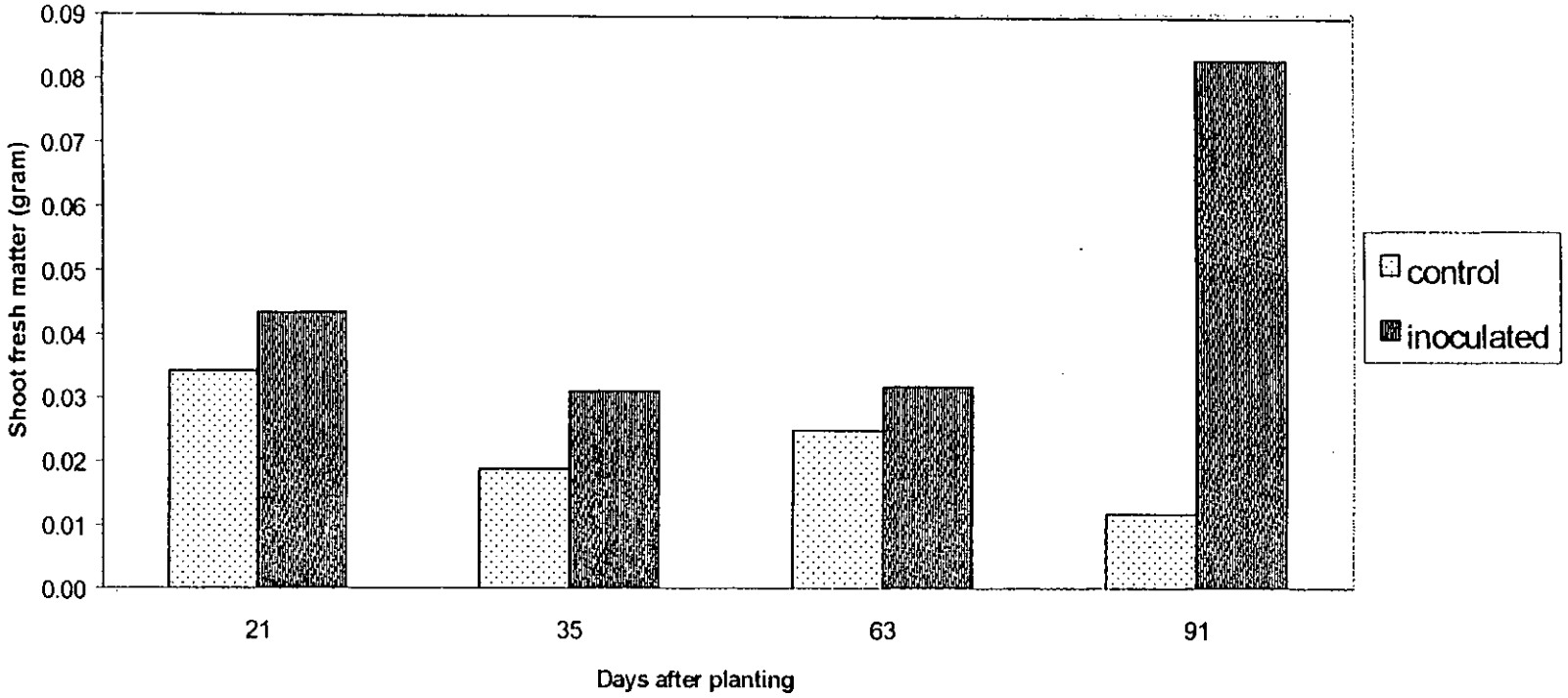


Fig. 3(i) Root/shoot ratio of *M. oreophila* to three different AM fungal inoculation levels

