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VESICULAR ARBUSCULAR MYCORRHIZAL FUNGI ASSOCIATED WITH AZADIRACHTA EXCELSA (JACK) JACOBS

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VESICULAR ARBUSCULAR MYCORRHIZAL FUNGI ASSOCIATED WITH *AZADIRACHTA EXCELSA* (JACK) JACOBS

By

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ABSTRACT

Mycorrhiza is known for its growth enhancement effect on plants. They are also important in soil ecology, such as protection of host plant against pathogens, soil stabilization and many more. This study was conducted to investigate the diversity of vesicular arbuscular mycorrhiza (VAM) in association with *Azadirachta excelsa* in two different habitats (natural stand and planted trees). Wet-sieving and decanting method was used and identification was done based on the characteristics of spores. The natural stand exhibited a higher value for species richness and Shannon-Wiener index, at 11 and 1.8607 respectively. While for planted trees, the values were 9 and 0.9537, respectively. The natural stand also showed a higher value for evenness, which was 0.776 compared to that of the planted trees at 0.434. However, the planted trees outnumbered the natural stand in spore density per 100g of soil at 3503 spores while the natural stand was only 2221 spores. Both study sites had moderate degree of similarity. Twelve species were identified with three species each from *Acaulospora, Gigaspora, Glomus* and *Scutellospora.* Among the species, *Glomus* sp. 1 was the most abundant in species found in both study sites. Results of the study showed that *A. excelsa* grown in the natural forest exhibited a more diverse biodiversity compared to planted trees.

ABSTRAK

Mikoriza dikenali kerana kesannya kepada pertumbuhan tumbuhan. Di samping itu, mikoriza juga memainkan peranan yang penting dalam ekologi tanah. Sebagai contoh, melindungi tumbuhan perumah daripada serangan pathogen, menstabilkan tanah dan lain-lain. Penyelidikan ini dijalankan untuk mengkaji kepelbagaian mikoriza vesikular arbusckular yang bersimbiosis dengan *Azadirachta excelsa* (Jack) Jacobs di dua habitat yang berlainan (dirian semulajadi dan pokok yang ditanam). Kaedah pengayakan basah dan penuangan digunakan dan pengecaman spesies adalah berdasarkan ciri-ciri spora. Dirian semulajadi menunjukkan nilai kekayaan kepelbagaian dan indeks kepelbagaian Shannon- Wiener yang tinggi iaitu masing-maisng 11 dan 1.8607. Untuk pokok yang ditanam, nilai yang diperolehi ialah 9 dan 0.9537. Dirian semulajadi juga mempunyai taburan spora yang lebih seragam berbanding dengan pokok yang ditanam. Walaubagaimanapun, kepadatan spora dalam 100g tanah untuk pokok yang ditanam menunjukkan nilai yang lebih tingi (3503) berbanding dengan pendirian semulajadi (2221). Indeks kesamaan di antara dua tempat kajian adalah sederhana tinggi. Dua belas spesies dikenalpasti di mana masing-masing tiga spesies dari *Acaulospora*, *Gigaspora*, *Glomus* dan *Scutellospora*. *Glomus* sp. 1 merupakan spesies yang paling banyak dijumpai di kedua-dua tempat kajian. Keputusan kajian tersebut menunjukkan *A. excelsa* yang tumbuh di habitat semulajadi mempunyai kepelbagaian yang lebih tinggi berbanding dengan pokok yang ditanam.

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I certify that this research project report entitled "Vesicular arbuscular mycorrhizal fungi associated with *Azadirachta excelsa* (Jack) Jacobs" has been examined and approved as a partial fulfillment of the requirement for the degree of Bachelor of Bioindustry Science in the faculty of Agriculture and Food Science, Universiti Putra Malaysia Bintulu Campus.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

The net forest loss from 1990 to 2000 was 8.9 million hectares per year (FAO, 2005). However, from 2000 to 2005, the net forest loss decreased to 7.3 million hectares per year. At the same time, the global demand for wood and wood products is projected to increase by approximately 50%, from 3.4 billion m^3 per annum in 1991 to 5.1 billion m^3 per annum by 2010, and this does not take into account the emerging markets of Asia (Anonymous, 1996).

In Malaysia, the projected annual demand for round wood by the year 2010 will be 26 million m^3 (Abdul Razak, 1997). Therefore, to meet the impending shortage of raw materials for the wood processing industry and to supply the rapidly expanding pulp and paper industry, plantation of indigenous forest trees and fast-growing exotics are being established (Lee, 1998).

Species selection is one of the main elements in forest plantation. As a member of Meliaceae family, A*zadirachta excelsa* (Jack) Jacobs is considered as a fast growing non-dipterocarp (Appanah and Weinland, 1993). A study carried out by the Forestry Department Peninsular Malaysia also indicated that *A. excelsa* is suitable for use as timber, and therefore has the potential to be planted as a forest plantation species to meet log demand in the future (Baskaran *et al.*, 1997).

From the statements mentioned earlier, *A. excelsa* plantation will definitely have potential to be utilized in Malaysia. However, conversion of primary forest into plantation causes a great loss of nutrient in soils (Lee, 1998). The regeneration of trees would be extremely slow due to the depletion of nutrient. Thus, it is under such conditions that mycorrhizas with their ability to enhance nutrient uptake and drought tolerance are expected to play an important role in the success of reforestation effort. Gehring and Connell (2006) suggested that the inoculation with mycorrhiza when tree seedlings are established might be a good idea in promoting growth of trees. However, the most compatible species of mycorrhiza has yet been found as fungi which are beneficial to the host in the natural forest may not be well adapted to the degraded sites where reforestation will be carried out. Brundrett *et al.* (1994) also urged that more research should be conducted in order to compare the capacity of fungus isolates from a range of habitats to promote the growth of associated plants in different soils. This study was therefore conducted to investigate the diversity of vesicular arbuscular mycorrhiza related to *A. excelsa* in the natural stand and planted trees.

CHAPTER II

LITERATURE REVIEW

Mycorrhiza

Literally, "myco" means fungus and "rhiza" means root. Agrios (2005) stated that the feeder roots of most flowering plants growing in nature are generally infected by symbiotic fungi that do not cause root disease but, instead, are beneficial to their plant hosts. The infected feeder roots are transformed into unique morphological structures, which are known as mycorrhiza.

Linderman (1997) defined the term "Mycorrhiza" as a mutual beneficial association formed between fungi and the roots of plants, where the absorption of mineral ions by the plant roots is enhanced by the presence of fungus, which benefits by obtaining soluble organic nutrients from the root cells. Mycorrhiza needs the host in order to grow and reproduce. In the absence of hosts, the fungi will remain in a dormant condition as spores or resistant hyphae (Agrios, 2005).

Vesicular arbuscular mycorrhiza (VAM)

Bolan (1991) stated that depending upon the plant and fungal species involved, mycorrhizas are grouped into two major types, ectomycorrhiza and endomycorrhiza. Ectomycorrhizas are characterized by dense mycelia sheaths around the roots and intercellular hyphal invasion of the root cortex. They are more common in temperate and boreal forest trees and number over 5000 species mainly within the Basidiomycetes (Sieverding, 1991, cited by Quilambo, 2003). On the other hand, endomycorrhiza is

where the fungi form external hyphal networks in the soil and grow extensively within the cells of the cortex (Bolan, 1991). According to Sieverding (1991), cited by Quilambo (2003), endomycorrhiza also form specific fungal structures, referred to as vesicles and arbuscules (Figure 1). This characteristic growth gives the endomycorrhiza the alternate name, vesicular arbuscular mycorrhiza (VAM). Besides, endomycorrhiza are also known as arbuscular mycorrhiza or endotrophic mycorrhiza. Sometimes VAM fungi are called Glomalean fungi (Brundrett *et al*, 1996).

