# GENETIC DIVERSITY OF ARBUSCULAR MYCORRHIZAL FUNGI INFECTED *Acacia mangium* Willd.

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# ความหลากหลายทางพันธุกรรมของเชื้อราอาบัสคูลาร์ไมคอร์ไรซาในกระถินเทพา

นายวีรวัตร นามานุศาสตร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree

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**วีรวัตร นามานุศาสตร์: ความหลากหลายทางพันธุกรรมของเชื้อราอาบัสคูลาร์ไมคอร์ไรซาในกระถินเทพา (GENETIC DIVERSITY OF ARBUSCULAR MYCORRHIZAL FUNGI INFECTED *Acacia mangium* Willd.)**
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การศึกษาลักษณะเบื้องต้นของการพึ่งพาอาศัยอยู่ร่วมกันของเชื้อราอาบัสคูลาร์ไมคอร์ไรซาจากไม้ยืนตระกูลถั่ว ทั้ง 5 ชนิด ได้แก่ แดง, ประดู่กิ่งอ่อน, สาธร, กระถินณรงค์ และกระถินเทพา ให้ผลแสดงถึงความเจาะเพาะจงต่ำต่อพืชอาศัย และลักษณะทางสัณฐานวิทยามีความแปรผันสูง ดังนั้นจึงได้ทำการคัดเลือกไม้กระถินเทพาเป็นไม้ต้นแบบในการศึกษา โดยศึกษาลักษณะและความหลากหลายทางสัณฐานวิทยา เพื่อยืนยันร่วมกับลักษณะทางพันธุกรรมในระดับโมเลกุล เพื่อให้ได้ผลครอบคลุมประชากรของเชื้อ โดยผลการศึกษาขั้นแรกจากตัวอย่างดินบริเวณรากของกระถินเทพา พบว่าสายพันธุ์ของเชื้อที่พบมากที่สุด อยู่ในสกุล *Gigaspora, Scutellospora, Glomus* และ *Acaulospora* ตามลำดับ ผลของการอ่านลำดับเบสบนยีน 18S และ 5.8S rDNA โดยตรงจาก single spore สามารถตรวจสอบสายพันธุ์ได้ดังนี้ *Gi. albida* (99% ความคล้ายคลึง), *Sc. persica* (96% ความคล้ายคลึง), *Gi. gigantea* (97% ความคล้ายคลึง), *G. mosseae* (94% ความคล้ายคลึง), *G. clarodeum* (90% ความคล้ายคลึง), uncultured *Glomus* (100% ความคล้ายคลึง; AY236250.1), uncultured AMF (100% ความคล้ายคลึง; AY267221), uncultured soil fungus (96% ความคล้ายคลึง; AF515414.1), uncultured soil ascomycete fungi (95% ความคล้ายคลึง; AJ515949.1), uncultured basidiomycete fungi (92% ความคล้ายคลึง; AJ515169.1) และ *Glonus* spp. 1 สายพันธุ์ (96% ความคล้ายคลึง; AF082581) ผลการตรวจสอบเชื้อราอาบัสคูลาร์ไมคอไรซาที่เข้าอยู่อาศัยในรากพืชโดยตรง พบสายพันธุ์ของเชื้อราอาบัสคูลาร์ไมคอร์ไรซา ดังนี้ *Sc. pellucida,* (98% ความคล้ายคลึง), *Sc. heterogama* (99% ความคล้ายคลึง), *Gi. decipiens* (100% ความคล้ายคลึง), *Gi. margarita* (100% ความคล้ายคลึง), *G. intraradices* (100% ความคล้ายคลึง), *A. laevi* (92% ความคล้ายคลึง), uncultured AMF (100% ความคล้ายคลึง; AY267221) และ unculturable ascomycete fungi (95% ความคล้ายคลึง; AJ515949.1) นอกจากนี้ในการทดสอบเปรียบเทียบผลกระทบของการใช้เชื้อราเป็นหัวเชื้อในกระถินเทพาที่มีอายุ 2 เดือน พบว่าต้นที่มีการใส่เชื้อมีการเจริญเติบโตที่ระบบรากอย่างมีนัยสำคัญมากกว่าต้นที่ไม่ได้ใส่เชื้อ ส่วนความสูงของต้นและน้ำหนักไม่มีความแตกต่างในต้นที่มีการใส่และไม่ได้ใส่เชื้อ เชื้อที่เข้าอยู่ร่วมอาศัยได้เป็นส่วนมาก และมีประสิทธิภาพในการเข้าอยู่อาศัยอยู่ได้แก่ สกุล *Glomus*

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2546

ลายมือชื่อนักศึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษา.....
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ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

**WERAVART NAMANUSART: GENETIC DIVERSITY OF ARBUSCULAR MYCORRHIZAL FUNGI INFECTED *Acacia mangium* Willd.**
**THESIS ADVISOR: ASSOC. PROF. NEUNG TEAUMROONG,**
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The preliminary investigation of AMF from rhizosphere of five tree legumes: *Xylia xylocarpa* Taub., *Pterocarpus indicus* Willd., *Millettia leucantha* Kurz., *Acacia auriculiformis* Cunn. and *Acacia mangium* Willd. showed the low host plant specificity and diverse genera were also found. Therefore, association between AMF and *Acacia mangium* was mainly focused in this study. The characterization of AMF was conducted in both morphological and molecular aspects. Most isolates from the rhizosphere of *A. mangium* belong to genera *Gigaspora, Scutellospora, Glomus* and *Acaulospora*, respectively. The DNA direct extraction from the single spore was developed. 18S and 5.8S rDNAs were used for sequencing. AMF from *A. mangium* rhizosphere were *G. albida* (99% homology), *Sc. persica* (96% homology), *Gi. gigantea* (97% homology), *G. mosseae* (94% homology), *G. clarodeum* (90% homology), uncultured *Glomus* (100% homology; AY236250.1), uncultured AMF (100% homology; AY267221), uncultured soil fungus (96% homology; AF515414.1), uncultured soil ascomycete fungi (95% homology; AJ515949.1), uncultured basidiomycete fungi (92% homology; AJ515169.1) and one strain *Glonus* spp. (96% homology; AF082581). Moreover, the DNA extraction from the plant root indicated the infection of *Sc. pellucida,* (98% homology),*Sc. heterogama* (99% homology), *Gi. decipiens* (100% homology), *Gi. margarita* (100% homology), *G. intraradices*

(100% homology), *Gi. margarita* (100% homology), *G. intraradices* (100% homology), *A. laevi* (92% homology), uncultured AMF (100% homology; AY267221) and unculturable ascomycete fungi (95% homology; AJ515949.1). In addition, the comparative studies on the effect of AMF of plant growth (2 months) indicated that almost selected fungi were respond to the root system of plant. The dry weight and total weight of plant were not statically different when compared them with control plant. The higher colonization and plant response were observed. The effective fungal strain most likely belongs to genus *Glomus.*

School of Biotechnology

Academic Year 2003

Student's Signature.....

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Co-advisor's Signature.....

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Weravart Namanusart

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**Morphological identification of selected 5 tree legumes (Fig. 4-8)**



**Morphological identification of *Acacia mangium* Willd. (Fig. 10-34)**

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# LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| AMF | = | Arbuscular Mycorrhizal Fungi |
| ATCG | = | nucleotide containing the base adenine, thymine, cytosine, and guanine, respectively |
| $^{o}C$ | = | degree celcleus |
| DNA | = | deoxyribonucleic acid |
| EDTA | = | ethylene diamine tetraacetic acid |
| Kb | = | Kilobase |
| mg | = | milligram |
| ml | = | millilitre |
| mM | = | millimolar |
| M | = | molar |
| $MgCl_2$ | = | magnesium chloride |
| ng | = | nanogram |
| µl | = | microlitre |
| µM | = | micromolar |

# CHAPTER I

# INTRODUCTION

## 1.1 Characteristics of Arbuscular Mycorrhizal Fungi

Arbuscular mycorrhizal fungi (AMF) belong to the order Glomales and form highly branched structures called arbuscules, within root cortical cells of many herbaceous and woody plant species, are obligate endosymbionts that colonize plant roots of almost 90% of terrestrial plants. Another name, Vesicular-Arbuscular Mycorrhiza (VAM), comes from structures which are formed within root cortical cells: vesicles, which are thought to be storage or reproductive structures; and arbuscules, branch multiple-tipped hyphal structures within the plant cell. Components of AMF associations were: (Figure 1)

A. **Structures in Roots**

- **Hyphae** - non-septate when they are young and ramified within the cortex.
- **Arbuscules** - intricately branched haustoria in cortex cells.
- **Vesicles** - storage structures formed by many fungi.

B. **Structures in Soil**

- **Hyphae** - A network of hyphae forms in the soil with thicker hyphae functioning as conducts and thin branched hyphae which are thought to absorb nutrients.

- **Spores** - Large (for a fungus) asexual spherical structures (20-1000 μm diameter) that form on hyphae in soil or roots.

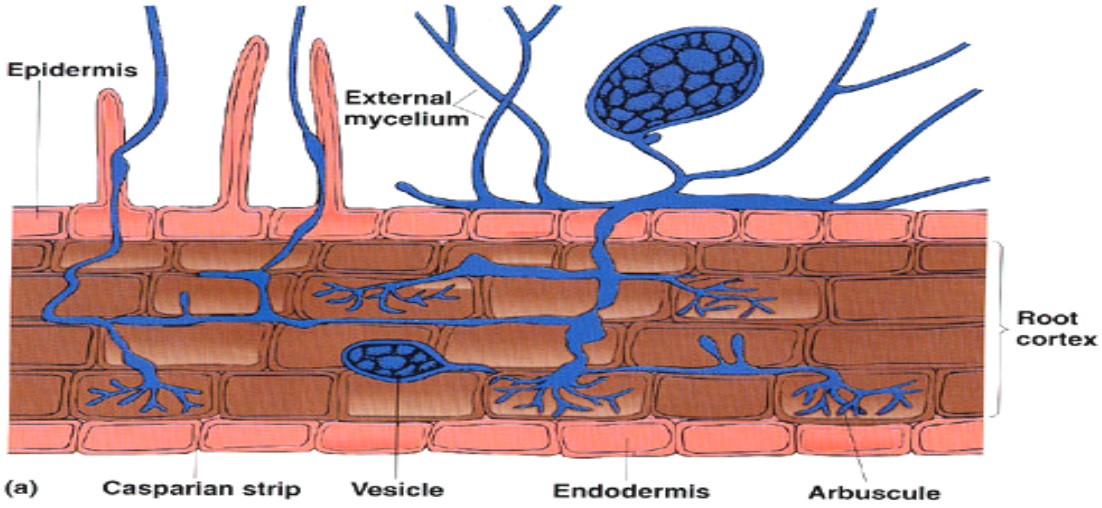


**Figure 1** Composition of arbuscular mycorrhizal fungal association in the plant according from General Botany, Biology 210, Fall (WWW, 2003)

AMF present in most natural and agricultural ecosystems, they are important for plant health, nutrient cycling, survival rate, and conservation of soil structure. AMF procure and transport phosphate and other nutrients from the soil to plant roots. On the other hand, the host plant provides fixed carbon to its fungal partner (Harrison, 1999). Furthermore, AMF could facilitate the management of metal contamination in soil for a restoration and/or bioremediation program. This information indicated the challenge of the transferring this ability to non-legumes such as cereals representing a very long term or the possibilities of this fungus opening the sustainable agriculture. As a consequence, AMF were crucial determinants of plant biodiversity, ecosystem

variability, and the productivity of plant communities (Heijden et al., 1998). However, due to these fungi are obligate symbionts and could not be cultured on nutrient media. Contrasting with the Rhizobium-legume association, there is very little host specificity in AM symbioses. The major problem of this fungal utilization is not host-specific; although evidence is grow that certain endophytes may form preferential associations with certain host plants.

Therefore, the selected study of AM fungal diversity may be the most important to need as components of forestation programs. In the same way, Moorland and Revees (1979) reported that the diversity of mycorrhizal fungi contributes to the "buffering capacity" of the forest ecosystem; or the ability of the system to withstand and recover from disturbance. Therefore, the mycorrhizal fungi are hoped for reforestation with rapid conversion of tropical forest. It is imperative to collect and assess indigenous mycorrhizal fungi for successful establishment of tree plantations.

## 1.2 Classification of Arbuscular Mycorrhizal Fungi

AMF belong to Order Glomales (as summerized in Table 1.) (Morton and Benny, 1990). Which have taxonomy subsequence are Suborder: *Glominecae* Family: *Glomaceae* (Morton, 1996; Sturmer and Morton, 1997), genus: *Glomus* and *Sclerocystis*; Family: *Acaulosporaceae* (Sturmer and Morton, 1998), genus: *Acaulospora* and *Entrophospora*; and new families (Morton and Redecker, 2000), Family *Archaeosporaceae* genus: *Archaeosporaceae*, and Family: *Paraglomaceae*, genus: *Paraglomus*; Suborder: *Gigasporneae,* Family: *Gigasporaceae* ( Franke and Morton, 1994: Morton, 1995, and Bentivenga and Morton, 1995), genus: *Gigaspora* and *Scutallospora*. Generally, we could find the AMF form multinucleate spores in the soil which vary in wall structure and morphology between species and different stages of development (Walker and Trappe, 1993). At the present, some of these fungi could be identified on the basis of morphological features such as the spore surface, spore color, spore shape, spore size, spore ornamentation etc. However, these morphological variations make it difficult to identify the species presenting in natural ecosystems (Gioivannetti and Gianinazzi-Pearson 1994). Besides, to ensure reliability in the identification of these fungi analysis should be completed on spores from single-spore pot cultures or a large number of specimens collected from the field (Walker, 1992), in order to have a range of spores at different developmental stages and to find spores with mature cell walls which can be used for classification (Morton, Franke and Bentivenga 1994). However, this methodology is time consuming. The morphology-based identification of AMF was limited when it was used in ecological settings because spore production was highly dependent on physiological parameters and may not be correlated with root colonization

(Merryweather and Fitter, 1991). Therefore, using of molecular techniques represents a powerful tool for identifying and understanding the genetic variation of these organisms.

**Table 1** Classification scheme for AMF taxa.

| Order | Suborder | Family | Genus | Reference |
|---|---|---|---|---|
| Glomales | *Glominecae* | *Glomaceae* | *Glomus*<br>*Sclerocystis* | Morton and Benny(1990) |
| | | *Acaulosporaceae* | *Acaulospora* | Sturmer and Morton (1998) |
| | | *Archaeosporaceae* | *Entrophospora*<br>*Archaeospora* | Morton and Redecker (2000) |
| | | *Paraglomaceae* | *Paraglomus* | |
| | *Gigaspornea* | *Gigasporaceae* | *Gigaspora*<br>*Scutallospora* | Morton (1995) |

## 1.3 Role of Arbuscular Mycorrhizal Fungi and minerals uptake

When a nutrient is deficient in soil solution, the critical root parameter controlling its uptake is surface area. Hyphae of mycorrhizal fungi have the potential to greatly increase the absorbing surface area of the root. For example, Rousseau et al. (1994) found that while extramatrical mycelia (aggregates of hyphae) accounted for less than 20% of the total nutrient absorbing surface mass, they contributed nearly 80% of the absorbing surface area of the pine seedlings. It is also important to consider the distribution and function of the extramatrical hyphae. If the mycorrhiza is to be effective in nutrient uptake, the hyphae must be distributed beyond the nutrient depletion zone that develops around the root. A nutrient depletion zone develops when nutrients are removed from the soil solution more rapidly than they can be replaced by diffusion. For a poorly-mobile ion such as phosphate, a sharp and narrow depletion zone develops close to the root. Hyphae can readily bridge this depletion zone and grow into soil with an adequate supply of phosphorus. Uptake of micronutrients such as zinc and copper is also improved by mycorrhizae because these elements are also diffusion-limited in several soils. For more mobile nutrients such as nitrate, the depletion zone wide is less like those hyphae grows extensively into the zone that is not influenced by the root alone.

Another factor contributing to the effective absorption of nutrients by mycorrhizae is their narrow diameters relative to roots. The steepness of the diffusion gradient for a nutrient is inversely related to the radius of the absorbing unit; therefore, the soil solution should be less depleted at the surface of a narrow absorbing unit such as a hypha. Furthermore, narrow hyphae can grow into small soil pores inaccessible to roots or even root hairs. Another advantage attributed to mycorrhizal

fungi is access to pools of phosphorus not readily available to the plant. One mechanism for this access is the physiochemical release of inorganic and organic phosphorus by organic acids through the action of low-molecular-weight organic anions such as oxalate which can be (Fox, et al., 1990): (i) replace phosphorus sorbed at metal-hydroxide surfaces through ligand-exchange reactions, (ii) dissolve metal-oxide surfaces that absorbs phosphorus, and (iii) complex metals in a solution and thus prevent precipitation of the metal phosphates.

## 1.4 AMF and plant symbiosis

AMF associations form when host roots and compatible fungi are both active in close proximity and soil conditions are favourable. The infection process consist of three different steps: (1) hyphal growth from a germinating spore, dependent initially upon its own nutritional supply; (2) stimulation of further fungal growth by root exudates and initiation of the infection process; and (3) fungal development of intracellular arbuscules which connect the fungus to the nutrient flux from the plant

### 1.4.1. Soil hyphae

Mycorrhizal associations were initiated by spore germination. Hyphae were also originated from fragments of roots. In many cases, there is already a pre-existing network of hyphae resulting from previous root activity. Hyphae resulting from spore germination have a limited capacity to grow and will die if they do not encounter a susceptible root within a week or more.

### 1.4.2. Root Contact and Penetration

Mycorrhizal associations start when soil hyphae respond to the presence of a root by growing towards it, establishing contact and growing along its surface. Next, one or more hyphae produce swellings called appressoria between epidermal cells. Root penetration occurs when hyphae from the appressoria penetrate epidermal or cortical cells to enter the root. These hyphae cross the hypodermis (through passage cells if these are present in an exodermis) and start branching in the outer cortex.

### 1.4.3. Hyphal Proliferation in the Cortex

Aseptate hyphae spread along the cortex in both directions from the entry point to form a colony. Hyphae within root are initially without cross walls, but these may be occurring in older roots. Gallaud (1905) observed that VAM associations in different species formed two distinctive morphology types, which name the **Arum** and **Paris** series after host plants. The differences between these two modes of spread within the root cortex both Arum and Paris type VAM associations are important in ecosystems (Smith & Smith 1997) (Fig. 2).

- In roots with **Arum** series associations, VAM hyphae proliferate in the cortex by growing longitudinally between host cells. This occurs because hyphae grow through longitudinal intercellular air spaces that are present (Brundrett et al. 1995).
- In **Paris** series VAM hyphae spread by forming coils within cells because there are no continuous longitudinal air spaces.