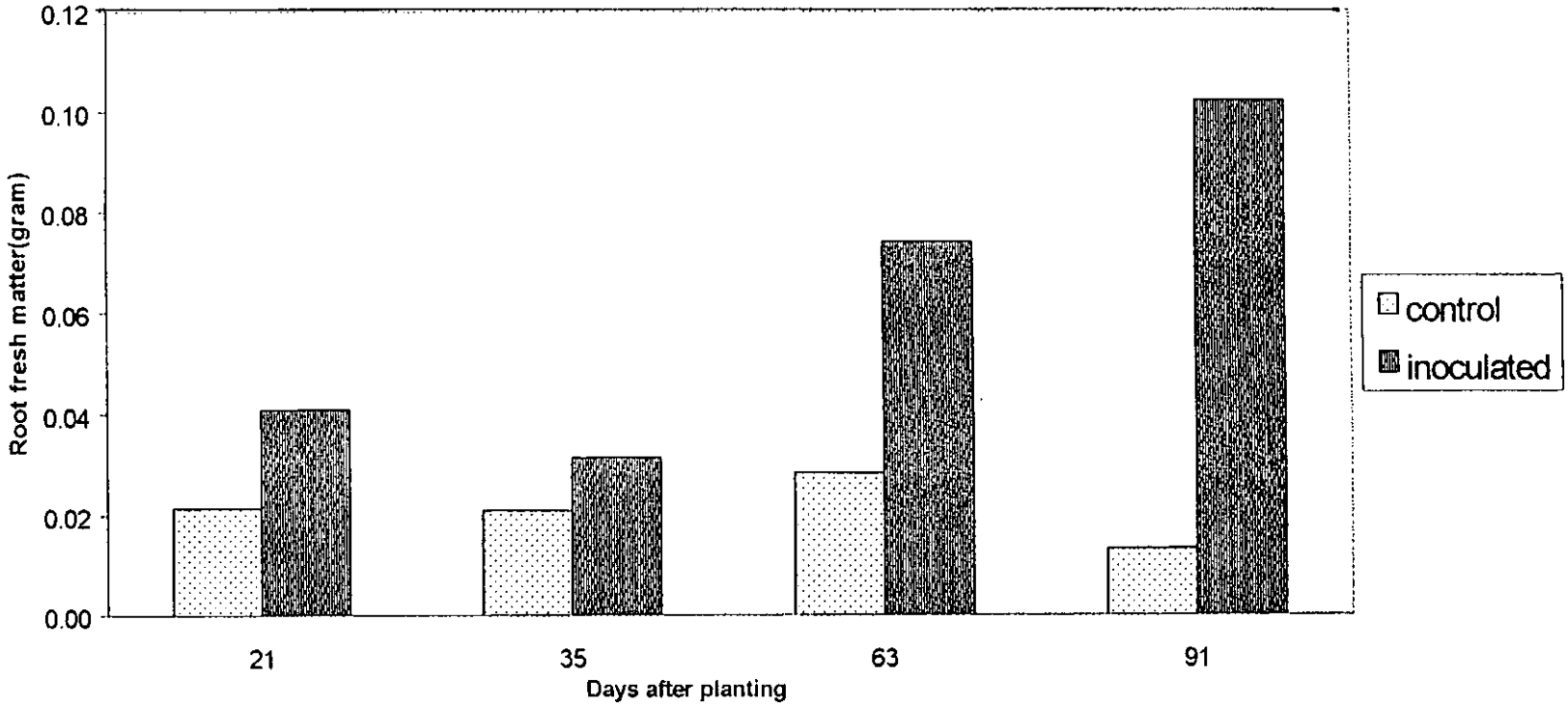
Fig. 4(a-c) Growth responses of *Astragalus sinicum* to AM fungal inoculation collected from the rhizosphere of *Machilus* species

Fig. 4(a) Shoot fresh matter of *A. sinicum* to AM fungal inoculation collected from the rhizosphere of *Machilus* species



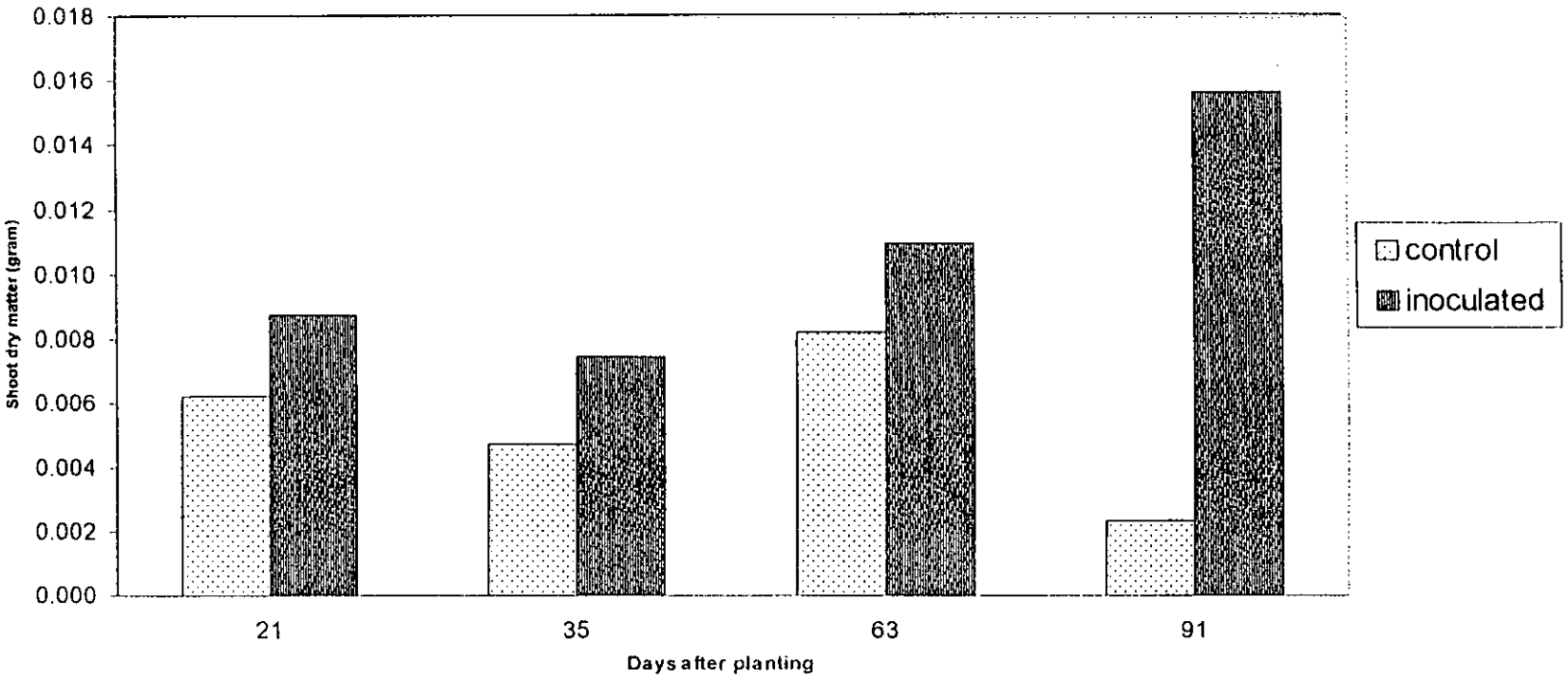
Due to the small size and light weight of the individual *A. sinicum* plants, 4 replicate plants were balanced together for their shoot fresh matter and the data represent means. Therefore no error bars are present.