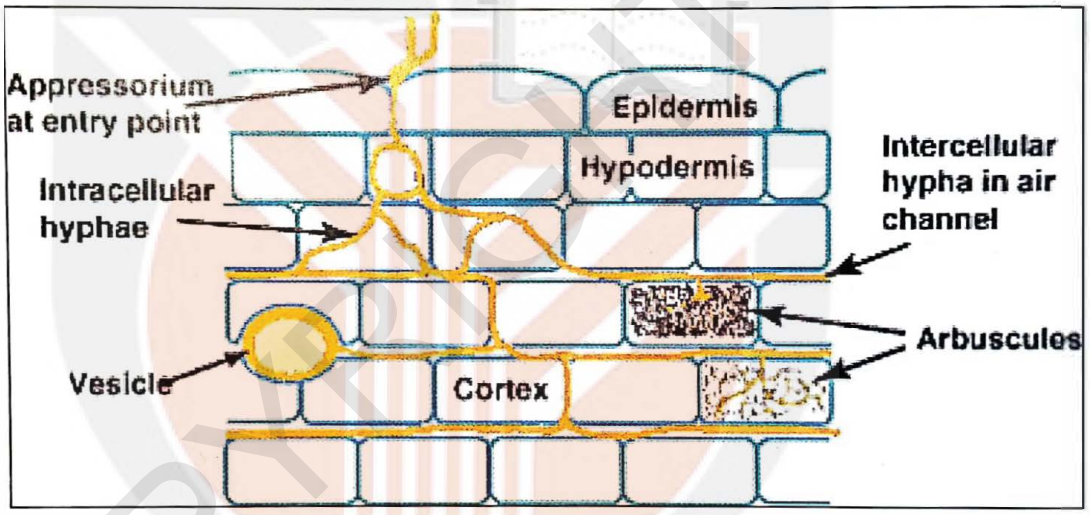

Figure 1: Structures formed by Glomalean fungi in mycorrhizal roots
(Source: Brundrett *et al.,* 1996)

Vesicular arbuscular mycorrhizal fungi produce a network of hyphae in the soil with relatively thin, highly branched hyphae (which are thought to absorb nutrients) and thicker hyphae which interconnect roots, spores and absorptive hyphae (Brundrett *et al.*, 1996). The hyphae is generally non-septate and it will penetrate into the plant root from the germinating spores, forming two types of structures: arbuscules and vesicles.

According to Brundrett *et al.* (1996), arbuscules are intricately branched haustoria that formed within a root cortex cell. They were named by Gallaud (1905), because they look like little trees. Arbuscules are formed by repeated dichotomous branching and reductions in hyphal width, starting from an initial trunk hypha (5-10 um in diameter) and ending in a proliferation of fine branch hyphae (< 1 um diameter) (Figure 2). Its formation follows hyphal growth, progressing outwards from the entry point. Arbuscules start to form approximately two days after root penetration. They grow inside individual cells of the root cortex, but remain outside their cytoplasm, due to invagination of the plasma membrane. Arbuscules are short-loved and begin to collapse after a few days, but hyphae and vesicles can remain in roots for months or years.

Figure 2: Mature arbuscule of *Glomus mosseae* with numerous fine branch hyphae (Source: Brundrett *et al.*, 1996)

Agrios (2005) defined vesicles as food-storing, large and swollen hyphal swellings. Brundrett *et al.* (1996) also stated that vesicles develop to accumulate storage products in many VAM associations. Vesicles are initiated soon after the first arbuscules, but continue to develop when the arbuscules senesce. It contains lipids and cytoplasm.

Some fungi produce vesicles which are similar in structure to the spores they produce in soil, but in other cases they are different (Brundrett *et al.,* 1996).

About 80% of all terrestrial plant species form association with VAM fungi (Smith and Read, 1997; cited by Quilambo, 2003). Brundrett *et al.* (1996) also suggested that many species of VAM fungi have worldwide distribution patterns and have apparently adapted to diverse habitats. They are found in a wide range of habitats usually in the roots of angiosperms, gymnosperm and ptarydophytes. They also occur in the gametophytes of some mosses, lycopods and Psilotales which are all rootless (Mosse *et al.,* 1981; Pocock and Duckett, 1985; cited by Quilambo, 2003). Recently, VAM are also found in aquatic plants (Beck-Nielsen and Madsen, 2001; cited by Quilambo, 2003).

Classification of VAM

According to Brundrett *et al.* (1996), the classification of the VAM fungi is based largely on the structure of their soil-borne resting spores. Rosendahl *et al.* (1994) also stated that the concepts used in the taxonomy of VAM fungi are based on the spore morphology, with the main criteria being spore size, shape, colour, ornamentation and wall or wall-layer structure.

Spore development is also used to define genera of Glomalean fungi. *Scutellospora* and *Gigaspora* species have spores which develop from a bulbous subtending hyphae, while those of *Glomus* species form on narrow or flaring hyphae (Brundrett *et al.* 1996)

Spore shape of most Glomalean fungi are globose (spherical), but some species have spores which are oval, oblong or occasionally other shapes. Subtending hyphae which remain attached to spores can be cylindrical, flared into a conical shape or swollen, and some spores have multiple or branched subtending hyphae (Brundrett *et al.*, 1996). Spore size is considered to be less useful than many other taxonomic criteria because of its variability, but substantial differences in spore sizes can help to distinguish species. According to Brundrett *et al.* (1996), Glomalean fungi have spores which fall within a range of sizes from very small (20-50μm) to very large (200-1000μm).

Walls of Glomalean fungus spore have one or more layers, that vary in their thickness, structure, appearance and staining reactions, and can be described using standardized terminology or diagrams (murographs). The wall characters have been useful for genera with spores in which several walls or wall layers are present (Rosendahl *et al.*, 1994), such as *Acaulospora* and *Scutellospora.* These wall layers can be observed on crushed spores with a compound microscope. For staining reactions, one or more wall layers may stain red or purple with the Melzer's reagent. Melzer's staining reaction may occur in inner or outer wall layers of spores in all genera, but typical staining reactions may not occur in spores that are old, damaged or have been stored in preservatives (Brundrett *et al.,* 1996).

According to Dodd (2000), VAM fungi are placed in a taxonomic order called the Glomales with six genera and three families namely: Glomacaea, Acaulosporaceae and Gigasporaceae. Species from the genus *Glomus* was the most researched. However,

Schüßler *et al.* (2001), followed by Walker *et al.* (2002), made some changes to the classification. With these changes, VAM and all the related fungi are placed under the phylum Glomeromycota, consisting of four orders: Glomerales, Paraglomerales, Archaeosporales and Diversisporales (Figure 3).