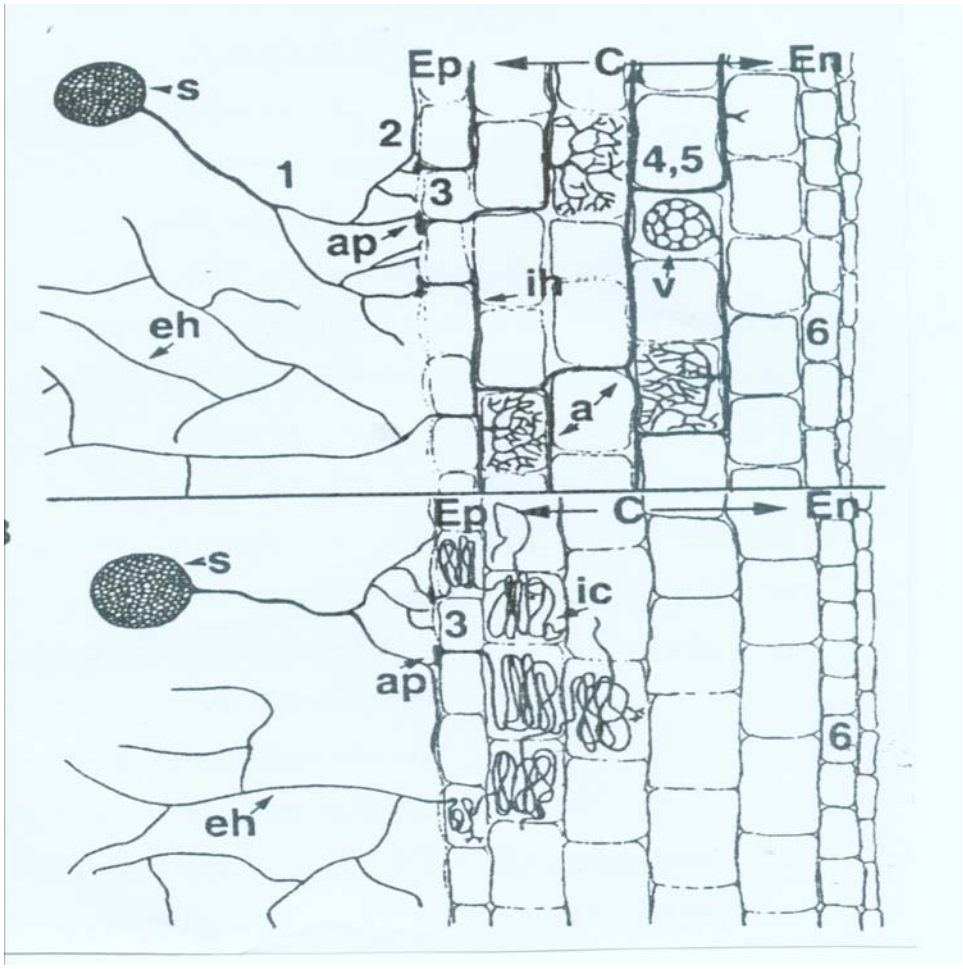


**Figure 2** Cartoon illustrations of AM fungal symbiotic morphologies and regulatory points. Number 1 to 6 indicates molecule or genetic control points described in the text. Ep, Epidermis; C, Cortex; En, endodermis; S, Spore; eh, external hyphae; ap, appressorium; ih, intercellular hypha; a, arbuscular; ic, intracekkukar coil; v, vesicle; A, Arum type and B, Paris type. Note that most control points have not been investigated in this symbiosis type.

### 1.4.4. Arbuscules

Arbuscules are intricately branched haustoria that formed within a root cortex cell. They were named by Gallaud (1995), 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). Arbuscules start to form approximately 2 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 considered the major site of exchange between the fungus and host. This assumption is based on the large surface area of the arbuscular interface, but has not been confirmed (Smith 1995). Arbuscule formation follows hyphal growth, progressing outwards from the entry point. Arbuscules are begun to collapse after a few days, but hyphae and vesicles can remain in roots for months or years.

### 1.4.5. Vesicles

Vesicles were developed to accumulate storage products in many VAM associations. Vesicles are initiated soon after the first arbuscules, but continue to develop when the arbuscules senesce. Vesicles are hyphal swellings in the root cortex that contain lipids and cytoplasm. These may be inter- or intracellular. Vesicles can develop thick walls in older roots and may function as propagules (Biermann & Linderman 1983). Some fungi produce vesicles which are similar in structure to the spores they produced in soil, but in other cases they are different.

### 1.4.6. Spores

Spores form as swellings on one or more subtending hypha in the soil or in roots. These structures contain lipids, cytoplasm and many nuclei. Spores usually develop thick walls with more than one layer and can function as propagules. Spores may be aggregated into groups called sporocarps. Sporocarps may contain specialised hyphae and can be encased in an outer layer (peridium). Spores apparently form when nutrients are remobilised from roots where associations are senescing. They function as storage structures, resting stages and propagules. Spores may form specialised germination structure, or hyphae may emerge through the subtending hyphae or grow directly through the wall.

## 1.5 Molecular Identification of Arbuscular Mycorrhizal Fungi

To investigate the biodiversity of AMF, molecular techniques have been developed, e.g. isozyme analyses (Rosendahl, 1989), specific antibodies (Hahn et al., 1993), RAPD-PCR (Lanfranco et al., 1995) and PCR and DNA fingerprinting of micro satellite regions (Gadkar et al. 1997; Longato and Bonfante 1997; Ze'ze' et al., 1997). The previous studied well-liked isozyme technique has some problems about genetic variation. Rosendahl and San (1992) reported that it could not use the malate dehydrogenase (isozyme taxonomy) for verifying the genetic relationship of *Glomus.* So this approach has limited in taxonomic resolution. For this reason, much effort has been focused on the application of PCR combined with the restriction analysis (Abbas et al., 1996; Redecker et al., 1997; Sanders et al., 1995). In side of the molecular genetic could analyzed studied the ribosomal gene which composes of 18S, 5.8S and 25-28S (conserved regions). These regions were separated by 2 sequences which

called non-translated sequences or intra-genic transcribed spacers: (ITS). These ITS regions (variable regions) found that there were more variation and different in species level (Gares and Bruns, 1993). So, it could be used to identify these fungi on genetic scheme. Furthermore, having described a method that were allows quick and easy PCR amplification and cloning of nearly complete small subunit rDNA (SSU rDNA) genes from AMF. The SSU rDNA has been extensively used in fungal taxonomy and biodiversity studies (Bruns et al., 1992; Berbee and Taylor, 1995). Due to 18S rDNA (SSU rDNA), gene are highly conserved region, and it is not easily changed by environmental factor. In 1998, Vandenkoornhuyse and Leyval elucidated the genetic variation of *Glomus moseae* by using SSU rDNA, and showed that the variation in 18S rDNA sequences in each organism indicates the genetic differences among individuals. As same as another group, Redecker et al., 2000 reported that ITS and 5.8S SSU rDNA could be used to verify the close phylogenetic relationship of species pairs between *Acaulospora leptotichum* NC176 , *G. occultum* HA771, and *A. trappei NB112.* These partial sequences were amplified, cloned and screened for the differences in restriction patterns by restriction-fragment length polymorphism (RFLP) and sequence selected clones to determine their phylogenetic position. The comparison of these patterns could distinguish individual species of AMF. In addition, using single spores for DNA extraction have some advantages over multispore-preparations; for example, it is less susceptible to the contamination with other organisms present in the cultures. The method must be used for the quick and reliable preparation of a large number of samples and is highly reproducible. It could also be used for genes other than the SSU rRNA gene (Schwarzott et al., 2000).

Therefore, the objective of this research was developed the detection strategies for AMF association with tree legume in Thailand. These characteristic was used on the basis of morphological and molecular techniques. This study was focus on *A. mangium* and AMF association as a model for widely used in the government reforestation program.

## Research Objectives

1. To obtain the biodiversity data of AMF infected *Acacia mangium*.
2. To achieve the reliable genetic or taxonomy database this can apply as the quality control program.

# CHAPTER II

# MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Arbuscular Mycorrhizal Fungi (AMF) strains

AM fungal strains were isolated from 5 selected tree legumes rhizosphere. Site selection was conducted in 2 regions, North and Northeast. The sampling site was Nakhon ratchasima, Lopburee, Lampoon, Phetchabun and Nakhon sawan province (Table 2). Some strains were obtained from Dr. Omsub Nopamornbodi, Department of Agriculture, Bangkok

**Table 2** Investigated site of AMF in preliminary study of five tree legumes

From plant rhizosphere

| Species of plant | Site selection |
|---|---|
| *Xylia xylocarpa* Taub. (XY) | Amphur Dankhuntod, Nakhon ratchasima Province<br>Amphur Srithep, Phetchabun Province<br>Amphur Noungbua, Nakhon sawan Province<br>Amphur Maetha, Lampoon Province |
| *Pterocarpus indicus* Willd.(PT) | Amphur Beungsamphun, Phetchabun Province |
| *Millettia leucantha* Kurz (ML) | Amphur Parsang, Lampoon Province |

From plant seedling

| Species of plant | Site selection |
|---|---|
| *Xylia xylocarpa* Taub. (XY) | Amphur Mueng, Nakhon ratchasima Province<br>Amphur Parsang, Lampoon Province |
| *Pterocarpus indicus* Willd.(PT) | Amphur Mueng, Nakhon ratchasima Province<br>Amphur Parsang, Lampoon Province |
| *Millettia leucantha* Kurz (ML) | Amphur Mueng, Nakhon ratchasima Province |
| *Acacia auriculiformis* Cunn (AA) | Amphur Parsang, Lampoon Province |
| *Acacia mangium* Willd. (AM) | Amphur Parsang, Lampoon Province |

## 2.1.2 Chemicals

All chemicals used were laboratory grade, or otherwise specified.

**1. For voucher specimen or spore mounting media**

Polyvinyl alcohol-lacto-glycerol (PVLG) mountant

(Koske and Tessier, 1983)

| | |
|---|---|
| Polyvinyl alcohol | 8.33 g. |
| Distilled water | 50 ml. |
| Lactic acid | 50 ml. |
| Glycerine | 5 ml. |

Melzer's reagent-mixed 1:1 (v/v) with PVLG

(Morton, 1991-1992)

| | |
|---|---|
| Iodine | 1.5 g. |
| Potassium iodide | 5 g. |
| Distilled water | 100 ml. |

**2. Reagents for roots clearing and staining reagents**

50 % ethanol-water (root preservative)

10% KOH (w/v) (exothermic reaction)

50% Glycerol-water (v/v) for destaining andstorage of roots

0.03% (w/v) Chlorozol Black E (CBE) in lactoglycerol (1:1:1 lactic acid, glycerol, CBE+water)

### 2.1.3. Media for pot culture (Brundrett et al., 1994)

**Solution A**: $KH_2PO_4$; $K_2SO_4$; $NH_4NO_3$/2 weeks

add to pot 36 mg/kg soil

**Solution B**: $CaCl_2.2H_2O$ add to pot 150 mg/kg soil

**Solution C**: $MgSO_4.7H_2O$ 20 mg/kg; $MnSO_4.7H_2O$ 10 mg/kg; $ZnSO_4.7H_2O$ 10 mg/kg; $CuSO_4.5H_2O$ 5 mg/kg; $H_3BO_3$ 0.8 mg/kg; $CoSO_4.5H_2O$ 0.4 mg/kg; $Na_2MoO_4.2H_2O$ 0.3 mg/kg soil (volume of addition in pot)

### 2.1.4. Reagents for DNA extraction

0.25 M NaOH

0.25 M HCl

0.5 N Tris-HCl

TE buffer: 10 min. Tris, 1 mM EDTA in distilled water (pH8)

### 2.1.5. Reagents for PCR amplification

Taq DNA polymerase in storage buffer B (Promega),

size: 500 U; concentration: 5u/µl

Magnesium chloride solution, 25 mM

10 X buffer Deoxynucletide triphosphate, 2.5 mM

### 2.1.6. Reagents for agarose gel electrophoresis

DNA marker: 100 bp. Ladder DNA (Promega)

Staining solution: 0.5 μg/ml ethidium bromide in distilled water

Tracking dye: 0.25% bromocresol purple in 50% glycerol

0.05 Tris-acetate (pH 7.9)

TBE buffer (5X): 54 g Tris base, 27.5 g Boric acid and 20 ml of 0.5 EDTA (pH 8)

### 2.1.7. Reagents for restriction analyses

| | |
|---|---|
| Sterilized, Deionized water | 5.7 μl |
| Restriction Enzyme 10X buffer | 3 μl |
| Acetylated BSA, 10 μg/μl | 0.3 μl |
| PCR product, 1 μg/μl | 20 μl |
| Restriction Enzyme, 10U/μl (*Hind* III) | 1 μl |
| Final volume | 30 μl |

## 2.2 Methods

### 2.2.1 Spore extraction from the soil

The most basic of spore isolation is wet sieving and decanting to remove the clay and sand fractions of the soil while retaining spores and other similar-sized soil and organic matter particles on sieves of various sizes. Spores of AM fungi were extracted by the wet-seiving and decanting method (Gerdemann and Nicholson, 1963) followed by sucrose centrifugation (Daniels and Skipper, 1982), and counted under microscope. Under a disecting microscope, spores were sorted twice with a micropipette into a petri dish with sterile tap water. Spores morphotypes were separated according to spore morphotype and colour.

### 2.2.2 Spore propagation

Since these fungi are obligate symbionts and unable to cultured on nutrient media these fungi were propagated by using the pot culture method (Brundrett, 1995). Pot culture was used by the common host plants as corn (Zea mays L.). This plant was watered by media for pot culture (Brundrett et al*.,* 1994) in everyday. After 3-4 months, newly produced spores were harvested and examined.

### 2.2.3 Spore staining/making permanent slide mountant

To obtain the information for basic identification, the specimen slides were prepared according to the method of Kosel and Gemma, 1989. After isolate the spores from fresh pot culture, a minimum of 20 spores per each host plant were collected in glass watch. These spores were analyzed in both forms of intact and

crushed after mounted with PVLG (polyvinyl lactoglycerol) and Melzer's PVLG (INVAM, 1997). The criterion of genus identification was summarized in Table 3.

**Table 3** Taxonomy and identification of arbuscular mycorrhizal fungi in genera level (Bernhard Kleikamp, 2002)

| |
|---|
| **Suborder Glomineae** - forms intraradicle vesicles |
| **Family Glomaceae** - Generally has simple wall structure, inner walls do not react in Melzer's reagent (iodine) |
| Genus *Glomus* - spores borne singly or in sporocarps; |
| subtending hypha present, straight, flared or recurved |
| Genus *Scelerocystis* - obligately sporocarpic, spores organized radially around a central plexus of hyphae |
| **Family Acaulosporaceae** - More complex wall structure (4-6 walls), inner walls may react with Melzer's; outer wall may be ornamented; Spore borne in or on neck of saccule |
| Genus *Acaulospora* - Spore borne on saccule neck; one scar in mature spores |
| Genus *Entrophospora* - Spore borne in saccule neck; two scares in mature spores |
| **Suborder Gigasporineae** - No intraradicle vesicles; huge spores (250 um); bulbous suspensor cell |
| **Family Gigasporaceae** |
| Genus *Gigaspora* - Inner walls present, may react with Melzer's; germination shield absence |
| Genus *Scutellospora*) - Inner walls present, may react with Melzer's; germination shield present |

### 2.2.4 Root staining

To investigate the spore colonization in the plant root (%colonization), fresh roots were placed in a 10% (w/v) KOH solution, warmed at 70°C for 7-8 h, or autoclaved for 2 hrs. Then transferred into 1%HCl solution for 10 min. The roots were then stained with tryphan blue (Phillips and Hayman, 1970). Slides were observed using a light microscope and then were photographed.

### 2.2.5 DNA extraction from the single spore

Before the DNA extraction, all the spores were rinsed twice with sterile distilled water, sonicated and surface sterilized with 250 mg/l kanamycin persulfate for 10 min, and then rinsed 2-3 times again with sterile distilled water before using. The DNA extraction method was partially modified from the protocol of Redcker in 1997. The cleaned spore was crushed within the microcentrifuge tube with a sterile pipette tip in 2 µl of 0.25 M NaOH prior to centrifugation at 3,000 rpm for 1 min. The aliquot were incubated in boiling water bath for 1 min, and then centrifuge at 3,000 rpm for 1 min. Then, 1 µl of 0.5 M Tris-HCl (pH 8.0) and 2 µl 0.25 M HCl were added. Then boiling this suspension for 2 min. The final solution was used as DNA template for PCR amplification.

### 2.2.6 DNA extraction from the plant root

The plant roots were carefully washed with water and the attached debris was removed. The roots were preserved in 50% ethanol until analysis. Plants root were frozen at -20°C and ground to a fine powder with liquid nitrogen in a prechilled (-80°C) mortar and pestle. This DNA extraction method modified according to Bonita

*et al* (1995). The DNA was performed by add 500 µl of 0.5 M Tris-HCl (pH 8.5) to the prepared fine powder that has 5% chelex x-100 (Biorad) before. Then, vortex immediately and incubated in water bath at 95°C 10 min. Next, incubated on ice immediately 5 min. The supernatant was separated from root fragments by centrifugation 30s at max speed. Then, diluted 1:100 or 1:500 in TE buffer and used as PCR remplate.

**2.27. PCR amplification of DNA fron single spore**

SSU rRNA (18 S rDNA) genes were amplified by the method of Redecker, 2000 using DNA primers which overlap with universal primers NS1 and NS2 (White *et al*., 1990). Sequences of these primers are NS1 (5'GTA GTC ATA TGC TTG TCT C'3; Tm 62°C) and NS2 (5'GGC TGC TGG CAC CAG ACT TGC'3; Tm 55°C) (primer sequences of this study was summarized in Table 3.). The PCR reaction was performed in a final volume of 50 µl, contained 0.1-10 ng of DNA template, 10 mM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 10 pmol of each primer and 2 units of taq polymerase. The amplification reaction was performed as follows: 95°C for 30s., then 35 cycles of 95°C for 3 min., 53°C for 30s. and 72°C for 2 min. Amplification was ended at 72°C for 10 min. in the extension step. Control reactions contained no DNA template. Forvisualization of the PCR product, 10 µl of the amplification products were separated by electrophoretically on 0.8% agrarose gels and stained with ethidium bromide prior to observe under UV-transiluminator.

**Table 4** DNA sequences of primers used in this study

| PRIMER | 5'-sequence-3' | Target | Reference |
|---|---|---|---|
| NS 1 | GTAGTCATATGCTTGTCTT | 18s rDNA of general fungi | (White, 1990) |
| NS 2 | GGCTGCTGGCACCAGACTTGC | | |
| GLOM1310 | AGCTAGGYCTAACATTGTTA | *G.mosseae/intraradices* | (Redecker, 2000) |
| GLOM5.8R | TCCGTTGTTGAAAGTGATC | *G. versiforme* | |
| LETC1670 | GATCGGCGATCGGTGAGT | *G.etunicalum/claroideum* | |
| ACAU1660 | TGAGACTCTCGGATCGG | *A. sensustrictoc* group | |
| GIGA5.8R | ACTGACCCTCAAGCAKGTG | *Gigasporaceae* | |
| ARCH1311 | TGCTAAATAGCCAGGCTGY | *A. gerdemannii/A. trappei* | |
| | | *G. occultum/G. brasilianum* | |
| | | *Geosiphon pyriforme* | |
| ITS1f | CTTGGTCATTTAGAGGAAGTAA | Fungal specific ITS regions | |
| ITS1 | TCCGTAGGTGAACCTGCGG | ITS regions of general fungal | |
| ITS4 | TCCTCCGCTTATTGATATGC | | |

### 2.2.8 Nested-PCR amplification of DNA from the plant root

The PCR condition was as same as the conditions of DNA amplify from single spore, except at the primer that used. This reaction used 2 pairs of primers for amplify the DNA (taxon-specific and universal primer) of AM fungi in 5 major phylogenetic groups (Redecker, 2000) and one pair primer for verify the PCR product (ITS1-4; universal primer) (White, 1990). The amplification reaction of first primer (ITS4-NS5; outer primer) was performed like NS1-2 except, different in annealing temperature 50-51°C. Then estimate the quantity of PCR product on 0.8% gel, if no PCR product visible, diluted 1:100/ visible diluted 1:1,000 or 1:10,000 for exceptionally bands. These dilution was tested with universal primer ITS1-4 to verifly amplifiabiliy (reaction same NS1-2) before use for the second PCR step. The next steps were various combinations of Glomales-specific primers, universal primers and ITS1F (specific for fungi): inner primer (Gerdes and Bruns, 1993). By the specific primers were combined into two sets. Set 1) comprised of the reverse specifc primes (GLOM5.8R, GiGA5.8 R) combined with ITS1F. Set 2) comprised of the forward specific primers (ARCH1311, ACAU1660, LECT1670) combined with ITS4.