Fig. 4(b) Root fresh matter



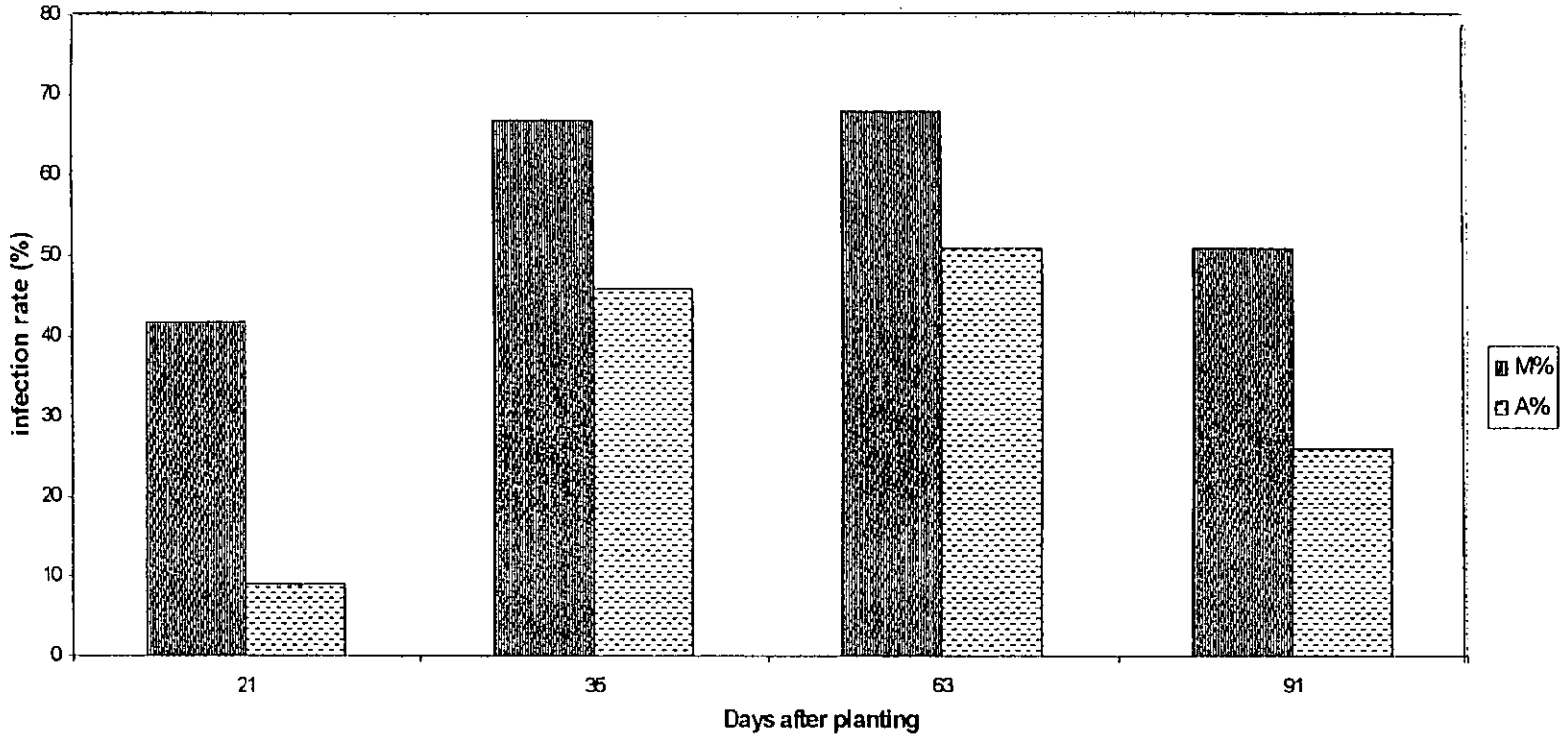
Due to the small size and light weight of the individual *A. sinicum* plants, 4 replicate plants were balanced together for their root fresh matter and the data represent the means. Therefore no error bars are present.