There are two families under the order of Glomerales, which are Glomeraceae 1 and Glomeraceae 2. Paraglomerales consists of a single family, Paraglomeraceae. As for Archaeosporaceae, it comprises of two families: Archaeosporaceae and Geosiphonaceae. Diversisporales consists of the most number of families. There are four families being placed under this order, which are Acaulosporaceae, Diversisporaceae, Gigasporaceae and Pacisporaceae (synonym Gerdemanniaceae). Currently, the eight families of VAM fungi consist of ten genera (Figure 3), namely *Acaulospora* (Acaulosporaceae), *Archaeospora* (Archaeosporaceae), *Diversispora* (Diversisporaceae), *Entrophospora* (Acaulosporaceae), *Geosiphon* (Geosiphonaceae), *Gigaspora* and *Scutellospora* (Gigasporaceae), *Glomus* (Glomeraceae), *Pacispora* (Pacisporaceae) and *Paraglomus* (Paraglomeraceae).

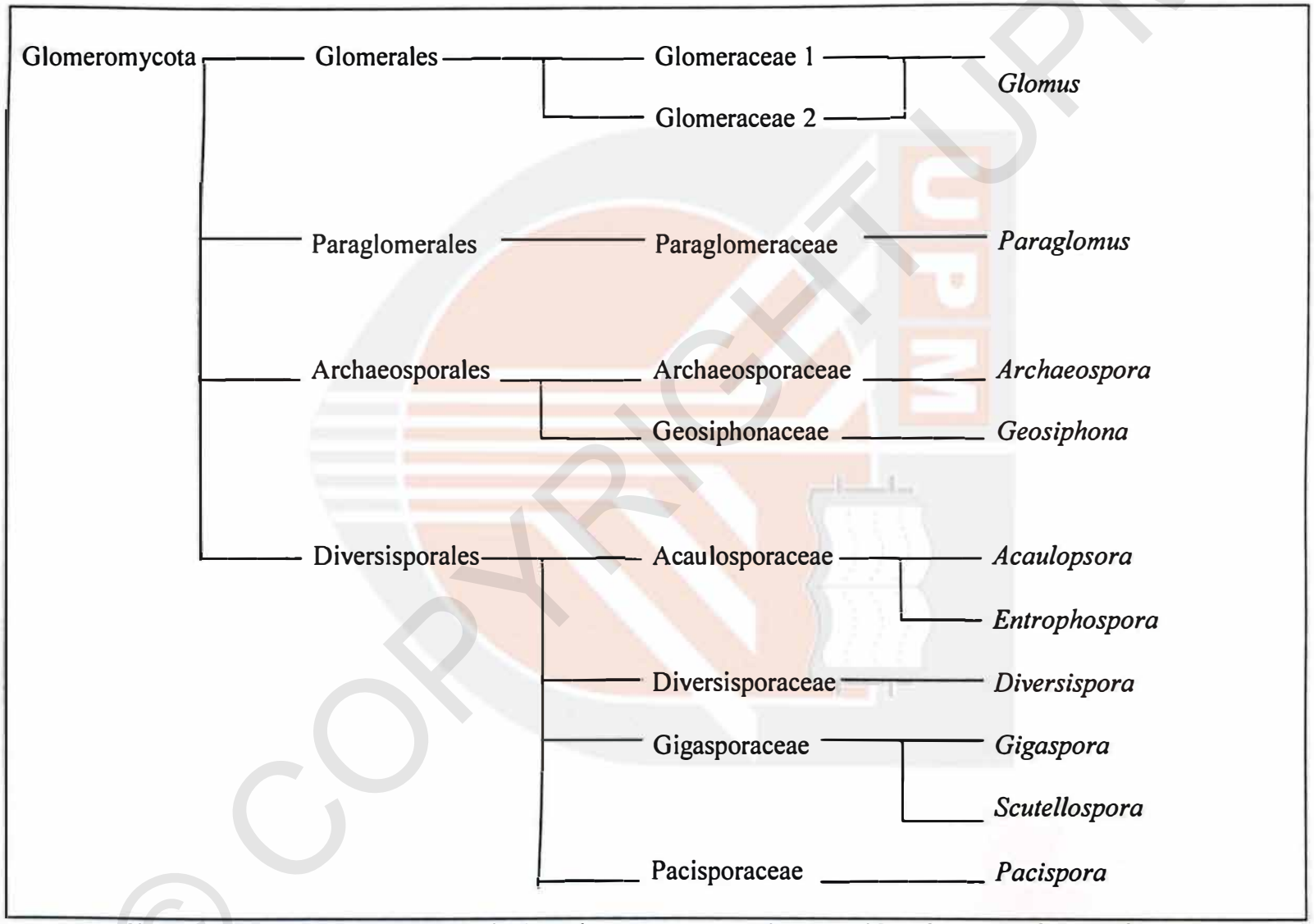

Figure 3: Current taxonomy of vesicular arbuscular mycorrhiza (modified from Schüßler *et al.*, 2001)

Importance of VAM

According to Bolan (1991), the beneficial effect of mycorrhiza on plant growth has mostly been attributed to an increase in the uptake of nutrients, especially phosphorus. However, Dodd (2000) claimed that apart from the increase in nutrient uptake, VAM also play an important role in soil aggregation, protection of plant against drought stress and soil pathogens and increasing plant diversity (Table 1).

Table 1: Important roles of mycorrhizal fungi in natural and managed ecosystems

Aspect	Importance
Benefits to plants	Plant nutrient supply through mycorrhizal roots Antagonism of parasitic organisms Non-nutritional benefits due to water relations, etc
Other roles in ecosystems	Nutrient cycling and conservation by soil mycelia Important food sources for many animals Improving soil structure Carbon transport from plant roots to other soil organisms
Values to people	Valuable food resources Medicinal uses Aesthetic values Fungal diversity is a bio-indicator of environmental quality

Source: Brundrett *et al.* (1996)

Linderman (1997) stated that soil hyphae of VAM fungi are able to bridge the zone of nutrient depletion around roots and acquire nutrient elements such as phosphorus, zinc and copper which are bound to soil particles and do not readily flow to the roots. Hence, it is said that VAM has "growth enhancement" effect on plants, especially plants that are under nutrient-deficient condition. Dodd (2000) focused more on phosphorus as he claimed that this is the nutrient most commonly associated with

mycorrhizal benefit as it is highly immobile in most soils and limiting to plant growth and reproduction. It is widely accepted that plants with highly branched root systems (Graminae) are less mycotrophic (less dependent on the fungus for normal growth) than those with coarser roots and that this determines the dependence of the plant on the symbiosis.

Vesicular arbuscular mycorrhiza has also been known to increase resistance to root-infecting pathogenic fungi such as *Phytophthora parasitica* or *Fusarium* spp. and root invading nematodes (Dodd, 2000; Agrios, 2005). There are other observations showing how the extraradical mycelium provides a surface for soil bacteria to use as a niche, the so-called "mycorhizosphere". The production of these "natural biofilms" on the surface of the hyphae may also play an indirect role in influencing pathogen levels and in aiding nutrient acquisition and soil stabilization. Extra radical mycelium provides a physical structure which can entangle soil particles and lead to micro- and macro-aggregate production. Recent finding indicated that a glycoprotein called "Glomalin" is produced by VAM soil-based mycelium and is a major binding agent in soils. This adds further weight to the importance of VAM in stabilizing soils and hence ecosystems (Dodd, 2000).

Quilambo (2003) suggested that the functions of VAM may range from stress alleviation to bioremediation in soils polluted with heavy metals. Schützendübel and Polle (2002) reported that mycorrhization stimulated the phenolic defence system in *Pinus*-mycorrhiza symbiosis. Cadmium-induced changes in mycorrhizal roots were found to be absent or smaller than those non-mycorrhizal roots.