The PCR reaction were preheated at 61°C and have annealing temperature 61°C, 5 cycles, then 60°C, 25 cycles. The annealing sites of these primers were summarized in Figure. 3.

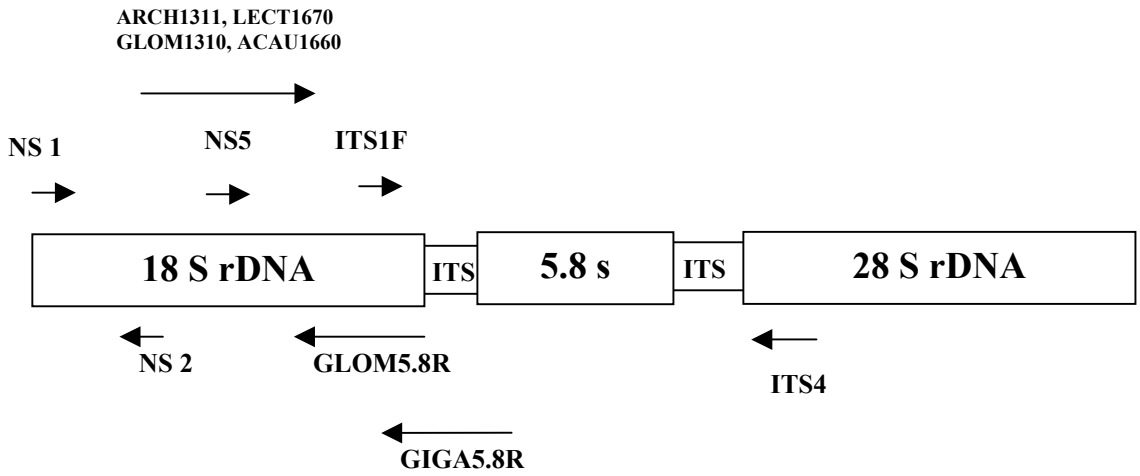


**Figure 3** schematic representations of ribosomal RNA genes with annealing sites of primers (Redecker, 2000).

### 2.2.9 Cloning Technique

The fresh PCR products were purified by using the QIAquick® PCR purification kit (Qiagen) and then cloned into pGEM-T easy vector (Promega) and transformed to the competent cell strain DH5α. The white colonies were pick up from Luria-Bertani (LB) medium containing with 40 mg/ml X-Gal and 50 μg/ml amplicilin and growth in 1 ml LB broth containing with amplicilin at 37°C overnight. Then extract the plasmid DNA was extracted by QIAprep® spin miniprep kit (Qiagen).

### 2.2.10 Sequencing analysis of 18s rDNA

The PCR products from 18S rDNA, and specific regions amplification were further analyzed by automate sequencer and transferred to a sequence analysis program. Blast program was used to search for homologous sequence in data bank (Gene Bank: America).

# CHAPTER III

# RESULTS AND DISCUSSIONS

## 3.1 Morphological study of Arbuscular Mycorrhizal Fungi (AMF)

### 3.1.1 The preliminary investigation of AMF in five tree legumes

AM fungal spores were isolated from soil collected from rhizosphere of tree legumes; *Xylia xylocarpa* Taub. (XY), *Pterocarpus indicus* Willd.(PT), *Millettia leucantha* Kurz (ML), *Acacia auriculiformis* Cunn (AA), and *A. mangium* Willd. (AM). The sites for soil sampling are Nakhon ratchasima, Lopburi, Lamphun, Nakhon sawan, Phetchabun, Chiang Mai provinces of Thailand. Spore isolated was conducted according to the modified wet sieving and decanting method of Germann and Nicolson (1963). Then the morphological study was carried out by staining with PVLG and Melzer's reagent to observe the shape and the ornamental of spore layer. In addition, diameter of spores, spore shape and other details were also determined. The data of all isolates were summarized in Table 4. and Figure. 4-8. The AM spores isolated from 5 tree legumes rhizosphere were identified into 4 genera; *Acaulosopora, Gigaspora, Glomus, Scutellospora* and one unknown *Glomus*-like species. The characterizations of these Genera were investigated. The preliminiary comparison for genera classification was distinguished by basic criteria (Table 3.) as, morphology of spore under microscope, reaction of staining reagent on spore wall. The colour is mainly determined by the relative amounts of the major content within spores, hydrophobic regions of macromolecules (in spore or germinal walls) and intensity of

the reaction is related in part to the length of carbohydrate chains. In most instances to date, the intensity of the reaction is directly correlated to plasticity of the structure in acidic mountants. Sizes of spore were varied from 106 to 330 μm. They performed of many forms such as globose, subglobose, ovoid and irregular shape.

The results from Table 5.implied that the AMF of 5 tree legume rhizosphere might have low plant host specificity. Thus, next study only AMF associated with *A. mangium* was selected for investigation the AMF diversity. Hence, *A. mangium* is fast-growing tree and widely used in the forestation program.

**Table 5** Morphological characteristics of AMF from 5 selected tree legume

| Isolates | Color of spore | | | Diameter (um) | Spore shape | | | | | | | | | other detail | Identification in genus level |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Without staining | Melzer | PVLG | | Globose | Subglobose | ovoid | ellipsoid | pyriform | irregular | oblong | reniform | elavate | | |
| XX1 | red | red | red | 200 | / | / | | | | | | | | germination shield, bulbous s | *Scutellospora* |
| | yellow | yellow | yellow | 110 | / | / | / | | | | | | | small size, globose | *Glomus* |
| PTc2 | yellow | yellow | yellow | 240 | / | / | | | | | | | | germination shield, bulbous s | *Scutellospora* |
| | red | red | red | 206 | / | / | | | | | | | | bulbous suspensor | *Gigaspora* |
| | hyaline | yellow | hyaline | 156 | / | / | | | | / | | | | pitted membrane | *Acaulospora* |
| XX3 | yellow | yellow | yellow | 130 | / | / | / | | | | | | | subtending hyphae | *Glomus* |
| | orange | orange | orange | 164 | / | / | | | | / | | | | pitted membrane | *Acaulospora* |
| | black | black | black | 243 | / | / | | | | | | | | subtending hyphae | *Gigaspora* |
| PT4 | red | red | red | 206 | / | / | | | | | | | | pitted membrane | *Acaulospora* |
| | yellow | yellow | yellow | 200 | / | / | | | | | | | | pitted membrane | *Acaulospora* |
| | red brown | black | brown | 195 | / | / | | | | | | | | subtending hyphae | *Gigaspora* |
| | yellow | yellow | yellow | 135 | / | / | | / | | | | | | subtending hyphae | *Glomus* |

Footnote: XX=*Xylia xylocarpa* Taub. , ML= *Milletia leucantha* Kurz, PT=*Pterocarpus macrocarpus* Willd,
1=Amphur Dankhuntod, Nokhon ratchasima Province, 2=Amphur Beungsamphun, Phetchabun Province and 3,4=Amphur Srithep, Phetchabun Province,

**Table 5** (continued)

| Isolates | Color of spore | | | Diameter (um) | Spore shape | | | | | | | | | other detail | Identification in genus level |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Without staining | Melzer | PVLG | | Globose | Subglobose | ovoid | ellipsoid | pyriform | irregular | oblong | reniform | elavate | | |
| PT5 | black | black | black | 205 | / | / | | | | | | | | subtending hyphae | *Gigaspora* |
| | red | red | red | 198 | / | / | | | | | | | | subtending hyphae | *Gigaspora* |
| | yellow | yellow | yellow | 135 | / | / | | | | | | | | sporocarpic | *Glomus* |
| | black | black | black | 196 | / | | | | | | | | | 2 subtending hyphae | Unknown like *Glomus* |
| PT6 | yellow | yellow | yellow | 200 | / | / | | | | | | | | subtending hyphae | *Glomus* |
| | red | red | red | 230 | / | / | | | / | | | | | subtending hyphae | *Gigaspora* |
| | yellow | yellow | yellow | 180 | / | / | | | | | | | | germination shield, bulbous s | *Scutellospora* |
| ML7 | yellow | yellow-brown | Yellow | 120 | / | / | / | | | | | | | sporocarpic | *Glomus* |
| | yellow | yellow | yellow | 170 | / | | / | | | | | | | subtending hyphae | *Glomus* |
| | red brown | red black | red | 235 | / | | | | | | | | | bulbous suspensor | *Gigaspora* |
| XX8 | hyaline | red | yellow | 250 | / | | | / | | | | | | germination shield, bulbous s | *Scutellospora* |
| | yellow | yellow-orange | yellow | 230 | / | | | | | | | | | pitted membrane | *Acaulospora* |
| | orange | orange | orange | 250 | / | | | | | | | | | germination shield, bulbous s | *Scutellospora* |
| PT9 | yellow | yellow | yellow | 120 | / | | | / | | | | | | pitted membrane | *Acaulospora* |
| | hyaline | hyaline | hyaline | 130 | / | | | | | | | | | germination shield, bulbous s | *Scutellospora* |

Footnote: XX=*Xylia xylocarpa* Taub. , ML= *Milletia leucantha* Kurz, PT=*Pterocarpus macrocarpus* Willd,

5,6=Amphur Nongbua, Nakorn sawan Province, 7=Amphur Phazang, Lampoon Province, and 8,9=Amphur Maetha, Lampoon Province

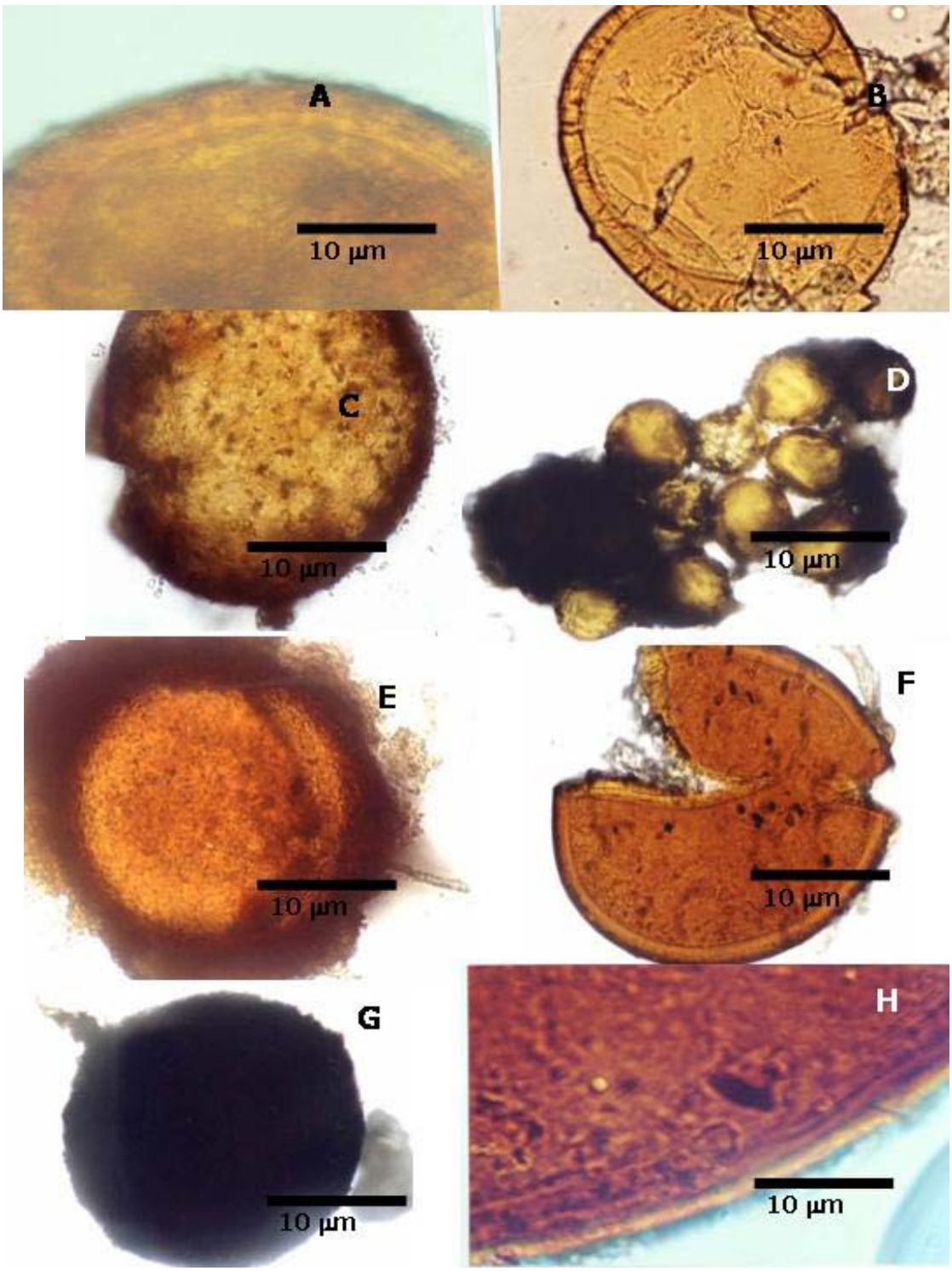


**Figure 4** Isolated spore from rhizosphere of various sites (A, D): *Pt. macrocarpus,* (B, C): *Ml. leucantha*, (E-H): *A. mangium* identified in genus *Gigaspora* (40X) showed typically morphological feature of AMF spore in Genus *Glomus* A-C) spores borne singly or in sporocarps (D); subtending hypha present, straight, flared or recurved (E-G), relatively small spores (A-H)**,** typical of the dead spores often found in field-collected soil (C&E). Isolated spore from 5 tree legumes (40X)

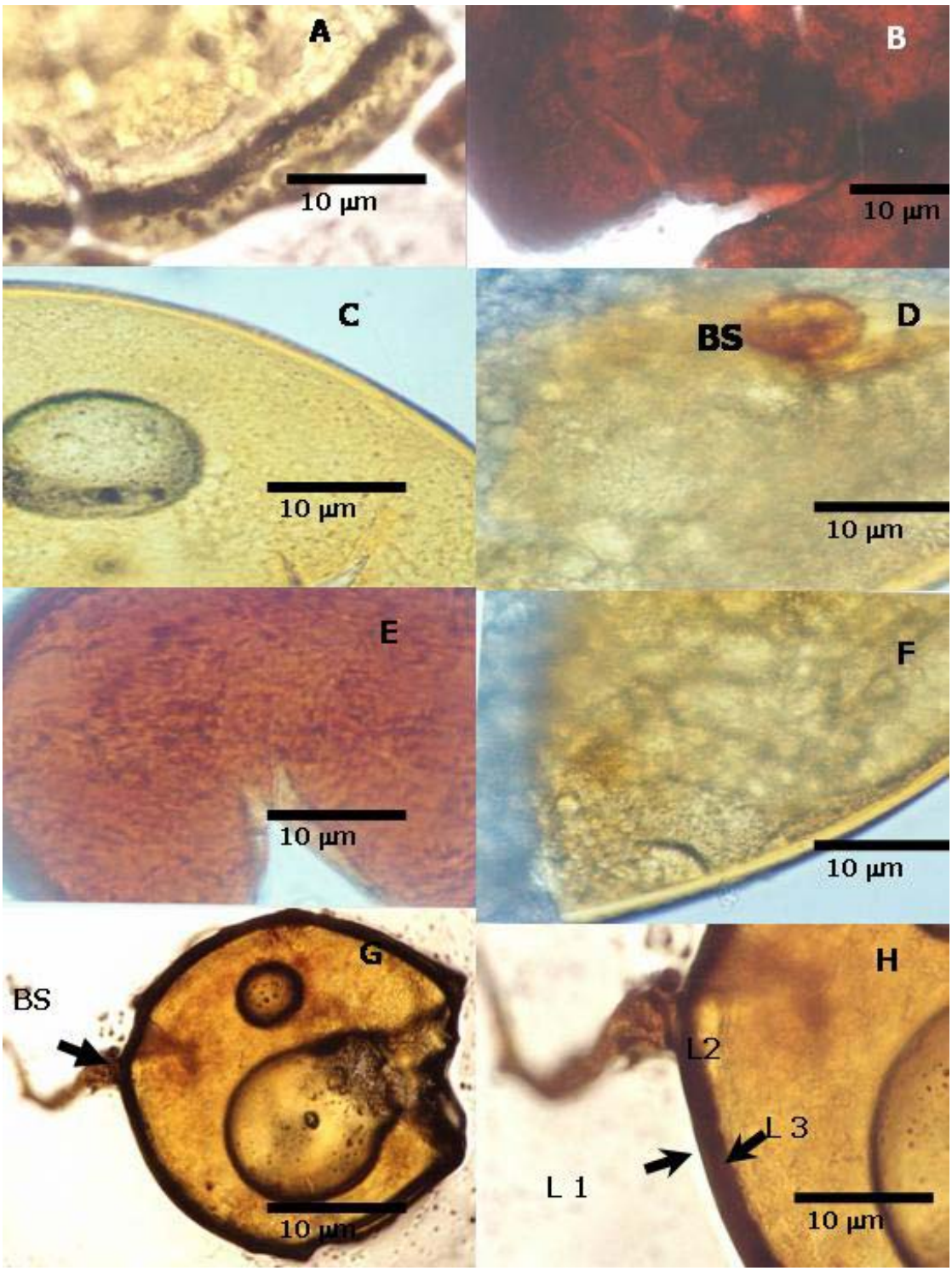


**Figure 5** Isolated spores from rhizosphere of *Pterocarpus indicus* Willd. collected from (A-F) Amphur Maetha, Lampoon Province, (G-H) Amphur Noungbua, Nakgon sawan Province, Show bulbous suspensor, 3 layer identified in Genus *Gigaspora* (40X), BS: bulbous suspensor