Fig. 4(c) Shoot dry matter of *A. sinicum* to AM fungal inoculation collected from the rhizosphere of *Machilus* species



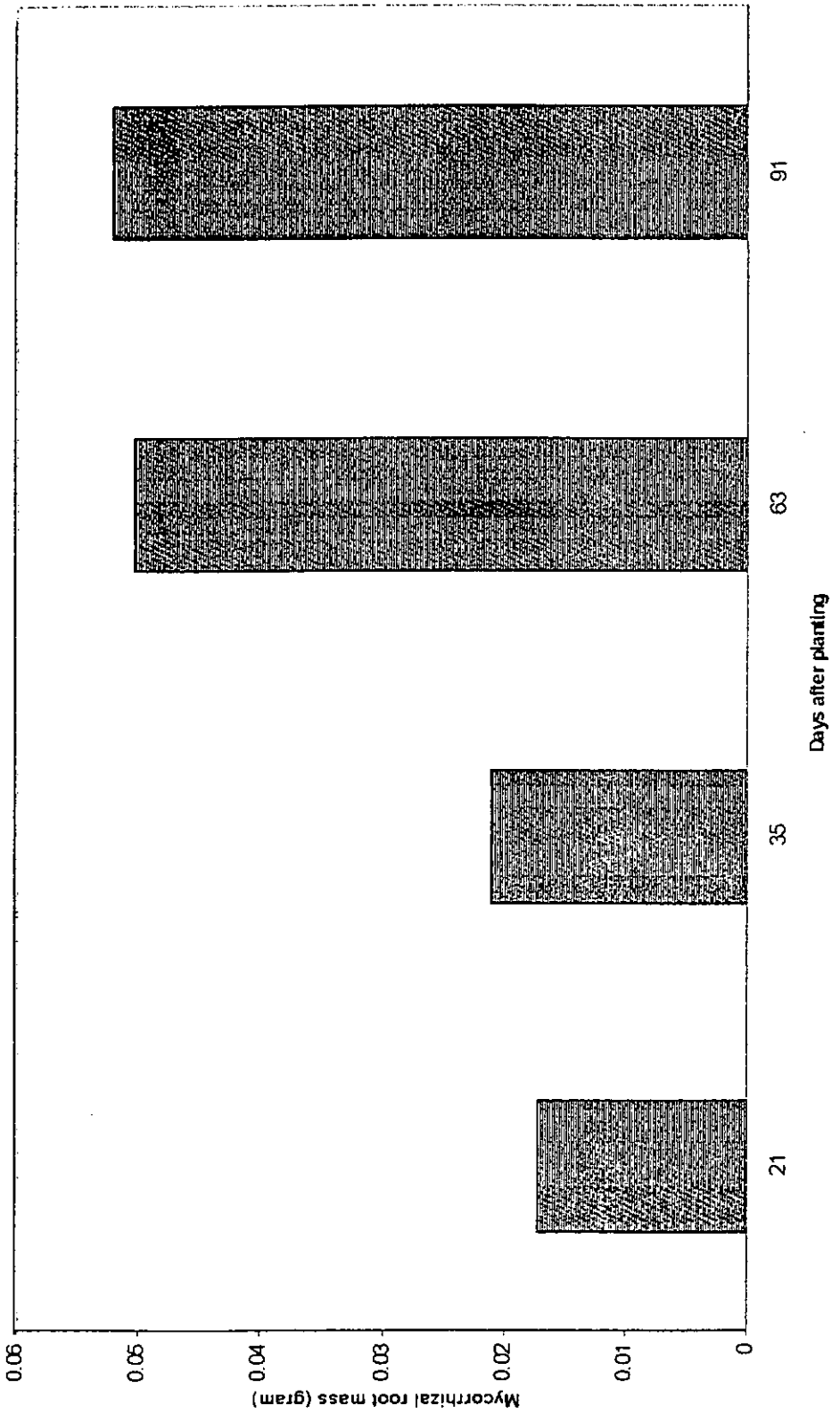
Due to the small size and light weight of the individual A. sinicum plants, 4 replicate plants were balanced for their shoot dry matter and the data represent the means. Therefore no error bars are present.

Fig 5. Mycorrhizal infection rate (M%) and arbuscule / vesicule infection rate (A%) in inoculated *Astragalus sinicum*



The infection rate was determined by evaluation of 100 root segments from 4 pooled subsamples in each harvest. Therefore no error bars are present.

Fig. 6 Development of *Astragalus siniaum* mycorrhizal root mass with inoculum from *Machilus* species