However, it should be noted that there may be many different host-fungus mycorrhizal associations, and each combination may have different effects on the growth of the plant. Some mycorrhizal fungi have a broad host range, whereas others are more specific. Also, some mycorrhizal fungi are more beneficial to a certain host than other fungi, and some hosts need and profit from association with a certain mycorrhizal fungus much more than other hosts (Agrios, 2005).

Forest Plantation in Malaysia

It is clear that the growing demand for wood with the proposed slowdown of harvest of natural forest will enhance the economic role of forest plantations of fast growing species (Baskaran *et al.*, 1997). According to FAO (2005), plantation forests are established at a rate of 2.8 million hectares per year. Lamb (1998) stated that forest plantation is one of the few methods where large areas of land can be reforested. This is also a method which is more efficient in producing commercial timber than the natural forest (Abdul Razak, 1997).

Species selection is one of the main elements in forest plantation. According to Baskaran *et al.* (1997), the concept of planting *A. excelsa* in Malaysia on a plantation basis for commercial timber production is relatively new and the awareness was created over the last four to five years. According to Appanah and Weinland (1993), *A. excelsa* has great potential in mixed plantation.

Azadirachta excelsa

Description

Azadirachta excelsa occurs scattered in lowland forest well within a 200-300 m altitude range. In plantations, this species can be considered a fast growing non-dipterocarp (Appanah and Weinland, 1993). The tree stands form a layered canopy. Phototropic insensitivity and apical dominance are moderate. The stem form is variable, with some that are straight, and others with bends and kinks (Figure 4). Planted at a regular spacing and respacing, trees should have excellent stem form. The crown is widespread, with moderately dense foliage (Appanah and Weinland, 1993).

Azadirachta excelsa requires site with good quality soil, preferably sandy loam soils with good drainage and aeration and an average annual rainfall of 1600-2000 mm. First two years of planting requires intensive labour in controlling weed competition. Once the seedlings are above the weeds, minimal care is required.

Distribution of *A. excelsa*

As a member of the Meliaceae family, *A. excelsa* is a well-known village tree in the middle and northern regions of Peninsular Malaysia. In 1820, it was described as *Melia excelsa* but was never recognized until 1937. It is native to Sumatra (Bengkulu, East Coast), Borneo (Sabah and Sarawak), Sulawesi, Celebes, Moluccas (Aru Islands), West New Guinea and the Philippines (Mindoro, Masbate, Samar, Palawan, Basilan).

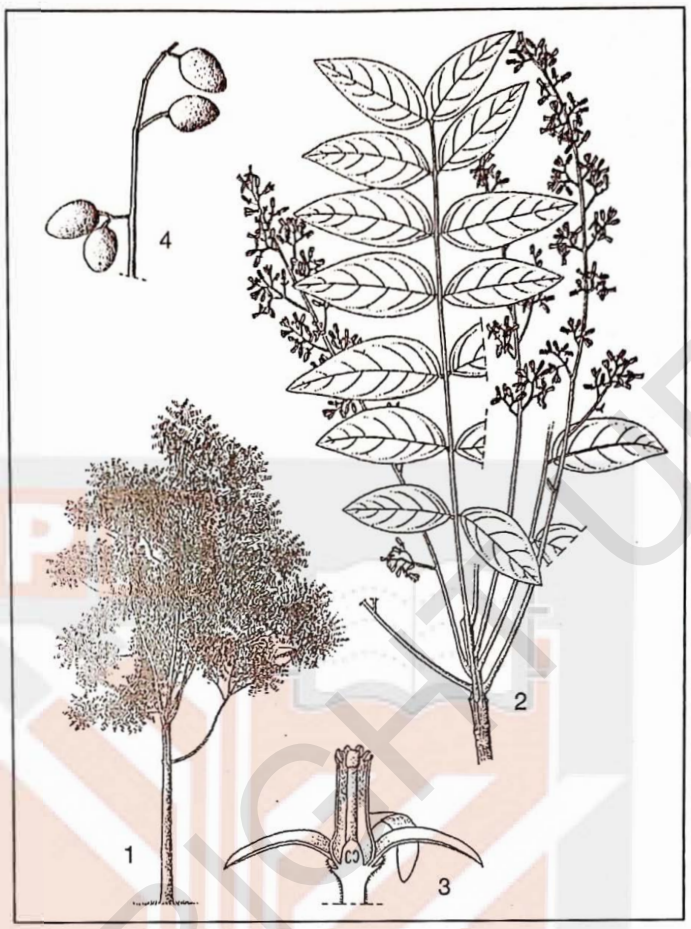

Figure 4: *Azadirachta excelsa* – 1, young tree; 2, flowering twig; 3, sectioned flower; 4, branchlet with fruits (Source: Noshiro *et al.,* 1995)

Study plots of *A. excelsa* have been established at Bukit Lagong Forest Reserve, Selangor and Relai Forest Reserve in Kelantan (Baskaran *et al.,* 1997).

Importance of *A. excelsa*

In Peninsular Malaysia and Thailand, timber of *A. excelsa* is used in house-building, light construction (under cover), joinery, furniture, interior finishing, panelling, partitioning, sliced veneer, flooring, decorative engraving turneries and matches (Noshiro *et al.,* 1995). The species is also commonly planted along roadsides and farm

boundaries or mixed in rubber plantations or mixed with other crops such as pineapple in these countries.

To the Malays the tree is important mainly because of its timber which is valued for house-building. The young shoots are also consumed as vegetable. The old leaves, which are intensely bitter, are sought after for medicine. The fruit is edible but not palatable. Seeds of *A. excelsa* yield azadirachtin, a derivative from *A. excelsa* oil which is used in producing insecticides (Noshiro *et al.*, 1995). Azadirachtin has been shown to be an effective compound in terms of insect feeding deterrent and as growth regulator for insects. The species also yield marrangin, another effective insect antifeedent (Baskaran *et al.*, 1997).

According to Kijkar (1997), woods of *A. excelsa* are also supplied to sawmills for general construction, furniture manufacturing, wood-carving products and interior paneling in Thailand. In Philippine *A. excelsa* is used for piano cases, matches, decorative engraving and cigar boxes, while in Papua New Guinea other applications include louvred doors and canoe making. It is also a promising, fast-growing source of fuelwood (Noshiro *et al.*, 1995).

Apart from timber utilization, Mohd Nor and Koh (1997, cited by Baskaran *et al.*, 1997) found out that *A. excelsa* with a fibre length of more than 1 mm and a thin cell-wall is comparable to the light hardwood species. Paper for printing, writing and wrapping can also be produced from *A. excelsa.*

Biodiversity

Many formal definitions of biological diversity or biodiversity have been proposed. Of these, the most important and far-reaching is that contained within the Convention on Biological Diversity. This landmark treaty was signed by more than 150 nations on 5th June 1992 at the United Nations Conference on Environment and Development, held in Rio de Janeiro, and came into force approximately 18 months later. The Convention states that "biological diversity" means the variability among living organisms from all sources including inter alia, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems (Gaston and Spicer, 2004).

The basic building blocks of biodiversity can be divided into three groups: genetic diversity, species diversity and ecological diversity (Dictionary of Biology, 2000). According to Gaston and Spicer (2004), genetic diversity encompasses the components of the genetic coding that structures organisms (nucleotides, genes, chromosomes) and variation in the genetic make-up between individuals within a species. It is due to the genetic diversity which is the biological heritage that species are able to respond to changes in the environment.