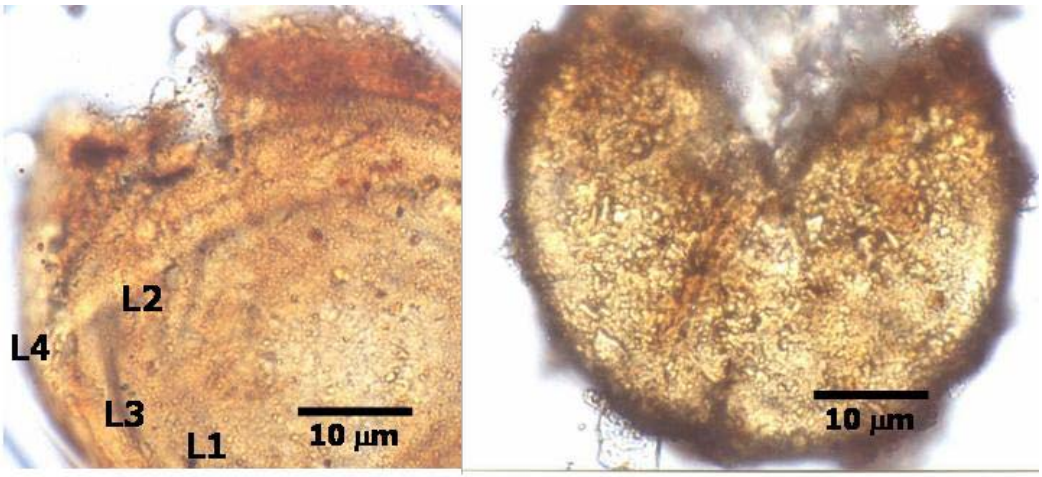


**Figure 6** Isolated spores from rhizosphere of *Pterocarpus indicus* Willd. collected from rhizosphere of *Pterocarpus indicus* Willd. Amphur Beungsampun, Phetchabun Province identified in Genus *Entrophospora* (40X)

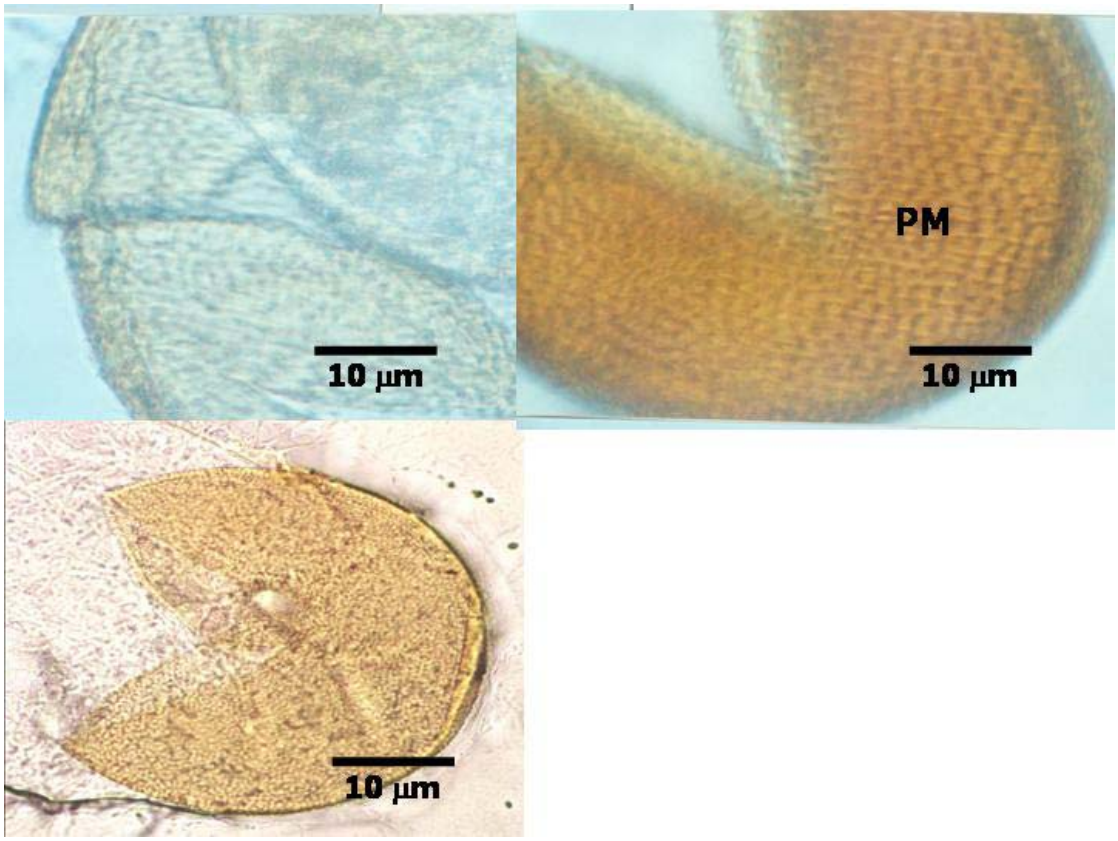


**Figure7** Isolated spores from rhizosphere of *Pterocarpus indicus* Willd. collected from Amphur Phazang, Lampoon Province, identified in Genus *Acaulospora*, show deep pits membrane, 3 layer spore wall (40X)

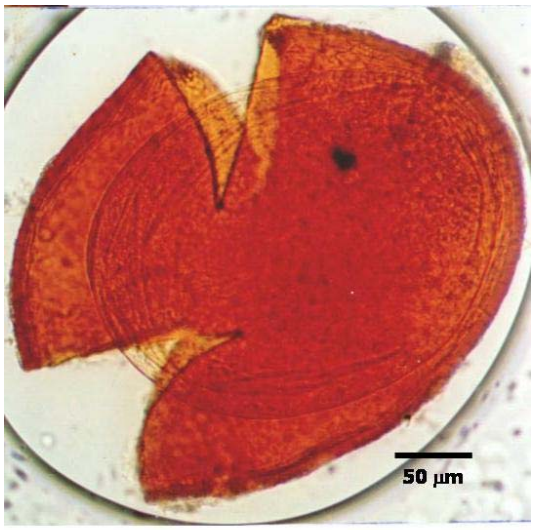


**Figure 8** Isolated spore from rhizosphere of *Xylia xylocarpa* Taub. collected from Amphur Maetha, Lampoon Province, identified in genus S*cutellospora*, show large black spore with deep pits and germination shields (40X)

### 3.1.2 The investigation of AMF in *Acacia mangium* Willd.

The AM fungal spores were isolated from *A. mangium* Willd. (AM) rhizosphere. Whereas the plant roots were extracted to stain for observe the root colonization pattern (Figure 9.). Oblong to irregular vesicles and attached hyphae often cluster within roots; they tend to stain more darkly and often are hard to see. Usually darkest near entry points and lighter near the outer edges of "infection units". The soil sampling was conducted at Nakhon ratchasima province such as the Sakaerat Environmental Research Station (SAK), Dankhuntod nursery forest station and Suranaree University of Technology area. Another collection site was at Lampoon province e.g. Parzang Nusery Forest Station.

The morphological investigation showed thirty different types of AMF found in soil samples (Table 6). From Sakaerat Environmental Research Station (SAK), AMF were found 4 genera containing 11 species. From Suranaree University of Technology (SUT) found 5 genera with 10 species; from Lampoon Nuresery Research Station (LUM) province found 5 genera with 8 species. And from Dankhuntod found 4 genera with 7 species. They were comprised of *A. laevi, A. koskei, Gi. gigantea, Gi. albida, Gi. margarita, Gi. decipiens,, Sc. pellucida , Sc. persica, Sc. heterogama, G. mosseae , G. claroideum , Scelerocystis coremioides* and 1 unidentified species. The species of AMF have been described on the basis of morphological features of the spore that were standardized by Walker (1996) and INVAM worksheet (1997).

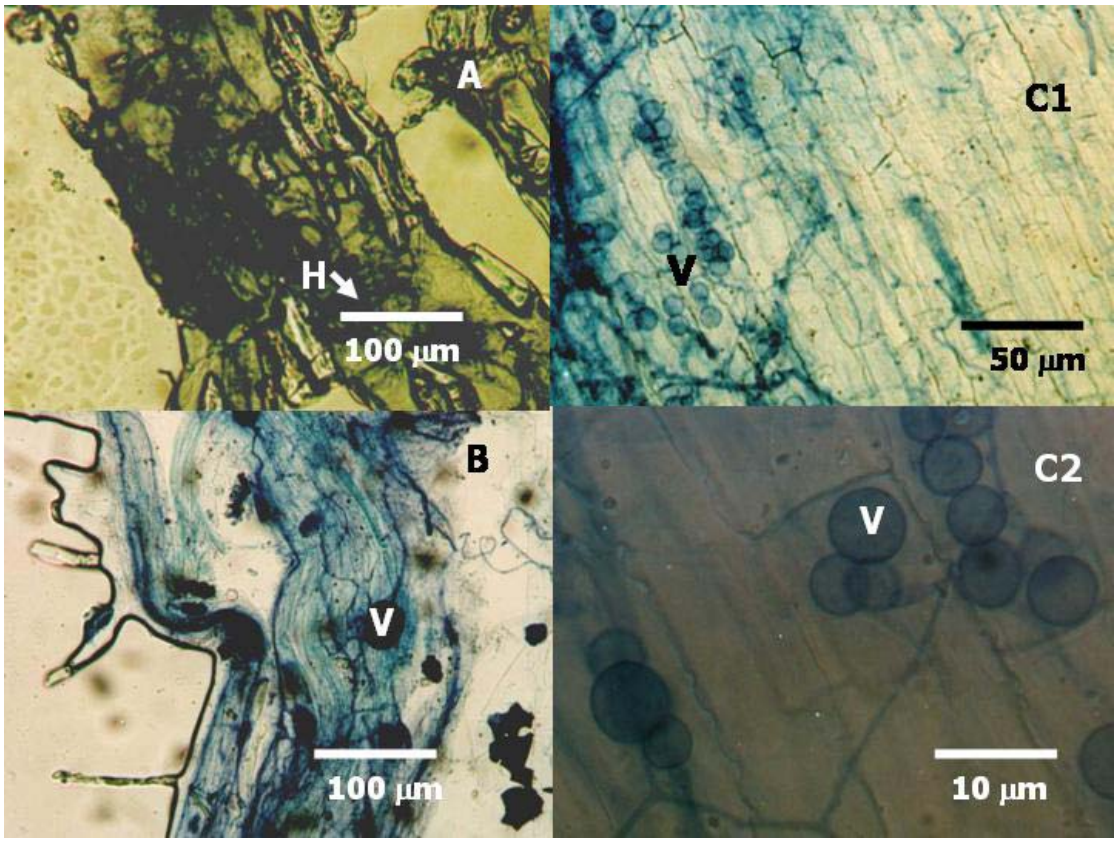


**Figure 9** Illustration of AMF roots infection strained with tryphane blue: A. *A mangium* from Nakhon ratchasima (3 months) (10X), B. *A. mangium* (5 months) from Ropburi (10X), C.1 Maize (4 months) (10X) and C.2 (40X)**,** H: hyphae, V: vesicle

Spores were sorted into morphological groups on the basis of their colour, size, and shape. The colour characteristics were depended on each strain, which may vary from cream, white, hyaline, pale white, yellow to various shades of red to brown or even dark brown.

The data of all isolates were summarized in Figure 10-34 and Table 6. Description of each species was indicated as following;

**1. *Acaulospora laevi*** (Gerd. & Trappe, 1974)

Typically, in *Acaulospora* Genera inner wall layers have been revealed by crushing spores and by staining with Melzer's reagent, but in this species no reaction in Melzer's reagent. This species found in all sites of this study. They have orange-brown color, globose, subglobose shape, size diameter 198 μm. Spores consist of a three-layered spore wall (L1, L2 and L3) and two bi-layered flexible germinal walls. The outer layers (L1) continue with the wall of the neck of the parent sporiferous saccule and inner two (L2) synthesized as the spore expands from the saccule neck **L1:** Hyaline, smooth **L2:** A layer consisting of very fine and often adherent sublayers, pale orange-brown to darker orange-brown,1.6-2.8 μm thick. The surface of this layer is smooth, with no reaction in Melzer's reagent. **L3:** Another layer of lighter yellow-brown color, consisting of very fine tightly adherent sublayers, 1.2-1.6 μm thick. (Figure 10-14).

**Table 6** Morphological characteristics of spores isolated from *Acacia mangium*

| | Color of spore | | | | Spore shape | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Isolates | water | Melzer | PVLG | Size (um) | Globose | Subglobose | ovoid | ellipsoid | pyriform | irregular | oblong | reniform | elavate | other detail | Identification in species level |
| SUTB | black | black | black | 330 | / | / | | | | | | | | bulbous suspensor | *Gigaspora decipiens* |
| SUTO | orange | orange | orange | 200 | / | / | | | | | | | | bulbous suspensor | *Gigaspora gigantea* |
| SUTY | yellow | orange | yellow | 180 | / | | | | | | | | | bulbous suspensor | *Gigaspora albida* |
| SUTH | hyaline | yellow | yellow | 183 | / | | | | | | | | | germination shield | *Scutellospora pellucida* |
| SUTW | white | cream | white | 150 | / | / | | | | | | | | germination shield | *Scutellospora heterogama* |
| SUTR | red | red | red | 184 | / | / | | | | | | | | bulbous suspensor | *Gigaspora gigantea* |
| SUTSc | brown | brown | brown | 206 | | | / | / | | | | | / | clevate wrap by peridium, | *Glomus* spp.. |
| SUTsmY | yellow | yellow | yellow | 130 | / | / | / | / | | | | | | sporocarpic, subtending hyphae | *Glomus claroideum* |
| SUTPW | pale white | white | yellow | 160 | / | / | / | | | | | | | subtending hyphae | *Glomus mosseae* |
| LUMsmY | yellow | yellow | yellow | 135 | / | / | / | / | | | | | | small size, subtending hyphae | *Glomus claroideum* |
| LUMB | brown | black | black | 350 | / | / | | | | | | | | bulbous suspensor | *Gigaspora decipiens* |
| LUMH | hyaline | yellow | yellow | 150 | / | / | / | | / | / | / | / | / | pitted membrane | *Acaulospora laevi* |
| LUMR | red | red | red | 230 | / | / | | | | | | | | bulbous suspensor | *Gigaspora gigantea* |
| LUMO | orange | orange | orange | 245 | / | / | | | | | | | | germination shield | *Scutellospora persica* |
| LUMSc1 | yellow | yellow | yellow | 200 | | | | / | / | | | | / | like sporocarp of Glomus | *Glomus* spp. |
| LUMSc2 | orange | orange | orange | 200 | | | | / | / | | | | | like sporocarp of Glomus | *Glomus* spp. |
| LUMSc3 | red | red | red | 200 | | | | / | / | | | | | like sporocarp of Glomus | *Glomus* spp. |

Footnote : SAK: Sakaerat Environmental Research Station; SUT: Suranaree University of Technology; LUM:Lampoon Province, Phazang Nursery Forest Station; DAN: Nakhon ratchasima Province, Dankhuntod Nursery Forest Station; Color: B=Black, R=Red, W= White, O=Orange, H=Hyaline, sm=Small size, Sc=*Sclerocystic* characteristic. Staining reagent: Melzer and PVLG (Polyvinyl lactoglycerol)

**Table 6.** (continued)

| Isolates | Color of spore | | | Size (um) | Spore shape | | | | | | | | | other detail | Identification in species level |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | water | Melzer | PVLG | | Globose | Subglobose | ovoid | ellipsoid | pyriform | irregular | oblong | reniform | elavate | | |
| SAKsmR | red | red | red | 108 | / | / | | | | | | | | small size, subtending hyphae | *Glomus pansihalos* |
| SAKbY | bright yellow | yellow | yellow | 200 | / | / | | / | | | | | | bulbous suspensor | *Gigaspora gigantea* |
| SAKR | red | red | red | 208 | / | / | | | | | | | | germination shield | *Scutellospora heterogama* |
| SAKO | orange | red | orange | 180 | / | / | | | | | | | | germination shield | *Scutellospora persica* |
| SAKW | white | yellow | white | 180 | / | / | / | | | | | | | bulbous suspensor | *Gigaspora albida* |
| SAKB | black | black | black | 156 | / | / | | | | | | | | bulbous suspensor | *Gigaspora gigantea* |
| SAKY | yellow | orange | yellow | 160 | / | / | | | | | | | | pitted membrane | *Acaulospora laevi* |
| SAKsmW | white | yellow | white | 106 | / | / | / | | | / | | | | subtending hyphae | *Glomus claroideum* |
| SAKSc | brown | black | black | 150 | / | / | | | | | | | | like sporocarp of *Glomus* | *Scelerocystis microcarpus* |
| DANY | yellow | red | yellow | 206 | / | / | | | | | | | | bulbous suspensor | *Gigaspora albida* |
| DANB | black | black | black | 208 | / | / | | | | | | | | globose, 3 wall layer | *Gigaspora decipiens* |
| DANW | white | yellow | white | 200 | / | / | / | | | | | | | germination shield | *Scutellospora heterogama* |
| DANsmW | white | yellow | cream | 107 | / | / | / | | | / | | | | small size, 3 layers wall | *Glomus claroideum* |
| DANR | red | brown | red | 250 | / | / | | | | | | | | germination shield | *Scutellospora persica* |
| DANRB | red | brown | brown | 208 | / | / | | | | | | | | bulbous suspensor | *Gigaspora decopiens* |

Footnote : SAK: Sakaerat Environmental Research Station; SUT: Suranaree University of Technology; LUM:Lampoon Province, Phazang Nursery Forest Station; DAN: Nakhon ratchasima Province, Dankhuntod Nursery Forest Station; Color: B=Black, R=Red, W= White, O=Orange, H=Hyaline, sm=Small size, Sc=*Scelerocystis* characteristic. Staining reagent: Melzer and PVLG (Polyvinyl lactoglycerol)

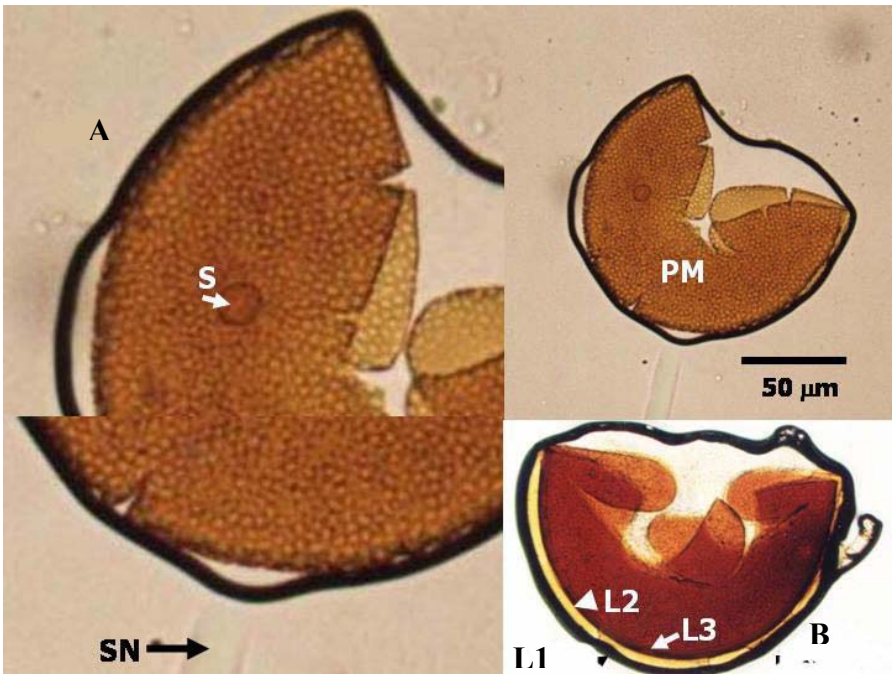


**Figure 10** Isolated spore from rhizosphere of *A. mangium* Willd. collected from Dankhuntod identified into genus *Acaulospora laevi* (40X), A) stained with PVLG, B) stained with Melzer, L: spore wall layer, PM: pitted membrane, SN: saccule neck, S: scar 1 position