Plates 13(a-c) A few types of newly-formed AM fungal spores from trap cultures

Plate 13(a) *Glomus* spores; × 50

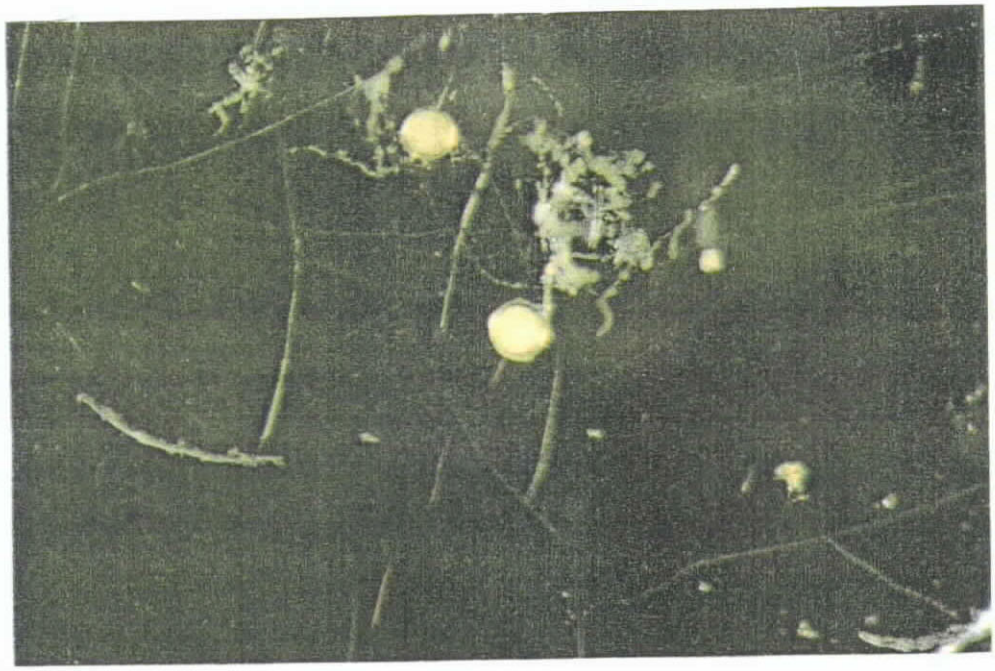


Plate 13(b) *Acaulospora* spores; × 50

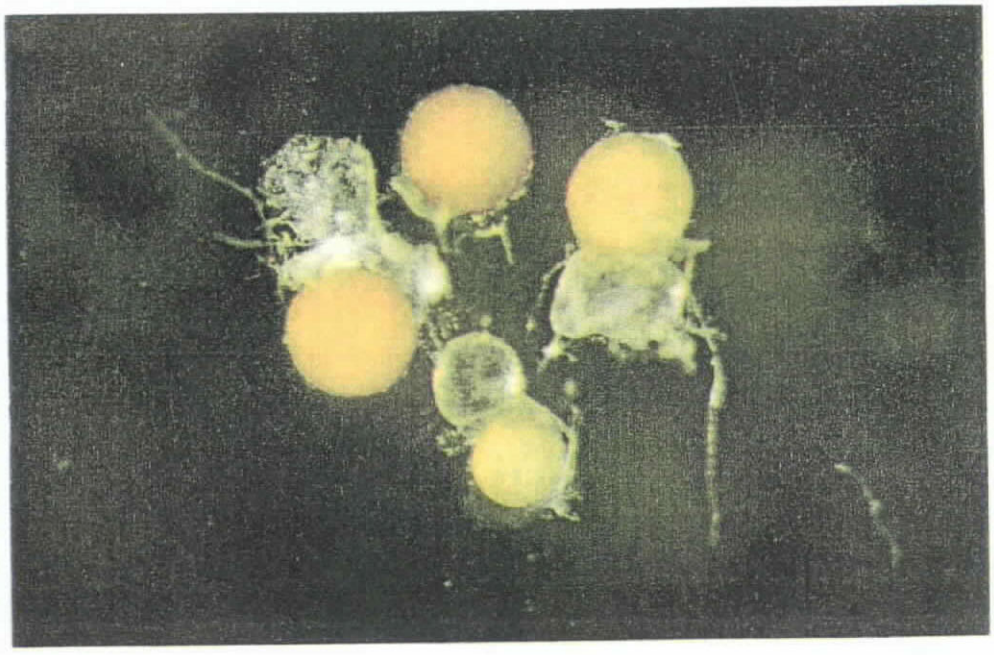
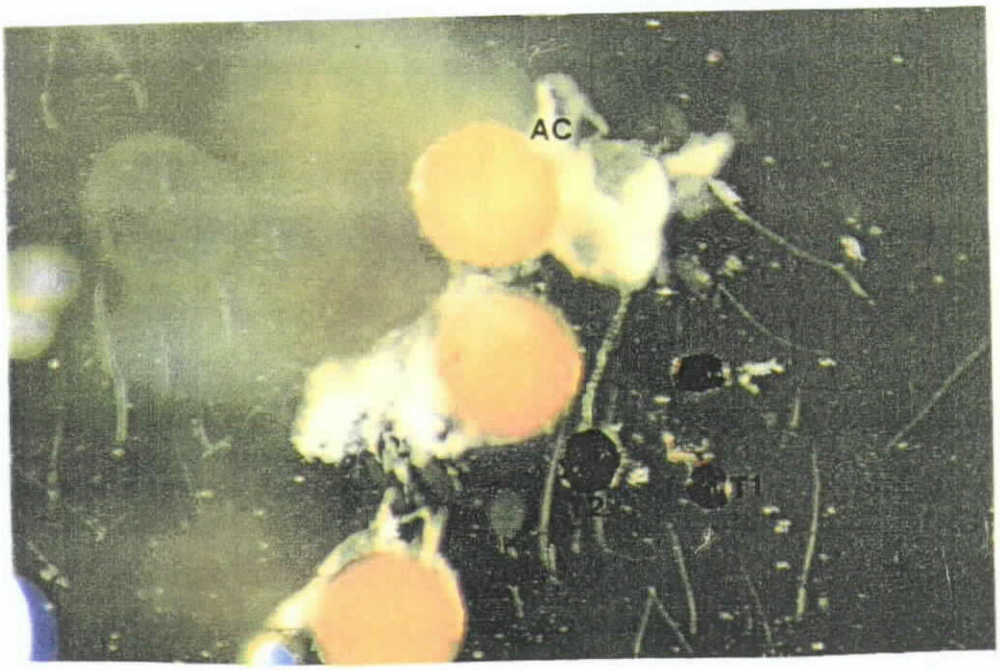