Species diversity can be referred to as the existence of a wide variety of species in a community or habitat (Dictionary of Biology, 2000). Species exist in a large variety of forms, with different sizes and biological characteristics. Operating individually or in groups within trophic webs, these properties influence the nature and magnitude of the

flow of matter and energy within the ecosystem. The different interactions among species, not only competition but also mutualism and symbioses, contribute collectively to the dynamics of an open system (Lévêque and Mounolou, 2003).

For ecological diversity, it encompasses the scales of ecological differences from populations, through niches and habitats, on up to biomes (Gaston and Spicer, 2004). Although presented separately, the three categories of biodiversity are intimately linked, and in some cases share elements in common.

There are numerous values of biodiversity and they can be divided into a few groups based on different viewpoints. However, these categories are not always clear-cut. Gaston and Spicer (2004) stated that in general, biodiversity provides food, medicine, biological control, industrial materials, tourism and ecosystem services (climatic regulation, atmospheric regulation, nutrient cycling, pollination and many more) to human.

Even though many of the elements of biodiversity may be difficult to define rigorously, and in some cases may have no strict biological reality (Gaston and Spicer, 2004), quantifying biodiversity is of practical importance when considering its evolution over time, geographical zones of interest and conservation strategies.

According to Lévêque and Mounolou (2003), different methodologies are adopted for measuring biodiversity and the choice of methods and scales tends to depend upon the objective pursued. Basically, there are two general components in the measure

of diversity: the number of entities and the degree of difference (dissimilarity) between those entities (Gaston and Spicer, 2004). The most common unit of measurement is the species richness determined for all the taxa, or subsets of taxa, identified in a given environment (Lévêque and Mounolou, 2003). However, confusion should be avoided between biodiversity and species richness. The former includes the latter but is not restricted to it. A high number of species in a given environment is likely to be a good indicator for a larger genetic, phylogenetic, morphological, biological and ecological diversity.

CHAPTER III

METHODOLOGY

Site Description

The study site for the natural stand of *A. excelsa* was located at a cemetery site in Sepupuk, Niah, Sarawak. The site was surrounded by vegetable gardens on the north and east, village on the south and Niah River on the west. The average annual temperature ranged from 25.3 to 26.8 °C with an annual rainfall of 2124 mm while the annual mean humidity varied from 83.0 to 88.6%. The site was undulating with slopes ranging from 1 to 25%. The stand comprises seven *A. excelsa* trees with diameter at breast heights (dbh) ranging between 2.21 – 4.70 m with heights varying from 23.3 to 35.4 m. There were other non-dipterocarp species left undisturbed within the cemetery site. The common ground vegetation included *Ageratum conyzoides, Borreria latifolia* and *Asystasia intrusa* (Ong *et al.,* 2006).

As for planted *A. excelsa,* the study site was located in an abandoned forest plantation and Botanical Garden in Universiti Putra Malaysia, Bintulu Campus. The abandoned forest plantation was established in 1986 and the *A. excelsa* trees were planted in 1989. Currently, the dbh of the trees were between 14.3 and 26.0 cm. Meanwhile, the *A. excelsa* tree in the Botanical Garden is two years of age with a dbh of 17.2 cm.

Collection of Soil Samples

Approximately 500 g of soil close to the roots of *A. excelsa* was taken using a soil sampler from a depth of 10-15 cm after removing the top litter layer (5-10 cm). Samples were collected in triplicate. Soil particles attached to the fine feeder roots were removed by generous shaking before being put into a plastic bag. The soil samples were brought to the laboratory and stored at 4°C. This was done to sustain the viability of the spores and to lower the activity of the rhizosphere micro flora (Chaurasia *et al.*, 2004).

Extraction of VAM Fungal Spores from the Soil Samples

Extraction of VAM fungal spores was done by wet-sieving and decanting method (Chaurasia *et al.*, 2004; Brundrett *et al.*, 1996). Soil sample from the plastic bag was placed on a flat surface for air-dried. A 20 g of air-dried soil sample was weighed and wetted by mixing it with appropriate volume of water for at least 30 minutes (Brundrett *et al.*, 1996). Frequent stirring was done to ensure the soil aggregates were broken apart. Meanwhile, 100 mL of sodium pyrophosphate was added to the soil sample in order to release the clay particles in the soil.

After allowing the heavy soil particles to settle for a few seconds, the soil sample was decanted through a series of sieves (53µm, 150µm and 710µm). The purpose of the coarse screen (710 µm) was to remove the roots, coarse debris and other soil particles while the finer screen (53µm) was used to capture the spores (Figure 5). The washing and decanting process was repeated two to three times until the spores were completely cleaned from all the soil particles.

Figure 5: Series of sieves that was used in the sieving process

The captured sieving was removed to a 15 mL centrifuge tube. Water was added to the tube and the sample was then centrifuged for 5 minutes at 3000 rpm. The floating debris and supernatant was discarded while the pellet was re-suspended in 50% sucrose by vigorously shaking the tightly stoppered tube (Brundrett *et al.*, 1996). The sample was then centrifuged again at 3000 rpm for two to three minutes. After centrifugation, spores in the sucrose supernatant were filtered by using a pre-wetted filter paper after being rinsed with water to remove excessive sucrose. Filter paper with the spores was kept in a petri dish and stored in refrigerator until further assessment.

Separation of Spores

A petri dish with spores was placed under a dissecting microscope. Spores were counted under a x30 magnification. The spores were separated into a few respective groups based on their colour and size by using paint-brush (Brundrett *et al.*, 1996) and placed in different petri dishes.

Preparation of Microscope Slides

Ten spores from each group of colour and size were selected by using paint-brush under dissecting microscope and were placed on a slide. The spores were mounted on glass slides in polyvinyl alcohol – lacto – glycerol (PVLG) + Melzer's reagent (Shi *et al.*, 2006). The spores were crushed to reveal the wall layers present in spores. The spore wall and the flexible inner walls have complex subcellular characteristics (Morton *et al.*, 2004) and without crushing, these characteristics could not be observed. Slides were left on a flat surface for four to five days before being sealed with colourless nail polish.

Slides were observed under a light compound microscope for resolution of subcellular layers (Morton *et al.*, 2004). Identification was done based on the characteristics of spores such as size, shape, cell wall, subtending hyphal wall and other spore structures. Identification was done up to genus level.

Data Analysis

Numbers and distribution of VAM fungal spores

Spore density, relative abundance and frequency were calculated according to Shi *et al.* (2006). Spore density was done by counting the number of VAM fungal spores in 100 g of soil. On the other hand, the relative abundance was calculated using the formula: (number of spores of a species/total spores) x 100. Meanwhile, frequency was determined by the equation: (number of samples in which the species was observed/total samples) x 100.

Species richness (S), Shannon-Wiener index of biodiversity (H), Evenness (E_H) and Sorensen's Index of community similarity (C) were also determined. Species richness is the most widely used parameter for evaluating aspects of fungal biodiversity. It is deceptively simple to define species richness as an enumeration of the species that are associated with a particular sample, area, habitat or substratum (Zak and Willig, 2004). The calculation of species richness is measured as the number of species whose spores are extracted from soil samples (Morton *et al.*, 2004).

Shannon-Wiener index of biodiversity, H was estimated using the following formula: H = - sum [Pi ln (Pi)], where Pi was calculated as the proportion of individuals of a given species to the total number of individuals in the community. Evenness (E_H) was calculated as H/ln S as a corresponding evenness index of H (Zak and Willig, 2004), where H is the Shannon-Wiener index of biodiversity and S is the species richness obtained.