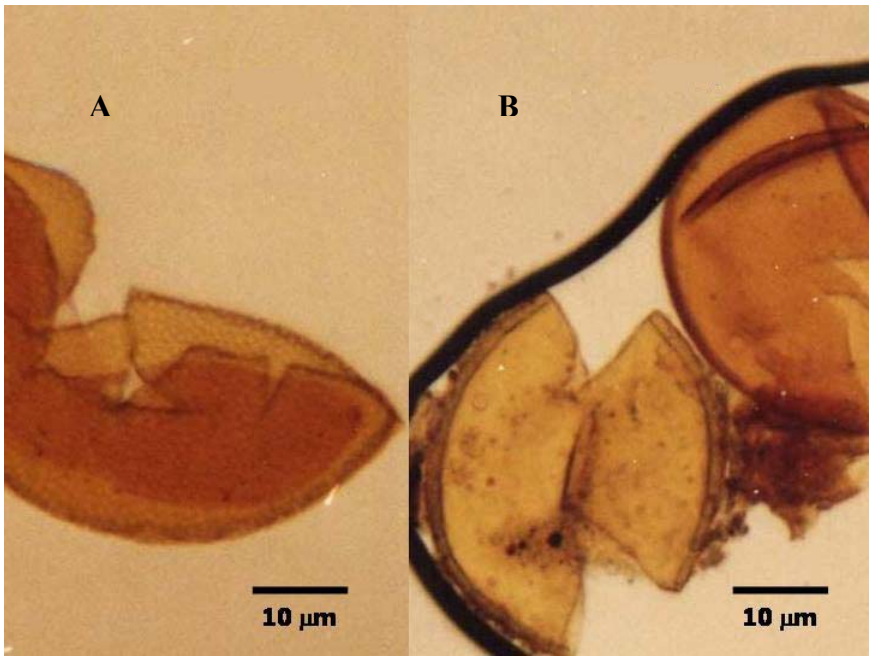


**Figure 11** Isolated spore from rhizosphere of *A. mangium* collect from Suranaree University of Technology identified into genus *Acaulospora laevi* (mature spore) (40X), PM: pitted membrane, A) stained with Melzer, B) stained with PVLG

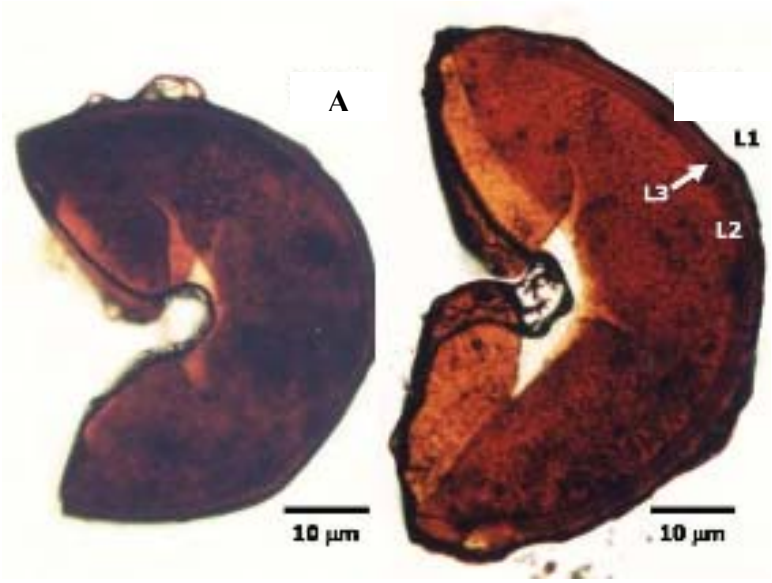


**Figure 12** Isolated spore from rhizosphere of *A. mangium* collected from Suranaree University of Technology identified into genus *Acaulospora laevi* (older or dead spore), bead membrane, 3 layer spore wall (40X), A) stained with PVLG, B) stained with Melzer

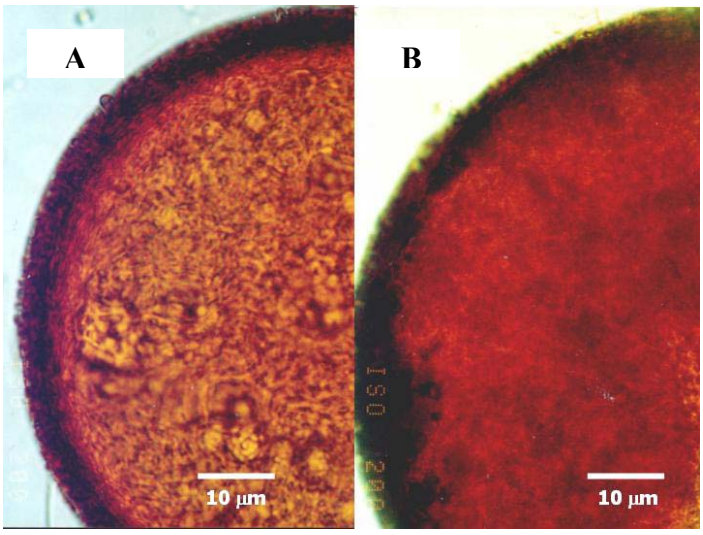


**Figure 13** Isolated spore from rhizosphere collected from *A. mangium* identified into genus *Aculospora laevi* (older spore), mucilaginous and bead membrane, red color in melzer and PVLG, A) stained with Melzer, B) stained with PVLG

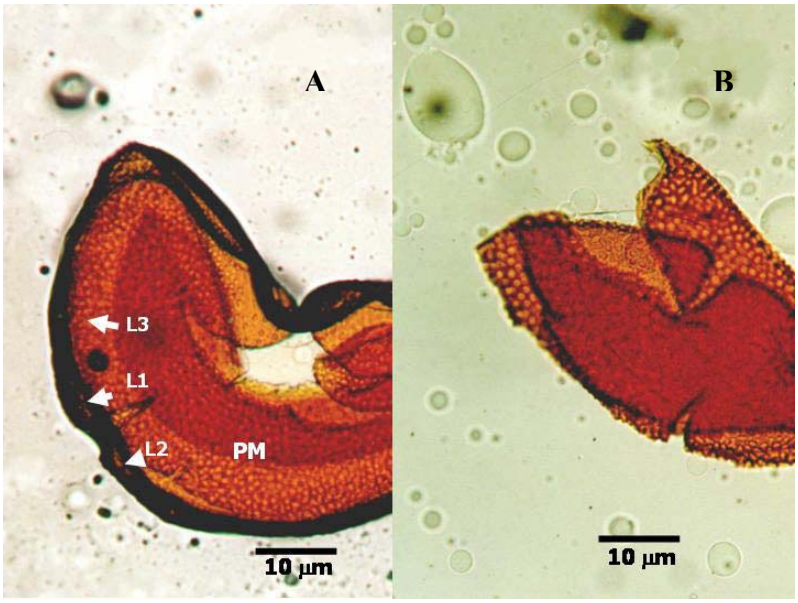


**Figure 14** Isolated spore from rhizosphere collect from *A. mangium* identified into *Acaulospora laevi* (immature spore), PM: pitted membrane, L: spore wall layer, A) stained with Melzer, B) stained with PVLG

**2. *Acaulospora koskei* (Błaszk, 1995)**

This specie found in Suranaree University of Technology site only. They have Pale yellow-brown to dark orange-brown, most pale orange-brown color, globose, subglobose, some oblong to irregular shape, size diameter 120-240 µm. Spore consists of three layers (L1, L2, and L3), the outer one continuous with the wall of the neck of the parent sporiferous saccule and inner two synthesized with expansion of the spore. This species closely resembles *A. laevis* in size and color; differing mostly in that it produces a reaction to Melzer's reagent in L3 of the spore wall (not reactive in spores of *A. laevis*) and L2 of gw2 (germinal wall) (not reactive or only faintly reactive in spores of *A. laevis*). Under a dissecting microscope, these two species are indistinguishable (Figure 15).

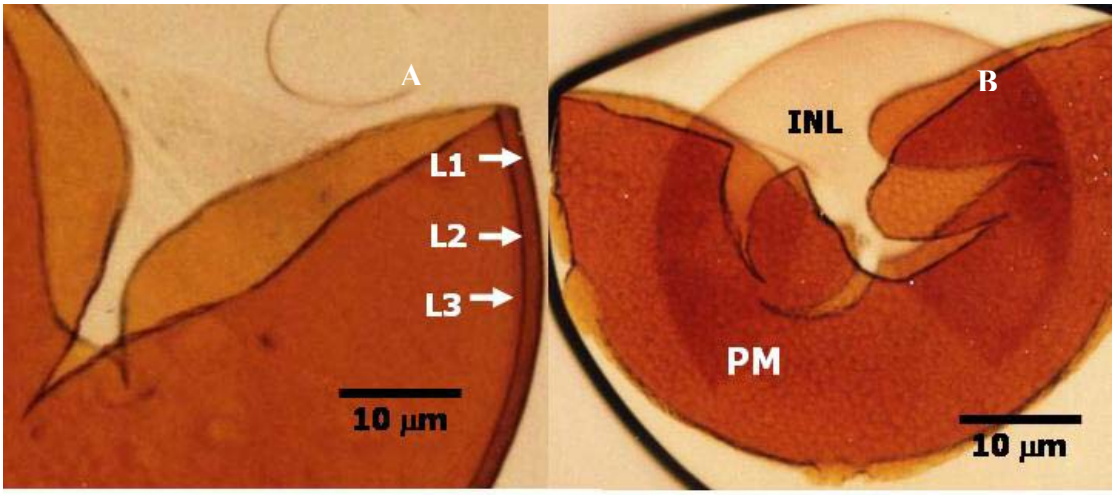


**Figure 15** Isolated spore from rhizosphere of *A. mangium* collected of Suranaree University of Technology identified into *Acaulospora koskei* (mature spore), bead membrane, when broken the spore, 2 spore were observed (40X) , INL: inner wall stained with Melzer, PM: pitted membrane, L: spore wall layer, A) stained with PVLG, B) stained with Melzer

**3. *Scutellospora persica*** (Walker et al., 1993)

This species was found in Lampoon, Dankhuntod and Suaranaree University of Technology not in Sakaerat. They have pale to dark red color, globose to subglobose shape, size diameter 313 µm. Their spore walls have two layers (L1 and L2) that are adherent in juvenile spores are of equal thickness, with L2 thickening as the spore wall grows and differentiates (**L1:** an outer permanent rigid layer, yellow-brown, 1.0 µm thick. The surface consists of many rounded warts 0.5 µm wide; **L2:** a layer consisting of orange-brown to dark orange-brown, sublayers (or laminae) that increase in number with thickness, 8.0 µm thick in mature spores. This layer stains an orange-red to red-brown color in Melzer's reagent. The germination shield has hyaline to pale Yellow, immature spores are pale cream (figure 16).

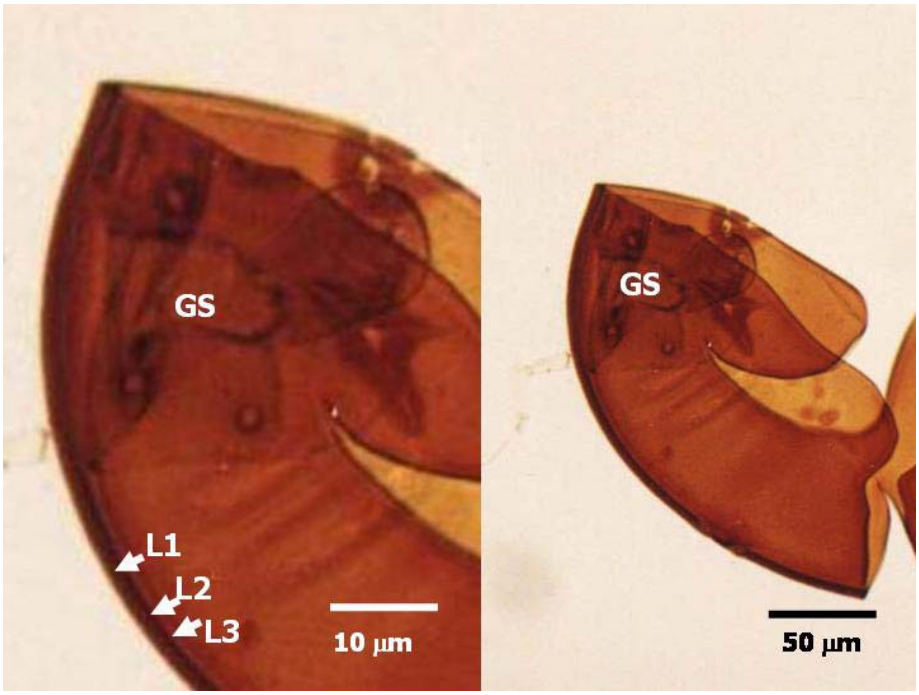


**Figure 16** Isolated spore from rhizosphere of Dankhuntod identified into *Scutellospora persica*, bulbous suspensor, germination shield have yellow color, L1, L2 and L3 have hyaline color both in PVLG and melzer's reagent (40X), GS: germination shield, L: spore wall layer

4. *Scutellospora pellucida* (Koske, R. E. and C. Walker. 1986)

This species was found in almost sites but not at Dankhuntod. The colors of spores are hyaline/white in younger spores to yellow-brown in older spores (especially those from field soils). The spore shapes are globose, subglobose, often elliptical or strongly oblong, spore diameter size 189 µm. The spore wall has three layers (L1, L2 and L3) that are adherent that in juvenile spores are of equal thickness, with the laminate layer (L2) thickening as the spore wall is differentiated. L1: An outer permanent rigid hyaline layer with a smooth surface, 1.8-5.0 µm thick and tightly adherent to L2. Easily distinguished from L2 when spores are placed in Melzer's reagent, where L1 is nonreactive and L2 stains dark red-purple. L2: A layer

consisting of very fine adherent hyaline to pale yellow, sublayers (or laminae), 3.0-8.8 µm thick in mature spores; staining dark red-purple to reddish-black in Melzer's reagent. **L3:** A very thin hyaline flexible layer, < 1 µm thick, can be seen in vigorously crushed spores. The germination shield has Hyaline to pale yellow-brown color, ovoid shape. Margins of shields are generally smooth, with few folds (each with paired germ holes). The shield is sufficiently robust to separate intact from the inner flexible walls when spores are broken. Older spores resemble those of *S. calospora* under a dissecting microscope, except they are more oblong and larger in size (Figure.17-18).

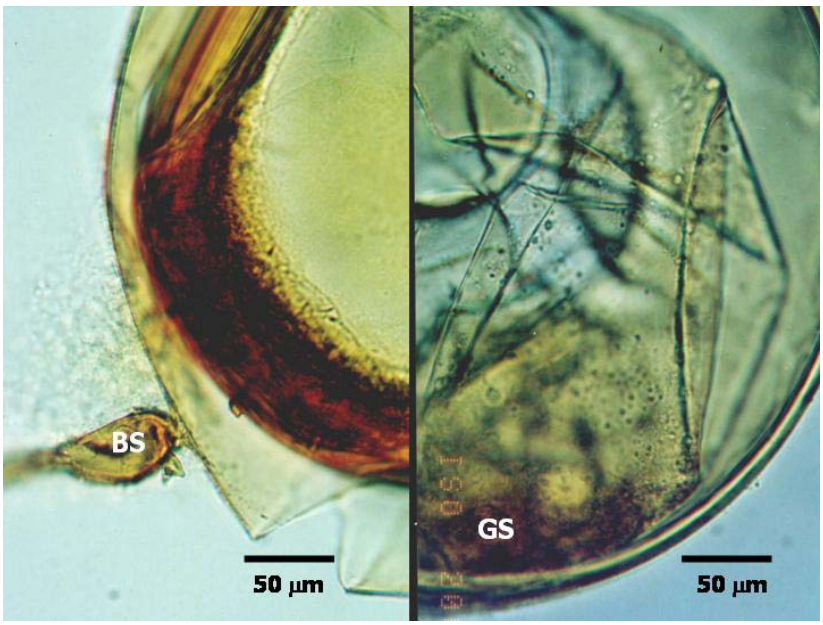


**Figure17** Isolated spore from rhizosphere collected of Suranaree University of Technology identified into *Scutellospora pellucida*, bulbous suspensor, germination shield have yellow color, L1, L2 and L3 have hyaline color both in PVLG and melzer's reagent (40 X), BS: bulbous suspensor, GS: Germination shield, broken spore stained with PVLG

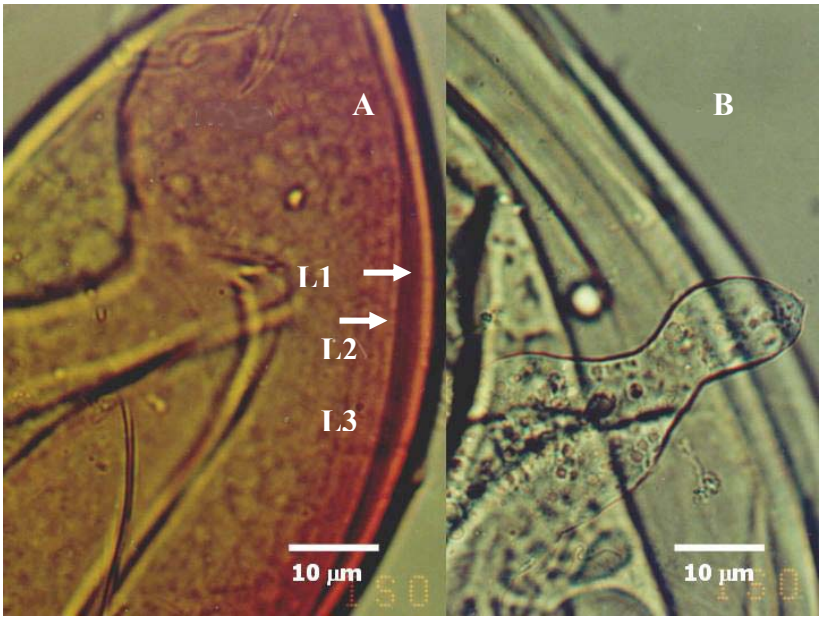


**Figure18** Isolated spore from rhizosphere of *A. mangium* collected from Sakaerat identified into *Scutellospora pellucida*, 3 layer spore wall, hyaline color in L1, L2 and L3, A) stained with Melzer, B) stained with PVLG

5. *Scutellospora heterogama* (Koske, R. E. and C. Walker. 1985)

This species was found only in Suranaree University of Technology. The spores have dark orange-brown to red-brown color, most tending toward the latter at maturity. Immature spores are white to cream with a rose tint under a dissecting microscope and in water (in Melzer's reagent they will turn almost black). The spore shape are subglobose to oblong, diameter size 120-200 µm. Spore wall was consisted of three layers (L1, L2, and L3) with L1 and L2 of equal thickness. **L1:** An outer permanent rigid layer has pale brown in color. **L2:** A layer consisting of fine orange-brown to red-brown sublayers (or laminae) in mature spores. This layer stains dark red-brown in Melzer's reagent. **L3:** A very thin hyaline flexible layer, < 1 µm thick,

can be seen in vigorously crushed spores, usually only where it attaches to the spore wall near the occluding plug and the sporogenous cell region. The germination shield was presented in spore in color pale yellow-brown to darker orange-brown, oblong, the margin of the shields is fairly smooth, with only a few folds and attendant paired germ holes. Shape of the shield resembles that of a violin. As the laminate layer of the spore wall (L2) becomes rigid, it then acquires orange and red pigmentation (Figure.19).