Plate 13(c) Type 1 (T1), Type 2 (T2), and *Acaulospora* (AC) spores; × 50



4.5 Discussion

In experiment 1, it was shown that the AM inoculum collected from the rhizosphere of *Machilus* species in the field were effective in infecting both *M. ichangensis* and *M. oreophila* in this study. Although the abundance of AM fungi within roots steadily increased with development of the root systems by time in both *Machilus* species, the AM inoculum showed different potential in colonising the roots of different *Machilus* species. In *M. ichangensis*, there were clear differences of the AM infection rate resulted from the different levels of inoculum (**Fig. 2a**). But the differences of those in *M. oreophila* (**Fig. 3a**) were not as obvious as in *M. ichangensis*.

There was also a trend in most cases that the growth responses of the inoculated plants were greater than that of the control plants. The inoculated plants always have better developments of shoots and roots although there was no clear relationships among different inoculum levels. Average increases in the growth of the two *Machilus* species resulted from inoculation showed that the formation of AM had positive growth effect on both *Machilus* species. However, the effects on *M. ichangensis* were much greater than that on *M. oreophila*. The inoculated *M. ichangensis* had average increases in plant total dry matter to 14.7% after 125 days planting, the *M. oreophila* had only 3.2%.

In a preliminary experiment, only the sandy soil was used as the pot medium and the plants failed to survive, probably due to the limited nutrient present (pot nutrient deficiency). Therefore a mixture of sandy soil and horse manure (6:1v/v) which was used to support *Machilus* in the local nurseries was chosen instead for our experiments described here. It was possible that this mixture of nutrients may be rich enough to exceed the nutritional requirement of the plants. If that was the case, this might explain the consistently better growth response with respect to all the parameters, except one, in low-level inoculated plants

compared with those in control plants. The low-level inoculation treatment contained more "nutrient-rich" soil mixture to compensate for the smaller volume of inoculum (**Table 8**). This may in turn support the plant growth best. The nutrient requirement of the *Machilus* and the soil nutrient analysis has not been performed, an omission that we hope to rectify soon.

Another drawback of this experiment was that most data of plant growth parameters were not significantly different at P=0.05 in the statistical analysis (**Table 11**). Owing to space limitation, only 3-5 replicates in each treatment group and no more plants could be grown in the growth chamber. In addition, the supply of seeds was not enough for setting up more uniform replicates. These may lead to large variance among the samples.

In conclusion, the present observation of experiment 1 showed that (1) the AM inoculum collected from the rhizosphere of *Machilus* species in the field were effective in infecting both *M. ichangensis* and *M. oreophila*, (2) the formation of AM had positive growth effect on the two *Machilus* species compared with the control plants, and (3) the AM inoculum had different effects on the two *Machilus* species in terms of both AM infection rate and plant growth responses.

In experiment 2, a nutrient solution was applied weekly to the supporting sandy soil when the nutrient deficiency symptom was observed in the growing plants 35 days after planting. That was why the growth of inoculated *A. sinicum* decreased at 21-35 days and increased thereafter. But the control plants had difficulty to survive though (**Fig. 4a-c**). The decrease of mycorrhizal infection rate from 63 days after planting (**Fig. 5**) was not consistent with the successive increase of mycorrhizal root mass (**Fig. 6**). This may be due to that the rate of root growth of the plant after 63 days growing was faster than the rate of AM fungal infection, but not on account of a decrease of mycorrhizal infection. The mycorrhizal infection includes arbuscule and vesicle infection as well as infection of other AM structures.

Therefore it was obvious that the mycorrhizal infection rate (M%) and frequency of arbuscule and vesicle (A%) had a similar development pattern and the value of A% was less than that of M% (**Fig. 5**). The present observation of experiment 2 showed that (1) the formation of AM significantly enhanced shoot and root growth of the inoculated *A. sinicum* compared with the control plants, the inoculated plants had average increases in plant shoot dry mass to 680% 63 days after planting (**Fig. 4c**), and (2) the inoculum collected from the rhizosphere of *Machilus* species had high effectiveness in infecting the *A. sinicum* plants, roots of *A. sinicum* plants were intensely colonized (42% of the root cortex) by the AM fungus 21 days after inoculation and the infection rate (M%) has reached 68% 63 days after planting.