Similarity, distance and dissimilarity coefficients represent an alternative analytical approach that can be used to quantify differences in species composition among sites (Zak and Willig, 2004). Sorensen's Index of community similarity (C) was used and calculated as: C = 2W / (a+b) where W is the sum of lower scores for each species, a is the sum of scores for Community A and b is the sum of scores of Community B.

Statistical analysis

Data were subjected to the Analysis of Variance (ANOVA) for unequal sample size using SAS version 6.12. Mean differences in VAM occurrence in the natural stand and planted trees of *A. excelsa* were separated using the Duncan Multiple Range Test.

CHAPTER IV

RESULTS

Total Spore

The spore density per 100 g of soil for all the VAM species found in this study is shown in Table 2. Total spore found in natural stand was 2221, which was lower compared to the planted trees (3503). The spore density of different VAM species in the rhizosphere of the natural stand ranged from 2 to 709, with an average of 202. Average spore density of VAM fungi found in the rhizosphere of the planted trees was 389. *Glomus* sp. 1 and *Glomus* sp. 2 produced the highest number of spores in both study sites.

Table 2: Spore density (per 100 g of soil) of VAM species in the study sites

Species	Spore density	
	Natural stand	Planted trees
Acaulospora sp. 1	50 a	50 a
Acaulospora sp. 2	259 a	190 a
Acaulospora sp. 3	115 a	27 a
Gigaspora sp. 1	44 a	0 a
Gigaspora sp. 2	183 a	13 a
Gigaspora sp. 3	47 a	0 a
Glomus sp. 1	709 a	2618 b
Glomus sp. 2	393 a	422 a
Glomus sp. 3	413 a	108 a
Scutellospora sp. 1	6 a	0 a
Scutellospora sp. 2	2 a	32 b
Scutellospora sp. 3	0 a	43 b
Total	2221	3503

Values in rows followed by the same letter are not significantly different by Duncan's Multiple Range Test ($p > 0.05$)

Genera and Species of VAM Fungi

A total of 12 species representing four genera of VAM fungi were distinguished in the soil samples from the two study sites (Table 2). Eleven species were found in the natural stand and nine were found in the planted trees. Eight species were present in both sites. *Scutellospora* sp. 3 was found to be absent from the natural stand. While *Gigaspora* sp. 1, *Gigaspora* sp. 3 and *Scutellospora* sp. 1 were not found in the planted trees' soil samples. Significant differences were detected among three VAM fungi species, namely *Glomus* sp. 1, *Scutellospora* sp. 2 and *Scutellospora* sp. 3.

Relative Abundance (%) and Frequency of Occurrence (%)

Relative abundance and frequency of occurrence for all the VAM fungi species obtained are shown in Table 3. The relative abundance of VAM fungi in the natural stand varied from 0.1 (*Scutellospora* sp. 2) to 31.9% (*Glomus* sp. 1) with an average of 9.1%. In the planted trees, the average relative abundance was 11.1%. *Glomus* sp. 1 was the most abundant species in both sites. *Glomus* accounted for 68.2 and 89.8% in the natural stand and planted trees, respectively.

In this study, the overall frequency of occurrence ranged from 14.3 to 100% (Table 3). Only *Glomus* sp. 1 achieved 100% occurrence in both study sites. *Acaulospora* sp. 2 and *Glomus* sp. 2 also recorded 100% of occurrence in the planted trees samples.

Table 3: Relative abundance (%) and frequency of occurrence (%) of VAM species in the study sites

Species	Relative abundance (%)		Frequency (%)	
	Natural stand	Planted trees	Natural stand	Planted trees
Acaulospora sp. 1	2. 3	1. 4	57. 1	33. 3
Acaulospora sp. 2	11. 7	5. 4	57. 1	100. 0
Acaulospora sp. 3	5. 2	0. 8	42. 9	33. 3
Gigaspora sp. 1	2.0	0. 0	42. 9	0. 0
Gigaspora sp. 2	8. 2	0. 4	57. 1	33. 3
Gigaspora sp. 3	2. 1	0. 0	28. 6	0. 0
Glomus sp. 1	31. 9	74. 7	100. 0	100.0
Glomus sp. 2	17. 7	12. 0	85. 7	100.0
Glomus sp. 3	18. 6	3. 1	71. 4	66. 7
Scutellospora sp. 1	0. 3	0. 0	14. 3	0. 0
Scutellospora sp. 2	0. 1	0. 9	14. 3	66. 7
Scutellospora sp. 3	0. 0	1. 2	0. 0	66. 7
Total	100.0	100.0	-	-

Shannon-Wiener Index of Biodiversity (H), Evenness (E_H) and Sorensen's Index of Community Similarity (C)

The natural stand of *A. excelsa* recorded higher H and E_H values when compared to the planted trees (Table 4). Higher value of E_H showed that the distribution of VAM fungi in the natural stand was more even. The C value suggested that the degree of similarity between the two study sites was moderately high.

Table 4: Vesicular arbuscular mycorrhiza status of *A. excelsa* at the two study sites

Site	Shannon- Wiener index of biodiversity	Evenness	Sorensen's index of community similarity
Natural stand	1.8607	0.7760	0.5684
Planted trees	0.9537	0.4340	

CHAPTER V

DISCUSSION

From the two study sites, a total of 12 species of VAM fungi were found, in which 11 species were recorded in the natural stand of *A. excelsa* while only nine were recorded for planted trees. This result was higher than what was reported by Shi *et al.* (2006), who investigated the association of VAM with Meliaceae on Hainan Island, China. However, this finding was lower than that obtained by Ong *et al.* (2002), who recorded 25 species of VAM spores in an *A. excelsa* plantation in Johor, Malaysia.

The natural stand recorded higher species richness compared to planted trees. This may have been resulted from the conversion of natural forest to plantation. The disturbance of soil may reduce VAM fungi diversity. Meanwhile, soils at the natural stand were left undisturbed which encourage the development of VAM. This was consistent with the results of Brundrett *et al.* (1994), where disturbed sites were found to have lower VAM. Geological condition of both sites in this study were very much different and the soil properties were not expected to be identical. According to Brundrett *et al.* (1994), the distribution of VAM fungi can be influenced by soil condition too. This was paralled to the finding by Morton *et al.* (2004). Differences in species richness among sites within a study area or between study areas of similar scale are likely the result of several factors, such as host or edaphic characteristics that influence colonization and sporulation; stochastic dispersal events over time and space; spatial and temporal differences in intensity of sampling; quality and quantity of fungal material collected for identification and many more.

The 12 species isolated consisted of four genera, namely *Acaulospora*, *Gigaspora*, *Glomus* and *Scutellospora*. All four genera existed in both study sites. According to Lee (1998), the majority of trees in the forests of South East Asia form VAM relationships with species such as *Gigaspora, Glomus, Acaulospora* and *Sclerocystis*. *Sclerocystis* was not found in the present study; instead, *Scutellospora* was found in both study sites. Ong *et al.* (2002) isolated five genera of VAM fungi in an *A. excelsa* plantation in Johor, Malaysia with an inclusion of *Entrophospora* that was not found in the current study.