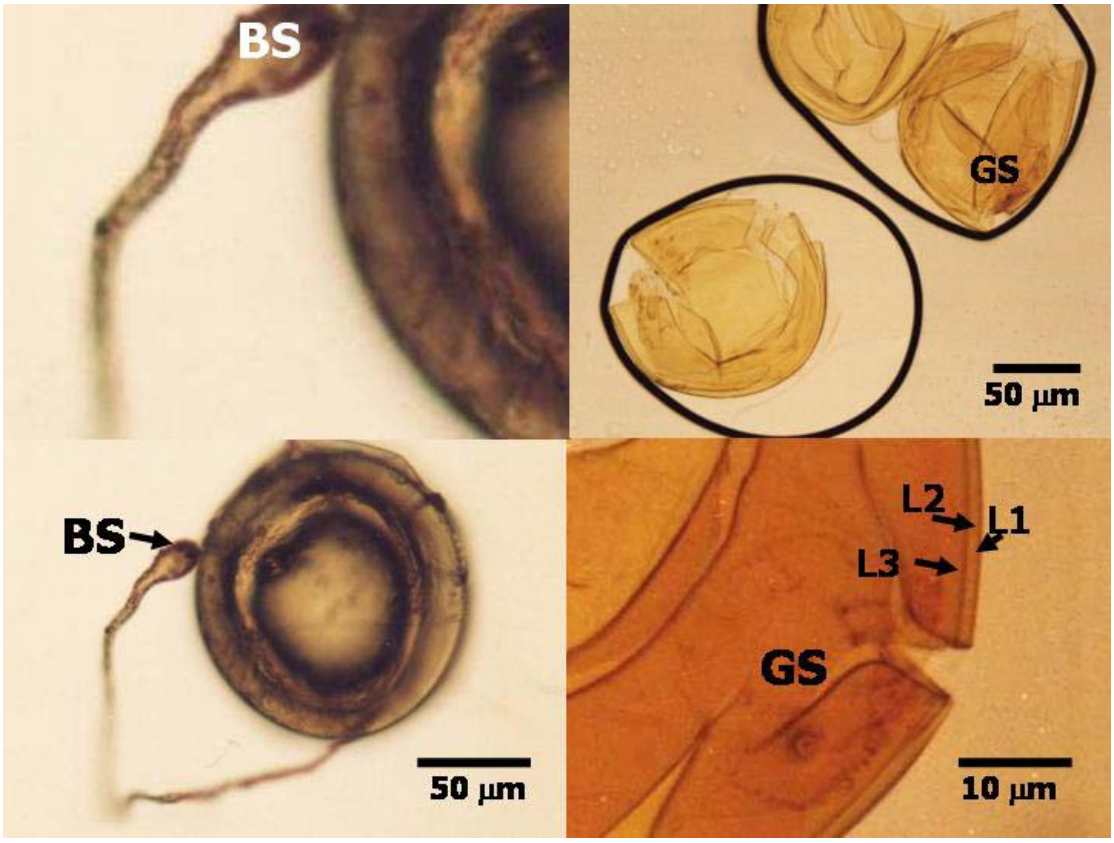


**Figure 19** Isolated spores from rhizosphere collected from Suranaree University of Technology identified into *Scutellospora heterogama*, bulbous suspensor, germination shield have hyaline color in melzer (40X) BS: bulbous suspensor, GS: germination shield, L: spore wall layer

6. *Gigaspora margarita* (W.N. Becker & I.R. Hall, 1986; Bentivenga, S. P. and J. B. Morton. 1995).

This species was found only in Dankhuntod, not found in the other sites of this study. The spore have white to cream in many spore to darker yellow in some generations or some isolates**,** globose to subglobose shape, diameter size 260-400 µm. The spore wall consist of three layers (L1, L2, and L3), the first two adherent and of equal thickness in juvenile spores, with L2 thickening as the spore wall is differentiated and L3 differentiating as a prelude to germ tube formation. **L1:** An outer permanent rigid layer, smooth, adherent to inner laminae, pale brownish-yellow **L2:** A layer consisting of hyaline sublayers (or laminae) that increase in number with thickness are rigid exhibit with some plasticity (swelling and spreading) when broken, yellow to brownish yellow in PVLG; staining dark red-brown to very dark red-purple in Melzer's reagent. In younger spores, sublayers merge and resemble "waves" without sharp transitions from ridge to trough. **L3:** A "germinal" layer that is concolorous and adherent with the laminate layer. Immature spores are salmon colored with a slight pink tint (Figure. 20-21).

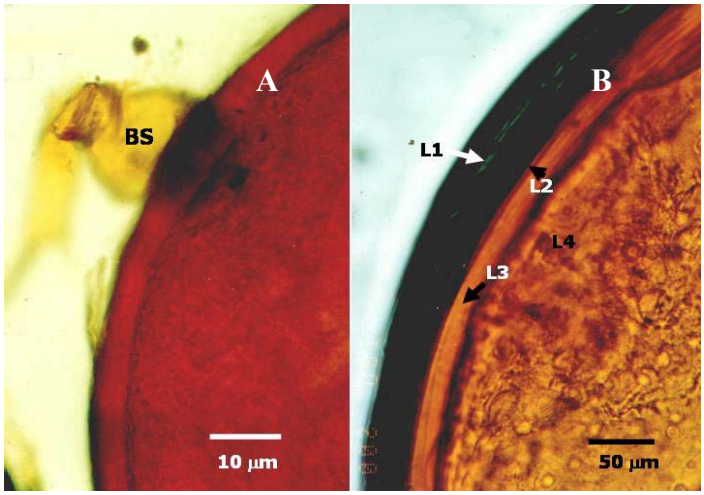


**Figure 20** Isolated spore from rhizosphere collected from *A. mangium* of Suranaree University of Technology identified into *Gigaspora margarita*, bulbous suspensor have yellow color in melzer, spore wall inner have red color in melzer, orange color in PVLG (40X), BS: bulbous suspensor, L: spore wall Layer, A) stained with melzer, B) stained with PVLG

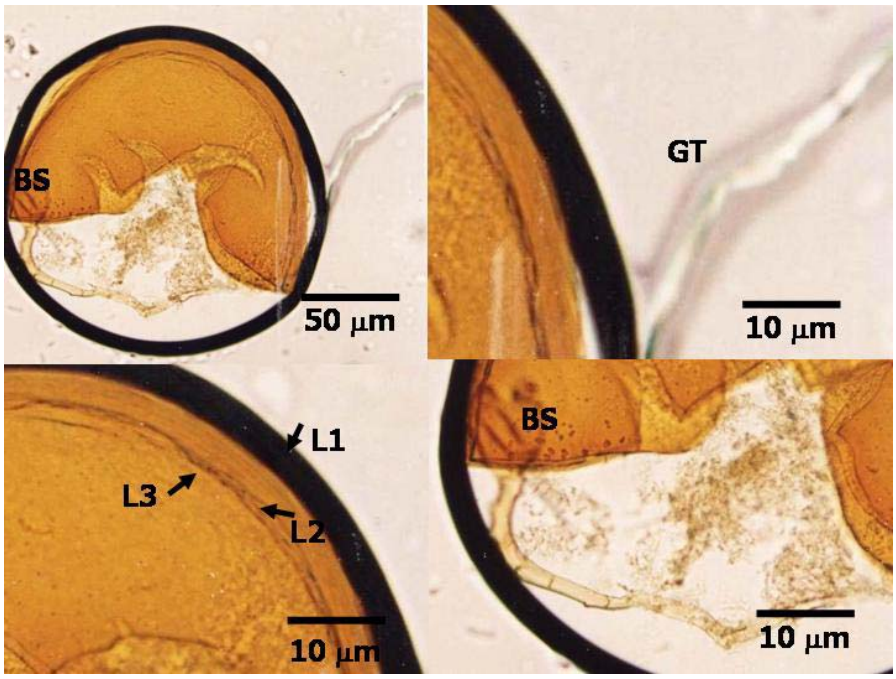


**Figure 21** Isolated spore from the rhizosphere of *A. mangium* collected from Dankhuntod identified in *Gigaspora margarita* show sporogenous cell or bulbous suspensor, spore wall 3 layer and germ tube (40X), BS : bulbous suspensor, GT : germ tube, L: spore wall layer, broken spore stained with PVLG

**7. *Gigaspora gigantea*** (Gerd. & Trappe, 1974)

This species has bright greenish yellow to bright yellow-green, globose to subglobose, rarely irregular shape, size diameter 260 µm. Spore consist of three layers (L1, L2, and L3), the first two adherent and of equal thickness in juvenile spores, with L2 thickening as the spore wall is differentiated and L3 differentiating as a prelude to germ tube formation. Some spores collected from the field appear leached out, and thus can be confused with *Gi. albida* at the small end of the size range (Figure 22-25).

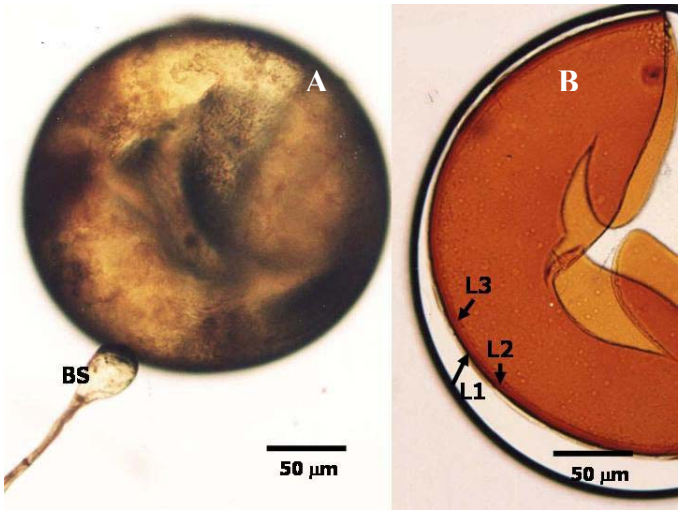


**Figure 22** Isolated spore from rhizosphere of *A. mangium* collected from Amphur Dankhuntod identified into genus *Gigaspora gigantea* showed bulbous suspensor and spore wall when stained the broken spore with Melzer's reagent (40X), BS : bulbous suspensor, L: spore wall layer, A) stained with PVLG, B) stained with Melzer

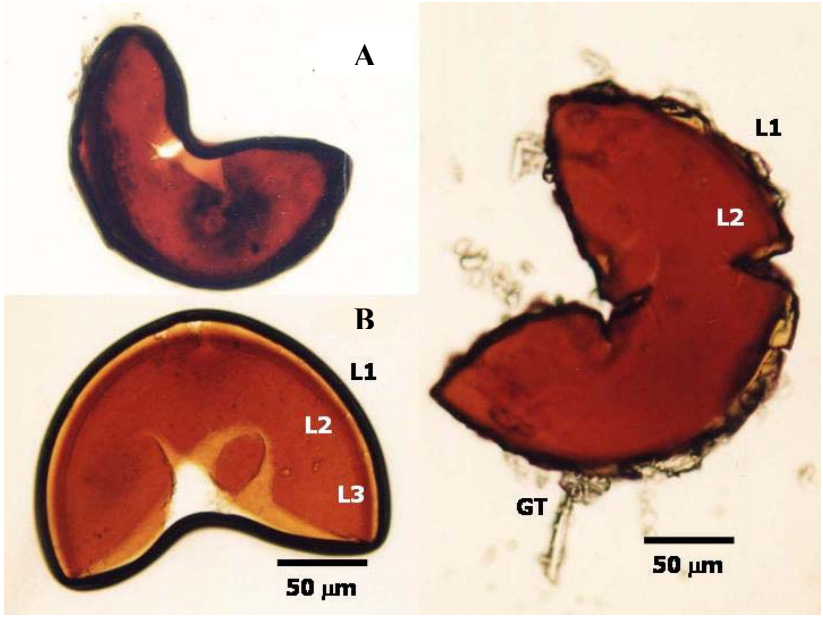


**Figure 23** Isolated spore from rhizosphere of *A. mangium* collected of Suranaree University of Technology, Nakhon ratchasima province identified into *Gigaspora gigantea* (immature spore), GT: germ tube, L: spore wall layer (40X), A) stained with Melzer, B) stained with PVLG

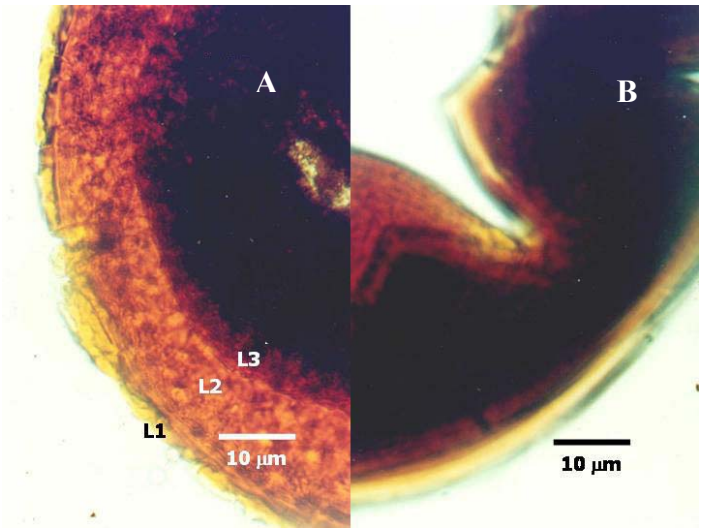


**Figure 24** Isolated spore from rhizosphere of *A. mangium* collected from Amphur Sakaerat, Nakhon ratchasima province identified into *Gigaspora gigantea*, L: spore wall layer, A) stained with PVLG, B) stained with Melzer

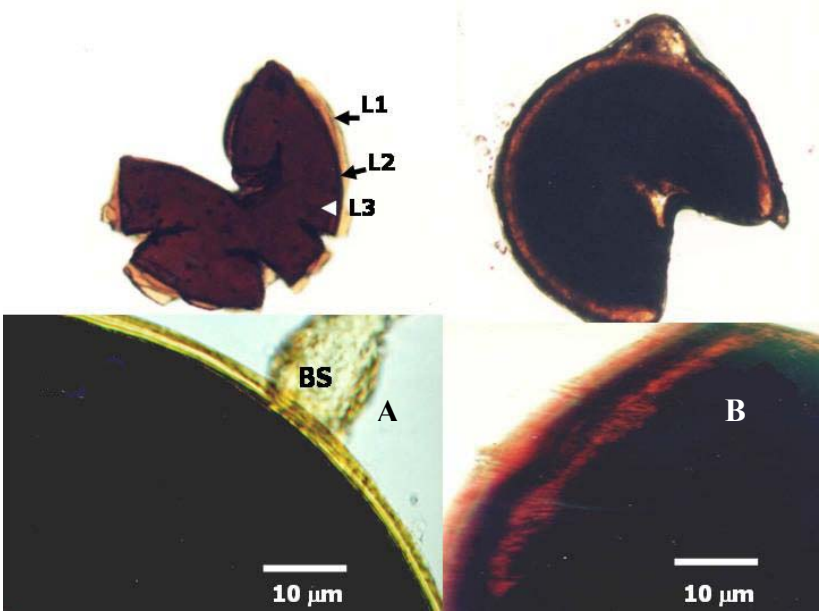


**Figure 25** Isolated spore from rhizosphere of *A. mangium* collected from Suranaree University of Technology identified into *Gigaspora gigantea* (mature spore), BS: bulbous suspensor, L: spore wall layer, A) stained with PVLG, B) stained with Melzer (40X)

8. *Gigaspora albida* (N.C. Schenck & G.S. Sm., 1982)

This species was found only in Sakaerat, not found in the other sites of this study. This spore has cream with pale green tint color, globose to subglobose shape**,** spore diameter size 200 - 280 µm. The spore wall has 3 layer**s; L1:** An outer permanent rigid layer with a smooth surface, hyaline to pale yellow, **L2:** A semi-plastic layer consisting of yellow to brownish yellow sublayers (or laminae) that increase in number with differentiation; varying considerably in thickness in mature

spores. Sublayers are pale brown in PVLG, staining dark red-brown to a very dark red-purple in Melzer's reagent. **L3:** A "germinal" layer that is concolorous with the L2 layer and is adherent. The Subtending hypha was presence, occlusion: Closure by a plug concolorous with L2 of the spore wall. Immature spores are often salmon-colored with a slight pink tint. Spores collected from the field can be dark brown to black from parasitism and colonization of the spore lumen by a wide range of soil microorganisms (especially actinomycetes and other saprobic fungi). This species is often confused with *Gi. gigantea* in the field because both can become brown with a green tint when old, parasitized, or dead (Figure 26).

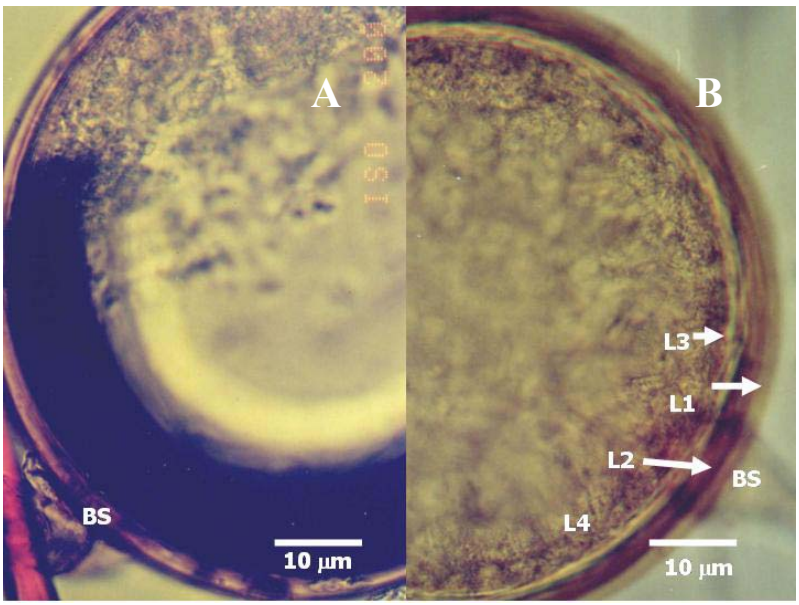


**Figure 26** Isolated spore from rhizosphere of *A. mangium* collected from Amphur Sakaerat, Nakhon ratchasima province identified into *Gigaspora albida*, L: spore wall layer, BS: bulbous suspensor, A) stained with PVLG, B) stained with Melzer

9. *Gigaspora decipiens* (I.R. Hall & L.K. Abbott , 1984)

This species was found only in Suranaree University of Technology and Lampoon province only. The spores have white to cream color, becoming yellow-brown with age or prolonged storage, spore shape**:** Globose to subglobose. Spore dimeter size**:** 358 μm. The spore wall was consisting of three layers (L1, L2, and L3). **L1:** An outer permanent rigid layer, smooth, adherent to sublayers of L2, **L2:** A layer consisting of hyaline sublayers (or laminae) that increase in number with thickness, are rigid, exhibit some plasticity when broken, pale yellow to yellow in newly formed spores, turning a darker brownish yellow with age and storage. Sublayers stain dark red-purple (almost black) in Melzer's reagent. In younger spores, sublayers merge and resemble "waves" without sharp transitions from ridge to trough. **L3:** A "germinal" layer that is concolorous and adherent with the laminate layer. Numerous "warts" or "papillae" form on the inner surface of this layer, and they are especially concentrated in regions where germ tubes form (usually in close proximity to the suspensor cell). Immature spores are salmon colored with a slight pink tint. Spores of this species are similar to those of *Gi. margarita* except for small differences in color (actually a continuum) and greater thickness and refractivity of the L2 layer of the spore wall (Figure 27-29).