In the first two experiments it was found that growth responses resulting from mycorrhizal formation was much greater in *A. sinicum* than in *Machilus*. The control *A. sinicum* had difficulty surviving even when a nutrient solution was supplied weekly, but the control *Machilus* grow very well in horse manure soil. *A. sinicum* is herbaceous plant and *Machilus* is woody plant. Because of the time limitation of this study, the *Machilus* could not be grown for any longer. The great and long-term mycorrhizal effect on *Machilus* growth may not be observable in a few months due to its long life spans. There were also obvious different growth effects between the two *Machilus* species resulted from the inoculation. The inoculation had different effects on the three host species suggested that the different host species had different trait for mycorrhizal effects in terms of enhancement of host growth.

A. sinicum is a good host plant for mycorrhizal researchers to study mycorrhizal physiology and make AM fungal cultures, owing to its easy survival, fast growing and effective AM infection (in experiment 2, the roots were found infected in just 21 days after inoculation). In experiment 3, therefore, *A. sinicum* was used as host plants in the trap culture for production of AMF spores. In experiment 3, all plants grown in P_0 fertilizer can not survive, suggesting

that phosphorus was very important for plant survival. This cannot be rescued even in the presence of mycorrhizal inoculation. Low phosphorus level (P_L treatment) led to high mycorrhizal infection. The hypha improved nutrient absorption of plants and resulted in better growth of the host plants. This observation was consistent with other literatures (Smith, 1980). The plants failed to survive may due to that the hosts happened not to be infected by the AMF. Without the supplementary nutrient supply by the AM fungi, the host plants thus in a poor nutrient condition (especially in phosphorus deficiency) and died at last. The phosphorus concentration in P_M treatment was the best for the development of both mycorrhizal plants and mycorrhizal fungi. All the plants survived well and have high mycorrhizal infection. The phosphorus concentration in the P_H treatment was good for the growth of host plants but not for mycorrhizal development. Such a high level of phosphorus may inhibit mycorrhizal formation, leading to a low mycorrhizal infection in the first two transplantations (infection rate < 30%). The high infection rate observed in P_H treatment in the last transplantation may be due to that at the last transplantation time there were more chance for the roots to be infected by more types of AMF of which some may stand the high phosphorus.

There were four types of spores found in the trap cultures transplanted at different time point after AM fungal inoculation. Four types of spores were yielded from the last transplantation, whereas only one type was observed in each of the previous transplantation. This may suggest that the six types fungi found in the field were different in their infection ability, spore production capacity or they were in different life stages e.g. some were in dormancy but others were not. The *Glomus* species found in the first transplantation pots may have the highest infection capacity, the *Acaulospora* species the next and the other two (Type 1 and Type 2) the least. However, care should be taken to draw this conclusion when the physiology experiments have yet to be done.

Of the four types of spores found in the cultures, two (Type 1 and Type 2) of which were the same as those from the field by gross morphological observation. The other two were different but belonged to the genus *Glomus* and *Acaulospora* which were included in the field-collected spore types. Were these two morphological different spore types the same as those found in the field except that they were in different stages of development or intact condition? Or were they really different spores? If it was the latter, there must exist some other forms of propagules of other types of AM fungi in the field soil, e.g. infective hypha, vesicles and roots but not spores. Molecular analysis may be used to differentiate between these two possibilities in the future.

It is thought that if the spores were not in dormant state they should all have a chance to infect the host plants after 13 weeks inoculation in these experiments. There were six types of spores found in the field soil but only four types found in the trap cultures. A possible cause was that some spores were in their dormant state. Another explanation was that different types of spores had different affinity to host plants (relatively host specificity) and there might be competitions for root colonization existing among the six types of AM fungi found in this study. There was also a speculation on that the types of spores found in the soil might be poorly related to the infective propagules in the soil. For those reasons further estimating the total number of infective propagules may give a more useful assessment of AM abundance.

This was not a conclusive experiment for studying the physiology of AM fungi although trap cultures were successfully performed from which some basic information of the physiology aspect of the AMF were obtained. Further experimentation will be necessary to confirm or test the assumptions in this study.

CHAPTER 5 GENERAL DISCUSSION AND CONCLUSION

Wide diversity exists within AM fungi in terrestrial ecosystems. Conservation and efficient utilization of their biodiversity are of crucial importance for sustainable plant production systems. However, there is relatively little knowledge concerning the morphological, physiological and genetic variability in AM fungi at different organizational levels (intra- and interspecies, population, ecosystem...etc.). This work examined the general biodiversity of AM fungi associated with three *Machilus* species in Hong Kong that has made a step forward to this end. Although there are important findings in this study, there are also many questions arising from the results. Such uncertainties and assumptions need further experimental confirmation.

Due to time limitations of this study, only Tai Po Kau Nature Reserve was selected as the studied site. The phosphorus concentration in the soil was very low, which was thought to give rise to the high AM infection rate (almost 100%) of *Machilus* species investigated. However, more sites should be investigated and in the future to confirm such finding. In this study, six morphological types of AM fungal spores were found in the rhizosphere of all the three *Machilus* species investigated. Five of these types were described for the first time in Hong Kong except Type 5. Soil samples were taken only from the *Machilus* species. No other plants or spots in the site were studied. Since all the six spore types were found in the rhizospheres of each *Machilus* species, they were thought to be suitable not only for the *Machilus* species, but also for the microecosystem studied. Therefore, more samples should be taken in the future to demonstrate the field variation and to identify the AM fungi specific to the *Machilus* species. Morphology of all the six spore types was recorded and some newly formed spore types were successfully obtained from trap cultures. This will form the basis

for the future work of strain selection, monospecific culture and exact identification of the AM fungi studied. In addition, the finding that Type 2 spores were the most dominant in the soil may indicate that they play the most important role in this microecosystem. Special attention should be paid to Type 2 spores in future studies. The extremely high spore density in the soil samples was in sharp contrast with the relatively few (only six) morphological species found. In another words, compared with the high spore population density, the species richness was relatively low. However, these results were obtained by observing only the spores in the soil. There may be other forms of propagules, besides spores, existing in the soil. Estimating the number of infective propagules may give more useful assessment of species richness.