Glomus was the most dominant genus found in both sites. This is consistent with the finding by Shi *et al.* (2006) where *Glomus* was the main genus found to be associated with Meliaceae in Hainan Island, China. Brundrett *et al.* (1994) in their study of mycorrhizal associations in disturbed and natural habitats in tropical Australia also found *Glomus* to be the dominant fungi in most soils even when living spores of these fungi were not present. *Glomus* sp. 1 was the most abundant species in both study sites. This was different from what was reported by Ong *et al.* (2002), who found *Acaulospora* to be the genus showing the highest spore density. According to Morton *et al.* (2004), sporulation could be affected by abiotic factors, such as temperature, rainfall, light and moisture. van Tuinen *et al.* (1994) also suggested that spore characteristics can vary depending on environmental factors and on the physiological status of the fungus.

Ludwig and Reynolds (1988) emphasized two properties of Shannon- Wiener index of biodiversity that make it popular. First, H = 0 if (and only if) the samples includes only a single species. Second, H reaches its maximum only when all species are

equally abundant. The magnitude of H is usually between 1.5 and 3.5 and is rarely greater than 4.5. In fact, to obtain a value of H of more than 5.0, the ecological unit would need to include 10^5 species (Zak and Willig, 2004). The value of natural stand (1.8607) was within the normal range whereas the value of planted trees (0.9537) was not within the normal range. This can be due to the lesser number of species present in the planted trees site.

Value of evenness in the natural forest was also higher than the planted trees, which means the distribution of different species of VAM was more even in the natural stand as compared to the planted trees. The extra high number of spore from *Glomus* sp. 1 in the planted trees samples was believed to be the contribution factor for the unevenness of the site. The Sorensen's index of community similarity calculated indicates that both study sites have moderate similarity in the community of VAM.

CHAPTER VI

CONCLUSIONS

This study showed that VAM fungi from the natural stand exhibited a more complex pattern of diversity compared to samples collected from the planted *A. excelsa.* However, the spore density per 100 g of soil under planted trees was higher than the number recorded for the natural stand. Hence, it could be concluded that the biodiversity of VAM fungi in association with *A. excelsa* in the natural stand is more diverse than the planted *A. excelsa.* However, this study is only a preliminary assessment of the status of VAM fungi associated with *A. excelsa* as the identification of VAM spores is only up to genus level and all the identification is based on visual observation. A more detailed study needs to be conducted to investigate the complex biodiversity of VAM associated with *A. excelsa* in the natural stand.

Future research should include the soil properties study to analyze the relationship between soil properties and the diversity of VAM. More samples should be collected at different time frame as some fungal species might have unique seasonal patterns. Besides, the influence of sampling climate and time should also be included. Research on VAM fungi in association with *A. excelsa* should be further carried out so that the most compatible VAM species can be identified and therefore aid in the commercial plantation of *A. excelsa.*

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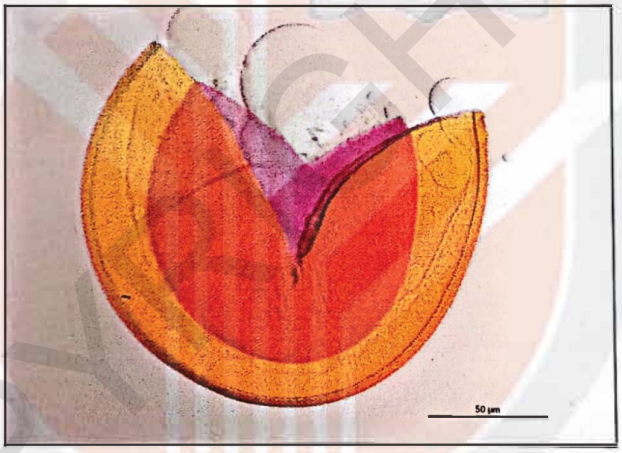
APPENDICES