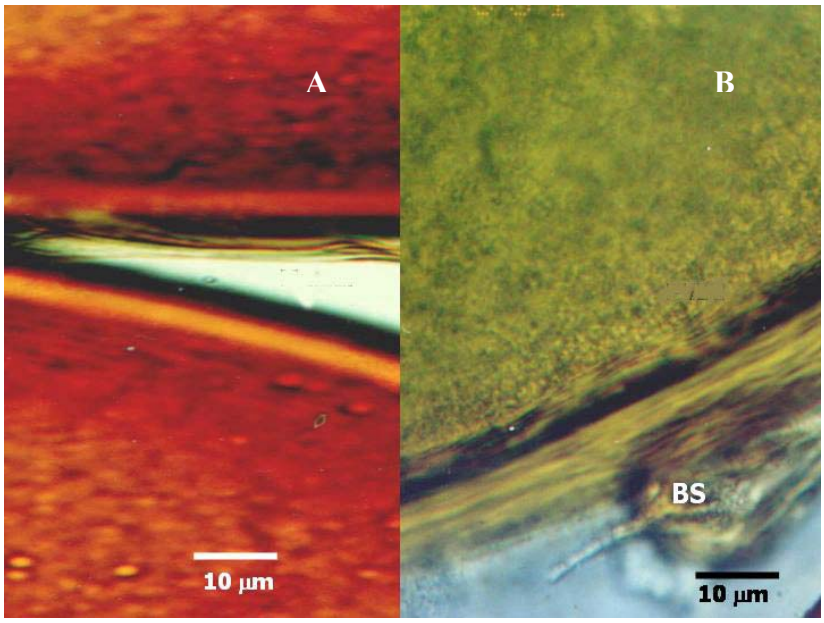


**Figure 27** Isolated spore from rhizosphere of *A. mangium* colleted from Suranaree University of Technology, Nakhon ratchasima province identified into genus *Gigaspora decipiens*, BS: bulbous suspensor (horizontal form), A) stained with Melzer, B) stained with

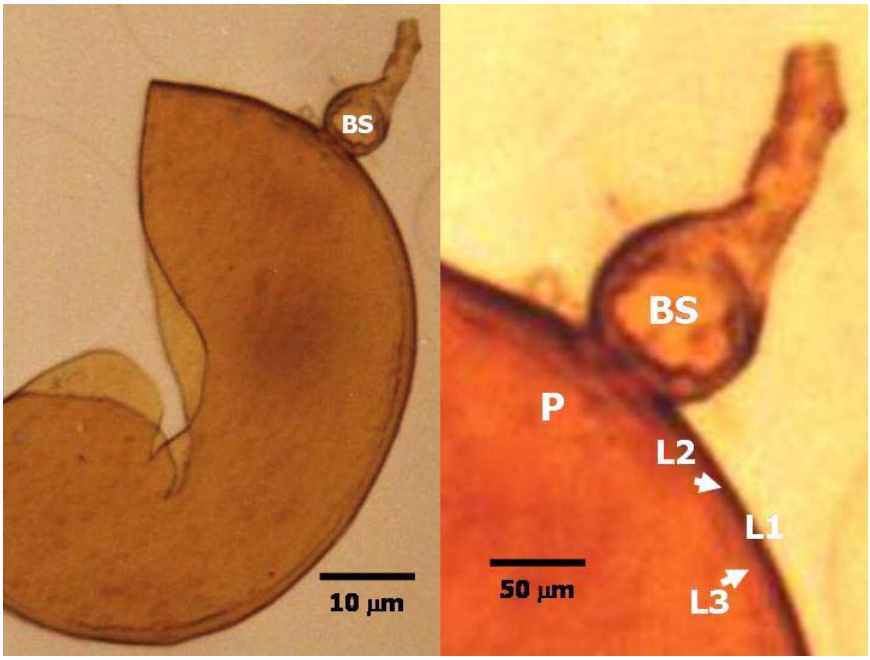


**Figure 28** Isolated spore from rhizosphere of *A. mangium*. from Amphur Sakaerat, Nakhon ratchasima province identified into genus *Gigaspora decipiens*, BS: bulbous suspensor P: plug, L: spore wall layer, L1: rigid membrane, L2: laminae wall, L3: geminal wall

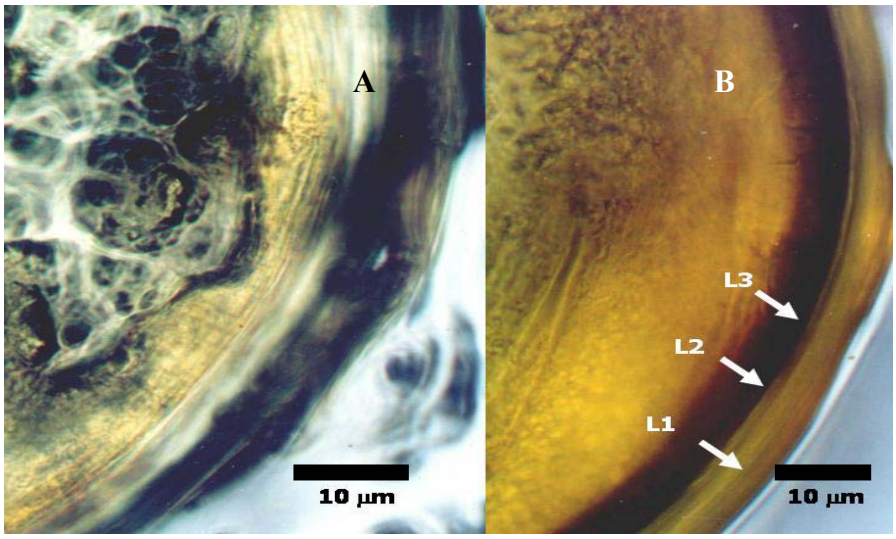


**Figure 29** Isolated spore from rhizosphere of *A. mangium* collected from Lampoon province identified into genus *Gigaspora decipiens*, A) parasitism attack spore, B) healthy spore, L: spore wall layer (40X)

**10. *Glomus caledonium*** (T.H. Nicolson & Gerd. and Trappe & Gerd, 1974)

This species was found only in Amphur Sakaerat, Nakhon Ratchasima province not found in the other sites of this study. They have pale orange-yellow to yellow-brown color or orange-brown color in the field or after long storage, globose to subglobose, rarely irregular shape, diameter size259 µm. The spore walls have four layers (L1, L2, L3 and L4); **L1:** Outer layer, hyaline, mucilagenous spores, stains a pale pink usually only on the wall of the subtending hypha near region of attachment to the spore. **L2:** A rigid hyaline layer, 1.5-3.5 µm thick in juvenile spores, no reaction produced in Melzer's reagent. **L3:** A hyaline layer of granular consistency, highly refractive in polarized light (differential contrast optics), usually attached to

L2, 0.8-1.6 µm thick when L2 is not separated from L4, otherwise 2-3.5 µm thick. **L4:** A layer consisting of pale yellow-brown sublayers (or laminae) that originally is one very thin (< 0.5 µm) sublayer and then thickens with synthesis of additional sublayers; 4-6.4 µm thick in mature spores.Under a dissecting microscope, spores of this species closely resemble *Glomus mosseae*. Published reports tend to confuse this species with both *G. mosseae* and *G. clarum* (which is of similar size and can be of similar color, but usually is more pale yellow to white) (Figure 30).

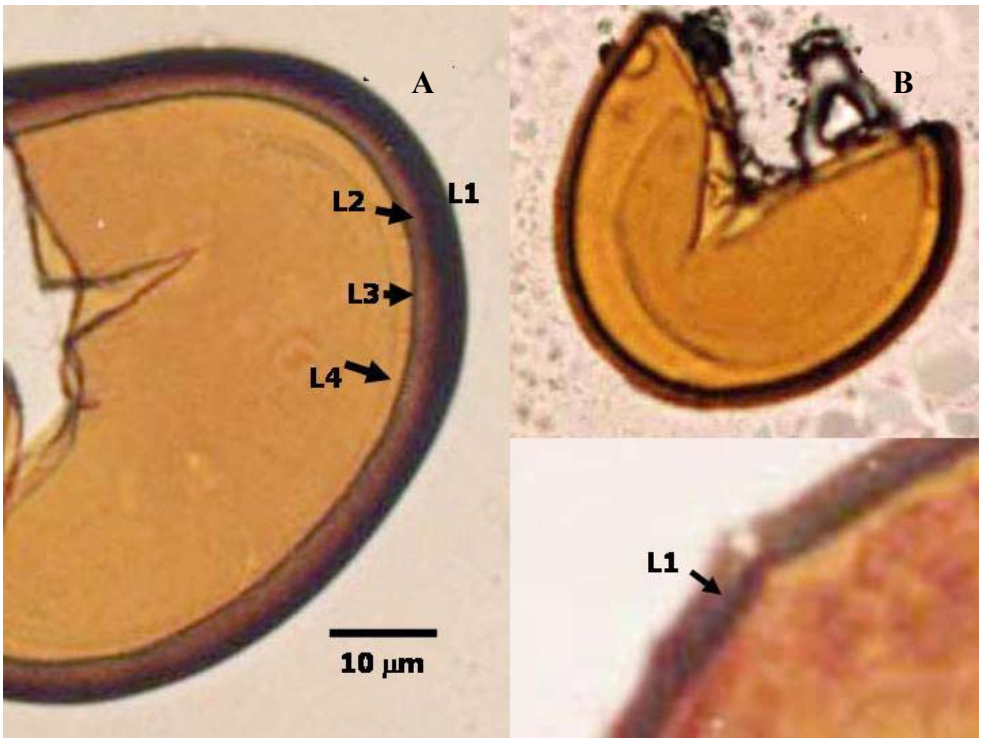


**Figure 30** Isolated spore from rhizosphere of *A. mangium* collected from Amphur Sakaerat, Nakhon ratchasima province identified into genus *Glomus caledonium,* L: spore wall layer showed 4 layer walls, colour: pale orange-yellow to yellow-brown orange-brown in the field or after long storage, shape: globose to subglobose, rarely irregular

11. *Glomus mosseae* (T.H. Nicolson & Gerd. and Gerd. & Trappe, 1974)

This species was found only in Amphur Sakaerat, Nakhon ratchasima province not found in the other sites of this study. The spores formed sporocarps with

yellow-brown to brown. While, the peridium surrounding these spores is 10-38 µm thick, with robust hyphae mixed with many finer branched hyphae. The whole spores have dark orange-brown or yellow-brown color, globose to subglobose, some irregular shape, spore diameter size 195 µm. The spore walls have three layers (L1, L2 and L3). The outer two often slough to varying degrees in mature or older spores (especially those in field soils). Hyaline color, mucilaginous membrane, 1.4-2.5 µm thick; staining pinkish-red in Melzer's reagent; often degrading and then forming a sloughing granular layer, sloughing in mature spores, appearing granular in advanced stages of degradation.**L2:** Hyaline, 0.8-1.6 µm thick,), very refractile when viewed with differential contrast optics, generally rigid and fracturing into sliver-like fragments observed when this layer separates from L3. It must be attached firmly to the underlying laminae because small irregularly-shaped and shallow pits appear as parts of this layer break away with application of pressure. Not reactive in Melzer's reagent. **L3:** A layer consisting of yellow brown to pale orange-brown, sublayers (or laminae), 3.2-6.4 µm thick; minute depressions cover the surface with separation of L1 and L2. The subtending hyphae, have flared to funnel-shaped. Another species often misidentified as *G. mosseae* is *G. caledonium*. However, spore wall structure of *G. caledonium* has four layers, each with distinct phenotypes (Figure 31).

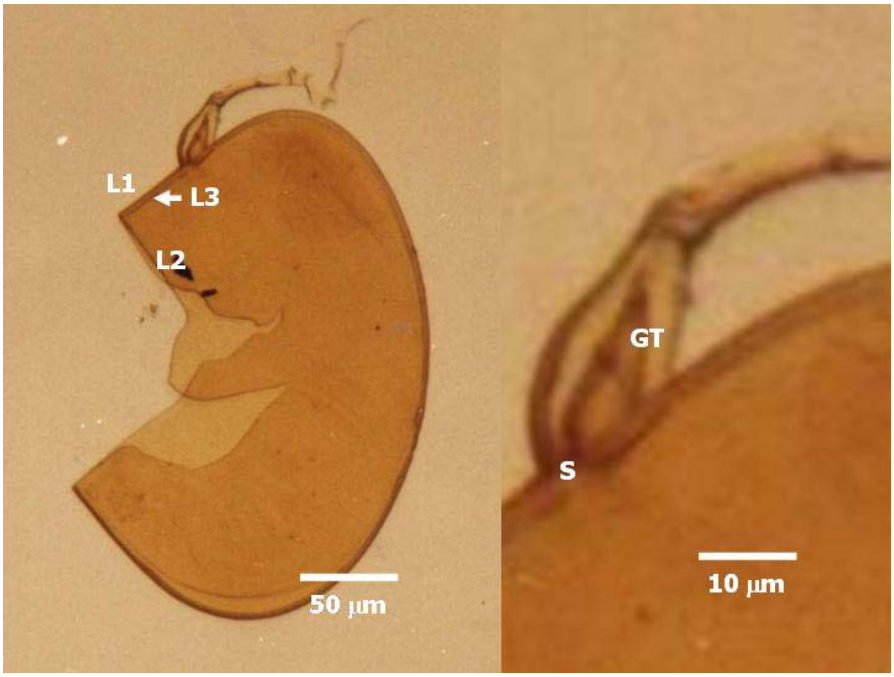


**Figure 31** Isolated spore from rhizosphere of *A. mangium* Willd. collected from Amphur Sakaerat, Nakhon ratchasima Province identified into genus *Glomus mosseae*, L: spore wall layer, GT: germ tube shape of subtending hyphae flared to funnel-shaped, occlusion**:** a recurved septum forms, S: septum (40X)

**12. *Glomus fasciculatum*** (The reference strain of this study) (Walker, C. and R. E. Koske. 1987)

The first germinal wall was redescribed (Koske and Walker, 1985) as having two "membranous walls" with some undefined adhesive causing them to stick together. The spores studied were neither fresh nor healthy, so the two layers of each wall were not seen. The colors of spore have pale yellow to pale yellow-

brown, globose, subglobose shape, diameter size 60-110 µm. The spore wall was consisting of three layers (L1, L2, and L3) which form sequentially, based on the pattern of spore wall differentiation observed in all other *Glomus* species. **L1:** An outer hyaline layer which was intact in the specimens examined, but which is reported to slough in descriptions of the species; producing a pinkish-red reaction in Melzer's reagent; adherent to L2. **L2:** A layer consisting of thin adherent sublayers (or laminae), light yellow-brown in color .All sublayers form a dark red to slightly purplish red color in Melzer's reagent. **L3:** A thin flexible layer < 1.0 µm thick which is continuous with the innermost layer of the subtending hypha. Subtending hyphae have cylindrical to slightly flared shape (Figure 32).

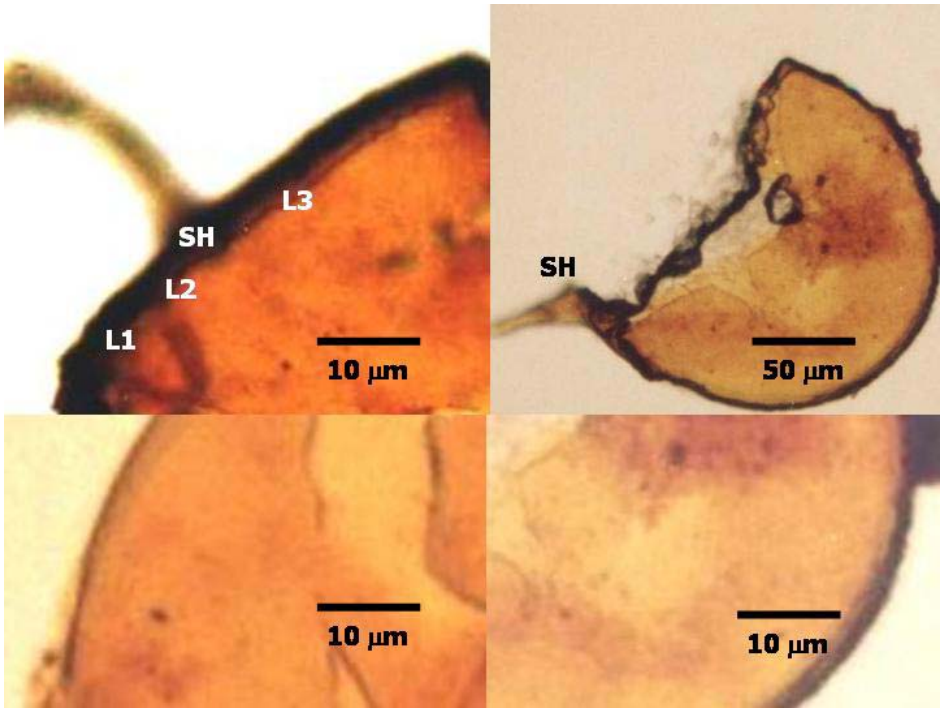


**Figure 32** AMF reference strain obtained from Kasetsart University; *Glomus fasciculatum* 40X

**13. *Scelerocystis coremioides*** (Wu, 1993).

This species was found in almost all sampling sites but different in the number of spore. The spores formed sporocarps are brown or yellow brown, 200-550 mm, subglobose, with or without multihyphal stipes, usually flattened at base; chlamydospores arranged radially in a hemispherical layer, ellipsoid, ovoid, triangular, or oblong ellipsoid to clavate, yellow brown, 50-80 mm; spore wall 1.5-2.5 mm and frequently thicked at base, subtending hypha long, single, plexal hyphae present. (Figureure33).