Preliminary results of the pot culture experiments showed that the AM inoculum from *Machilus* species has positive effects, despite to different extents, on all three types of host plants tested. These results demonstrated a rough profile of the AM inoculation effect on host plants. Four types of newly formed spores were successfully obtained from trap cultures. However, spores from pot cultures had different morphology from field-collected spores. In addition, different types of field-collected spores showed different ability in infecting hosts and different spore production capacity in the pot cultures. Future studies should be focused on to testing the physiology of the individual type of spores. This will be useful for selecting the best strains that benefit the specific host plant most. Moreover, morphological and genetic techniques can be used to compare the differences between spores from the field and from the cultures. DNA probes can be developed for detecting the studied fungi in both natural sites and plant roots.

There are deficiencies in the experimental design of this project. The seeds used in pot cultures were not uniform, the replication of the seedlings was not adequate, and the growth

period was not long enough. These affect the statistical significance of the data obtained. A soil nutrient analysis should be carried out for the potting mixture in section 4.3.1 before the experiment begins. This is an omission that should be rectified in later research. Nevertheless, our findings delineate the different aspects of AM fungal diversity and are important for the future study of biodiversity of AM fungi in Hong Kong, their associations with host plants, and their role in the development and conservation of valuable and important terrestrial ecosystems.

In summary, the field survey of Tai Po Kau Nature Reserve helped to understand the basic mycorrhizal conditions of *Machilus* in this area. The soil characteristics, mycorrhizal infection rate of roots, spore density in the rhizosphere soil were determined. Soil in the studied site was found to be acidic, deficient in phosphorus, and contained moderate amount of organic matter. The spore density in the rhizospheric soil (500-800 spores/g soil) and the mycorrhizal infection of *Machilus* (infection rate almost 100%) were rather high. The high spore density was in sharp contrast with the relatively few (only six) morphological species found. This field survey provided the foundation for studying the fungal-plant relationships in the local forest.

The second part of this work dealt with the morphological characterization and identification of the spores found in the field. Six types of AM fungal spores (spore Type 1 – 6) have been isolated from the rhizosphere soil of three *Machilus* species in the forest. The morphological characteristics of each type were described and recorded. Three of these types (spore Type 1, 3 and 4) belong to the genus *Glomus* and one (spore Type 5) belongs to the genus *Sclerocystis*. Two types (spore Type 2 and 6) remain unidentified but could possibly be *Acaulospora* species. It was notable that the distribution of the spore abundance was not even in the six spore types within the soil. Type 2 spore accounted for more than half of the

total spores whereas Type 3 and 6 were of the lowest abundance. Whether Type 2 spores play the most important role in this microecosystem and how were they selected to adapt to this very soil condition merit further study.

In the last part of this work, the indigenous AM fungi associated with *Machilus* species collected from the field were used as inoculum to assess their impact on the plant growth responses. Different growth parameters in *Astrugalus sinicum*, *Machilus ichangensis* and *Machilus oreophila*, treated with various levels of fungal inoculum, were monitored. In general, the inoculation has a positive effect, despite to different extents, on all the three types of plants. Although the inoculation effects on the *Astrugalus Sinicum* were much greater than that on the *Machilus*, the different effects resulted from inoculation between the two *Machilus* species were obvious. Trap cultures of the AM fungi were set up and four types of spores were successfully produced. The effects of different phosphorus levels on AM fungal infection and spore production were also roughly assessed. It was found that the effect of medium level phosphorus applied were the most effective for both the survival of the hosts and the production of AM fungal spores in this trap culture. Of the four types of spores found in the cultures, two of which (Type 1 and Type 2) was the same as those from the field by gross morphological observation. The other two were different and belonged to the genus *Glomus* and *Acaulospora*. They were included in the field-collected spore types. In the future, molecular analysis may be used to differentiate the differences between spores from the field and from pot cultures.

This work provided the fundamental information of the morphological, biological and physiological aspects of diversity in the AM fungi associated with three *Machilus* species in Hong Kong. However, many questions arise from the results and there is still a lot more to be done. First, more *Machilus* communities in Hong Kong should be investigated. Second,

morphological characterization should be done more carefully by using spores from pot culture and the spore development should also be observed. Third, the difference between spores in the field and in the cultures should be detected using molecular tools for further ecological studies. This will fill the gaps in current knowledge of the genetic diversity in AM fungi. Finally, more thorough study of the biological and physiological diversity in AM fungi should be investigated by using spores from the monospecific pot cultures.

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