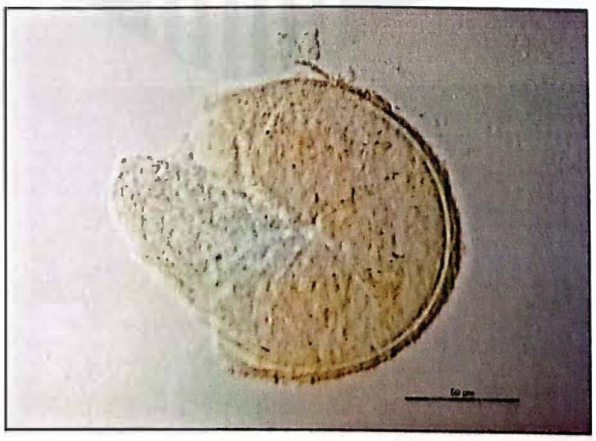
Appendix A

Acaulospora

Acaulospora sp. 1



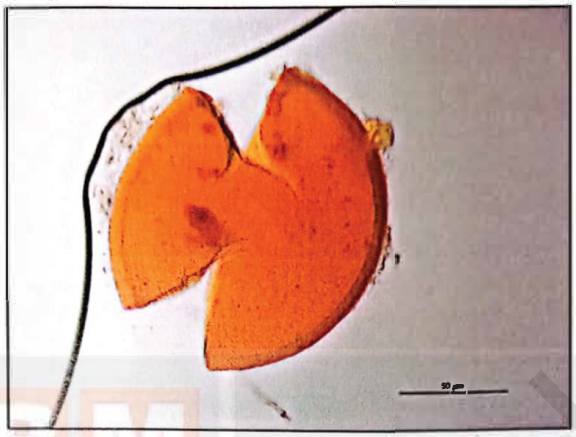
Acaulospora sp. 2



Acaulopsora sp. 3

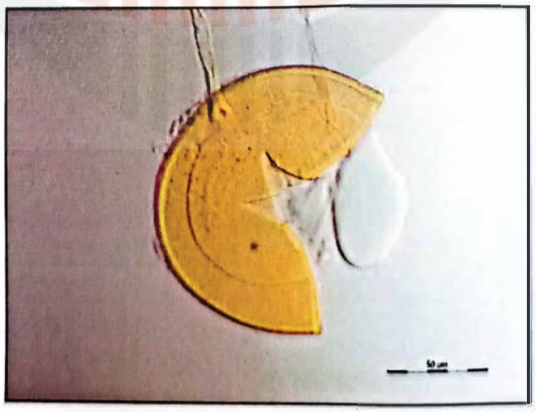
Appendix B

Gigaspora



Gigaspora sp. 1

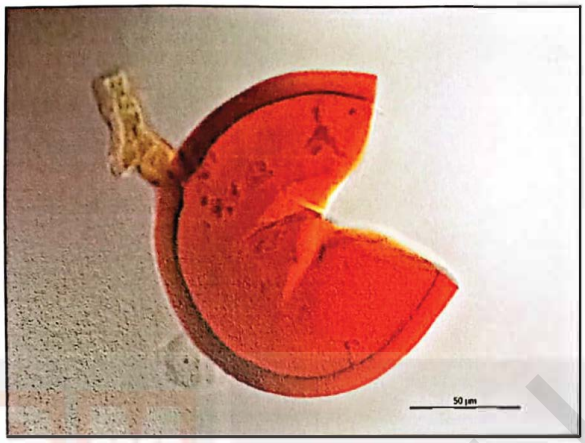
Gigaspora sp. 2



Gigaspora sp. 3

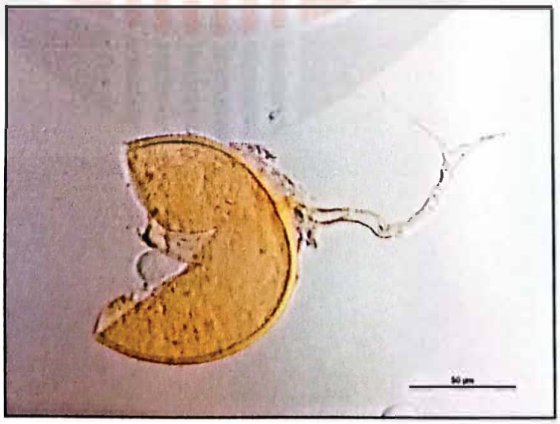
Appendix C

Glomus



Glomus sp. 1

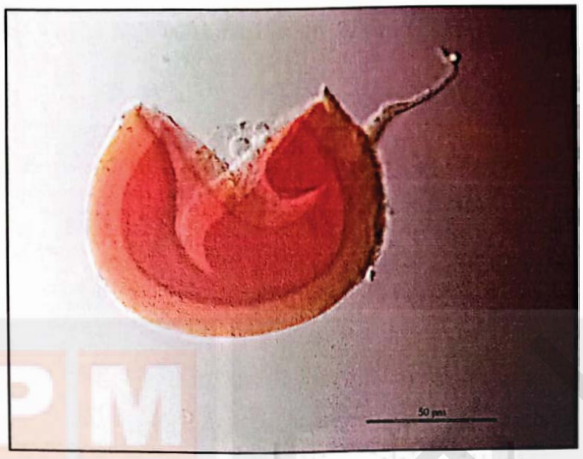
Glomus sp. 2



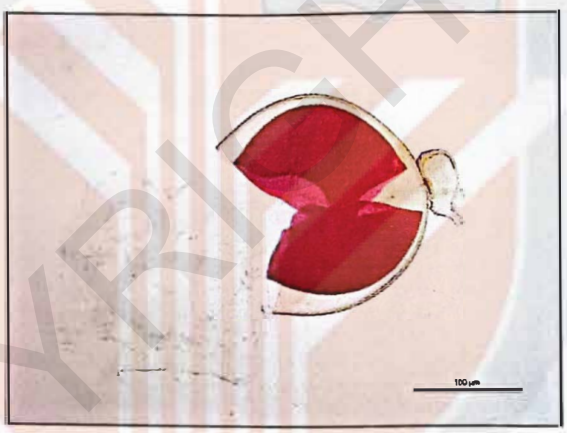
Glomus sp. 3

Appendix D

Scutellospora



Scutellospora sp. 2



Scutellospora sp. 3

Appendix E

Spore Count and Diversity Index

Table: Spores extracted from the soil samples of natural stands of *A. excelsa* sorted by colours

Sample	Red	White	Yellow	Total
S1	177	100	245	522
S2	177	90	562	829
S3	188	243	40	471
S4	135	33	31	199
S5	188	213	183	584
S6	139	68	22	229
S7	128	58	89	275
Total	1132	805	1172	3109

Table: Spores extracted from the soil samples of planted *A. excelsa* sorted by colours

Sample	Red	White	Yellow	Total
SI	1107	61	203	1371
SII	189	6	84	279
SIII	305	40	107	452
Total	1601	107	394	2102

Table: Biodiversity index of planted *A. excelsa*

Suspected species	Total	Pi	Pi ln (Pi)
Acaulospora sp. 1	30	0.0143	-0.0607
Acaulospora sp. 2	114	0.0542	-0.1580
Acaulospora sp. 3	16	0.0076	-0.0371
Gigaspora sp. 2	8	0.0038	-0.0212
Glomus sp. 1	1571	0.7474	-0.2176
Glomus sp. 2	253	0.1204	-0.2549
Glomus sp. 3	65	0.0309	-0.1074
Scutellospora sp. 2	19	0.0090	-0.0424
Scutellospora sp. 3	26	0.0124	-0.0544
Total	2102	1.0000	-0.9537

Table: Biodiversity index of natural stands of *A. excelsa*

Suspected species	Total	Pi	Pi ln (Pi)
Acaulospora sp. 1	70	0.0225	-0.0854
Acaulospora sp. 2	363	0.1168	-0.2508
Acaulospora sp. 3	161	0.0518	-0.1533
Gigaspora sp. 1	62	0.0199	-0.0779
Gigaspora sp. 2	256	0.0823	-0.2055
Gigaspora sp. 3	66	0.0212	-0.0817
Glomus sp. 1	992	0.3191	-0.3645
Glomus sp. 2	550	0.1769	-0.3064
Glomus sp. 3	578	0.1859	-0.3128
Scutellospora sp. 1	8	0.0026	-0.0155
Scutellospora sp. 2	3	0.0010	-0.0069
Total	3109	1.0000	-1.8607

Appendix F

Changes in Extent of Forest in South and South East Asia

Table: Changes in extent of forest and other wooded land 1990-2005

Country/area	Forest							Other wooded land		
	Area (1 000 ha)			Annual change rate				Area (1 000 ha)		
	1990	2000	2005	1990–2000		2000–2005		1990	2000	2005
				1 000 ha/year	%[a]	1 000 ha/year	%[a]			
Bangladesh	882	884	871	n.s.	n.s.	-2	-0.3	44	53	58
Bhutan	3 035	3 141	3 195	11	0.3	11	0.3	566	609	611
Brunei Darussalam	313	288	278	-2	-0.8	-2	-0.7	142	155	160
Cambodia	12 946	11 541	10 447	-140	-1.1	-219	-2.0	335	298	270
India	63 939	67 554	67 701	362	0.6	29	n.s.	5 894	4 732	4 110
Indonesia	116 567	97 852	88 495	-1 872	-1.7	-1 871	-2.0	-	-	-
Lao People's Democratic Republic	17 314	16 532	16 142	-78	-0.5	-78	-0.5	2 875	4 053	4 643
Malaysia	22 376	21 591	20 890	-78	-0.4	-140	-0.7	-	-	-
Maldives	1	1	1	0	0	0	0	0	0	0
Myanmar	39 219	34 554	32 222	-466	-1.3	-466	-1.4	10 219	10 629	10 834
Nepal	4 817	3 900	3 636	-92	-2.1	-53	-1.4	1 180	1 753	1 897
Pakistan	2 527	2 116	1 902	-41	-1.8	-43	-2.1	1 191	1 323	1 389
Philippines	10 574	7 949	7 162	-262	-2.8	-157	-2.1	2 230	3 292	3 611
Singapore	2	2	2	0	0	0	0	0	0	0
Sri Lanka	2 350	2 082	1 933	-27	-1.2	-30	-1.5	0	0	0
Thailand	15 965	14 814	14 520	-115	-0.7	-59	-0.4	-	-	-
Timor-Leste	966	854	798	-11	-1.2	-11	-1.3	-	-	-
Viet Nam	9 363	11 725	12 931	236	2.3	241	2.0	0	1 816	2 259
Total South and Southeast Asia	**323 156**	**297 380**	**283 127**	**-2 578**	**-0.8**	**-2 851**	**-1.0**			

Source: FAO, 2005

PUBLICATION OF THE PROJECT UNDERTAKING

This is to certify that I have no objection to publish the project entitled "Vesicular Arbuscular Mycorrhizal Fungi Associated with *Azadirachta excelsa* (Jack) Jacobs" by the supervisor in a joint authorship. However, it has to be evaluated by the Faculty of Agriculture and Food Science, Universiti Putra Malaysia Bintulu Campus and published in the form approved by the faculty.

Lee Jia Huey

Date: 10th May 2007