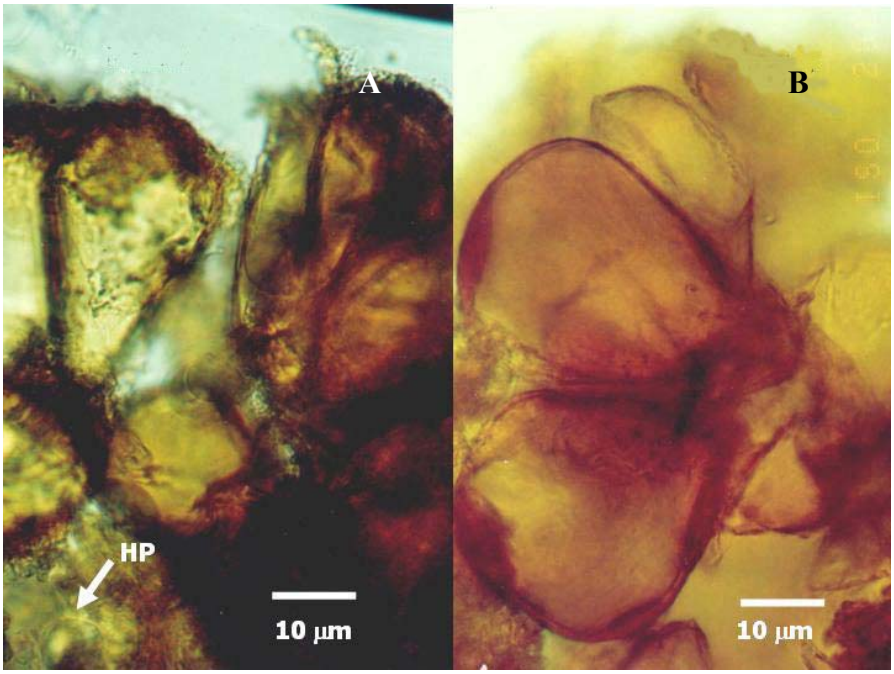


**Figure 33** Isolated spore from rhizosphere of *A. mangium* identified into *Scelerocystis coremioides* from Lampoon province, showed sporocarps reddish brown, brown or dark brown, globose, subglobose or ellipsoidal, 200-300 x 180-280 mm, with chlamydospores formed radially in a single, tightly packed layer around a central plexus of hyphae with peridium now it classify into *Glomus fasiculatum* (Almeida, R. T. and N. C. Schenck. 1990), A) stained with PVLG, B) stained with Melzer, HP: hyphal plexus (40X)

**14. *Glomus* spp.** (this study)

This species was found in many numbers in the Lampoon Province site. The characteristic was likely the sporocarpic of *G. mosseae* (Figure 34).

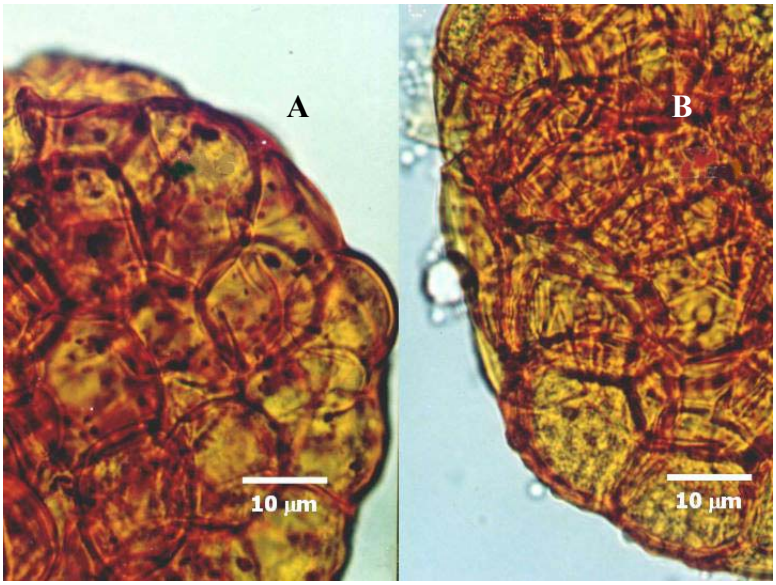


**Figure 34** Isolated spore from rhizosphere of *A. mangium* collected from Lampoon province, unidentified species like sporocarpic of genus *Glomus*, orange red and yellow-brown color, globose subglobose shape (40X), A) stained with PVLG, B) stained with Melzer

## 3.1.3 Limitation of Morphological investigation of AMF

In this study, investigations of AMF species diversity have been hindered by difficulties in species identification. Identification of AMF is based on characteristics of their spores may be difficult to discern and are subject to alterations during spore ontogeny or by parasitsm. The hyphal connections of the spores, normally needed for genus determination, can be lost. Another limitation of morphological identification is the fact that field-collected spores are often parasitized or degraded and therefore unidentifiable. This, the lack of comparative material is a constant problem with the investigation of field samples. These tend to become degraded in the field and ideally

identification should be made from pot-cultured material (each culture derived from a single spore) or from a large series of field-collected specimens (Walker, 1992). The soil from the field site were brought into contact with suitable plant hosts of AMF under controlled conditions in order to propagate the species occurring at the field site and to obtain fresh spores of all developmental stages. However, the plant species used in the trap cultures may have an influence on which AMF are detected (Jansa et al. 2002). Producing pot cultures is time consuming and the obtaining single spore cultures from spore extracted from the field is unpredictable, partly owing to the small numbers of viable spores in the soil. In addition, the identification of healthy spores may pose problems, because morphological characters are scarce in some AMF. For example, *Glomus* and *Paraglomus* cannot be discerned by spore morphology but are distantly related. Even after the separation of the genus from *Paraglomus* (Morton and Redecker, 2001), the genus *Glomus* is still polyphyletic, because it comprises two independent lineages according to molecular criteria (Schwarzott, et al. 2001). Dimorphic AMF species (e.g. *Archaeospora leptotich*a, *G. dimorphicu*m) can cause further confusions in morphological identification.

**3.2. DNA extraction from the AMF single spore**

At present, there is little information existing on species diversity in communities of AMF, mainly owning to difficulties in identification of field extract spores on the basis of morphology. The possibility was explored to identify AMF spores from the field on the basis of a molecular marker. Thus, the DNA extractions from multiple spores were employed in the preliminary study. Initial test, the least spore 1, 5, 10, and 15 spores were used for DNA extraction and amplification. Five to Ten spores were found that were suitable for DNA extraction. However, this study failed to yield amplified product from single spore. Nevertheless, multispore-preparation is time-consuming and does not reproducible. On the other hand, using of single spores for DNA extraction has some advantages over multispore-preparations, e.g. it is less susceptible to contamination with other number of samples and is highly reproducible. In addition, in 1997 Zeze' found the heterokaryotic status of the nuclear population within a spore azygospores or chlamydospores. Mostly, fungi were uninucleate but AMF multinucleate number of nuclei ranging from 2,000 to 20,000 depending on species and investigation.

Therefore, attempt on the DNA extraction from single spore was carried out. Using contaminate prevention was conducted with fewer spores. According to the methodology of Redecker, 1997, was modified by using sterile pipette crushing combined with freeze-thawing and followed by alkaline treatment. The critical step is inducing of spore germination before the DNA was extracted approximately 10-15 minutes. Total protocol rapid procedure took at least 1 hour for completion. The results (Figure 35) showed that the size of PCR product is approximately 500-bp. The both of PCR amplification products were generated from ITS1-4 region (variable

region) and NS1-2 region (conserve region). The yield of PCR product is enough to be amplified and allow cleavage with the restriction enzymes (Figure 37). The RFLP pattern of ITS1-4 region (Figure. 37A) indicated that the sum of the fragment sizes was sometimes greater than the size of the original PCR product (undigested). This phenomenon was supported by the numerous reports (Hosny, et al., 1996; Sanders et al., 1995) which found that the restriction analysis of ITS sequences of AMF have a relatively high level of heterogeneity even within single spore. So, the use of the ITS region has been rejected for this purpose because of sequence heterogeneity, which has been detected even within single spore (e.g. Antoniolli, et al. 2000).

In this study, many factors were involved in DNA extraction such as inducing of spore germination (temperature and antibiotic reagent) and spore quality. In the term of spore germination, this study was to be consistent with the report of Franken in 1997, who found that the RNA extraction of AMF was activated by using of antibiotic inducing combined with incubation at 4°C before use. Usually, spores were stored at 4°C before use but not excess than 1 month. Moreover, numerous investigate about the breaking of spore dormancy was employed. The decreasing of dormancy time of spore was suggested by Brundrett and Juniper in 1993. This condition are 4°C, 14 days after that, incubated at room temp 14 days before use for DNA extracted. Furthermore, the preparations of spore material for DNA isolation are more important. One easily accessible and realible source of information to aid with preparation of spores for molecular analyses can be used from page World Wide Web site with the URL address: http://invam.caf.wvu.edu/ established by J. Morton and S. Bentivenga of INVAM (the International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi, USA). The freshly sieved spores were observed under

a binocular microscope and sorted into two groups according to color and appearance of the spore contents. One group of spores was chestnut in color, had a shiny surface, and contained clearly visible lipid globules. Spores having these characteristics were assumed to be healthy spores, since a preliminary test showed that a high proportion of these spores were able to germinate (Figure 37B). The second group of spores had contents with a cloudy white appearance. It assumed that these were dead, parasitized spores that were not viable, as it have never observed their germination in previous experiments. However, this study yet has a little percentage of spores failed to yield an amplification product, probably because they had lost their contents.

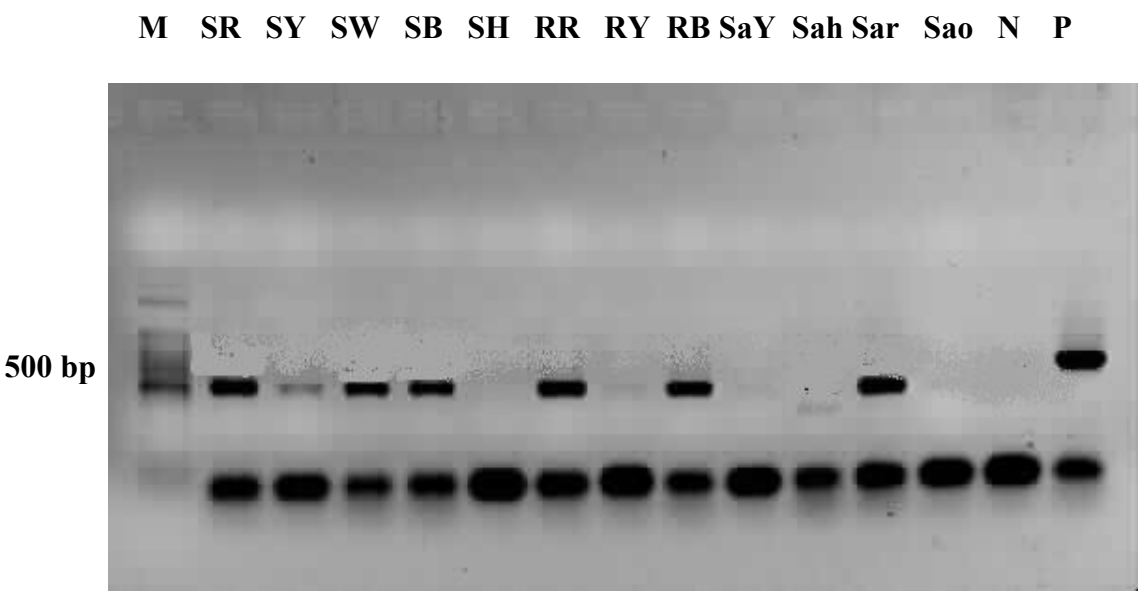


**Figure 35** NS1-2 PCR fringerprint pattren of AMF single spore from *A. mangium* rhizosphere M: DNA marker100 bp, (SR) : SUT red spore, (SY): SUT Yellow spore, (SW) : SUT white spore, (SB) : SUT Black spore, (SH) : SUT Hyaline spore, (LR) :Lopburi Red spore, (LY) : Lopburi Yellow spore, (LB) : Lopburi Black spore, (SaY) :Sakaerat Yellow spore, (Sah) : Sakaerat Hyaline spore, (Sar) : Sakaerat Red spore, (Sao) : Sakaerat Orange spore, (N) : Negetige control (without DNA) (P) : Positive control (containing mycorrhizal DNA)

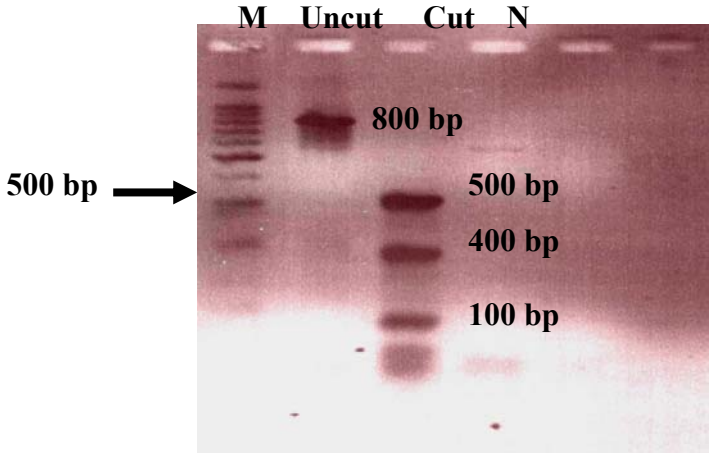


**Figure 36** PCR-RFLP pattern of ITS1-4 region from AMF single spore. M: DNA marker 100 bp., RFLP pattern Uncut : PCR template without cutting, Cut : PCR template cutting with *Hind*

### 3.3. DNA extraction from the plant root

The previous experiment might include the AMF that unformed within the plant root. In recently, numerous reports found that lack of sporulation does not necessarily mean absence from a site. For example, mycelium of some AMF was detected in roots of plant, but spores were not found in any of the soil samples. Furthermore, the identification of infrastructure of this fungus in the root was difficult because some AMF strains are difficult to detect by root staining procedures. In the present study staining was observed in only about 5% of the root system despite the fact that roots were strongly colonized by mycorrhizal fungi. This problem was reported previously for *G. Occultum* (Morton, 1985) and *A. gerdemanni*i/*Gl. leptotichum* (Morton et al., 1997). Intraradical structures stain more weakly with direct blue, trypan blue (Koske and Gemma, 1989), or chlorazol black E (Brundrett et al*.,* 1984) than other *Glomus* or *Acaulospora* species. The only exception among the species tested may be *G. brasilianum,* but it stains with variable intensity in plant hosts tested thus far (Morton, unpublished). Therefore, the ecological role of these fungi has been largely ignored in all previous studies based on staining methods. Thus, detecting AMF community from the plant root by using the development of molecular techniques has been focused.

The DNA extraction from the root was done according to the method of Simon et al. 1996 by using Chelex resin combined with the Nested-PCR techniques. DNA extracted from mycorrhizal root fragments was used as a template for nested PCR reactions. The result of first primer (ITS4-NS5) which is the outer primer showed no any bands (Figure 37A.). Thereafter, the band can further be detected with application of the next primer (ITS1-ITS4: verify primer) (Figure 37B.). The ITS1-ITS4 region

was existed in many bands because it might contain many copy number of the ITS gene. Then PCR product of specific primer was generated in size of 200-bp and 300-bp by GLOM5.8R-ITS1F and GIGA5.8R-ITS1F, respectively (Figure 37C.). Which only these primers could be amplified the PCR product, whereas the other primers showed no exist band. By the target group of the GIGA 5.8 R and GLOM5.8R primer (data showed in Figure 37C) are subgroup of the genus *Gigaspora* and *Glomus*, respectively. Primer GIGA5.8 R and GLOM5.8R were successfully tested with roots of *A. mangium* colonized by *Gigaspora, Scutellospora* and *Glomus*. The PCR results obtained with non colonizatized has never been detected this product, even after second amplification. In addition, the target gene was also detected with other primers as VANS1 and NS21.

The DNA fragment was sequenced by indirect methods. Because they generated mixed sequences, therefore all sequences were obtained from cloned DNA. Clones were selected for sequencing on the basis of Blue-White screening techniques. A total of 15 clones from the AMF were sequenced, and a further compare with the database. These came from a total of 10 roots sample that yielded enough PCR product for cloning. Alignment of the partial SSU fragments generated using the fungal-specific primers corresponding to the three families, *Acaulosporaceae*, *Gigasporaceae* and *Glomaceae*. Almost of them gave the 96-98% homology with responding the sequence from database. Furthermore, sequencing analysis of AMF DNA from plant root has so far shown that single plants may be colonized by many AMF simultaneously, sometimes including representatives of all three families in the order. The Nested-PCR techniques help to identify individual fungal strains. It can

open the new opportunity for study of these fungi in the community level. They appears to be a difference in the fungal community among host plants. These were combination systematic help to monitor the AMF biodiversity in field-area.

Since, the obtained DNA could not be further amplified by direct PCR. Until, the application of Nested-PCR was employed together. Part of the difficulty may be due to the recovery of fungal DNA from plant root in soil: polysaccharides, polyphenolic compounds, and humic substances all have to be efficiently removed to obtain high-quality DNA; also, because fungal tissue is embedded deeply within the roots in AMF and therefore DNA extraction can be more of problem. Thus, specific PCR primers have been developed to amplify AMF rDNA directly from colonized roots while eliminating amplification of plant or nonAMF DNA (Simon et al., 1992; Helgason, et al., 1998; Van Tuinen, et al., 1998). The primer in this study were designed group-specific primer from Redecker, 2000 for five major phylogenetic lineages of AMF to amplify the highly variable ITS (primer map shown in Figure 2 and target group of primer in Figure 2). This method is promising to reveal the structure of AMF communities in natural ecosystems especially in field-area.

The addition of chelex resin to the tris-HCl buffer when the colonized roots were boiled affected to increased amplification of the AMF rDNA product from reaction mixtures (Figure 37). Usually, the inhibition of PCR by phenolic substances, polysaccharides, or humic acids has often been reported in association with DNA from plant tissue or environmental samples (Demeke and Adams, 1992; John 19992; Tsai and Olson, 1992), and the presence of plant phenolics in roots colonized by AMF

has been documented (Grandmaison et al, 1980). The Chelex resin and moreover, dilution of the template DNA does appear to be indirectly purification (required 100-fold or 500-fold dilutions) usually provided positive results. The optimum dilution of this study was 100-fold dilution. This result was consistently the report of Bonita et al. in 1995 who found that this technique could detect *G. intraradices* in the roots of five plant species such as Lettuce, Zinnia, Endive, Pepper and Leek. This AM fungus was detected in the first few weeks of growth on roots of different species and age when the colonization was at least 30%. The extent of root colonization and not the harvest date appeared to be critical for detection. The role of Chelex was the chelating reagent which binds with cations including $Mg^{+}$. By binding with the magnesium ions, the Chelex renders DNAases inoperable, thus protecting DNA from their action. The method was described does not required any lengthy DNA extraction procedure, yet it does provide suitable DNA template for PCR. Root samples were simply boiled with a buffer containing Chelex resin. Optimal results were obtained when root were processed for PCR immediately after harvest and DNA samples were then used in the PCR assay within a few days. Bonita et al, 1995 reported that storing the roots in the refrigerator or the processed DNA samples in the freezer (-20°C) for more than 2 weeks diminished or eliminated amplification of the rDNA product. The availability of a simple method to confirm AM fungal colonization on a broad range of species and in the first few weeks of growth would be useful in the application and evaluation of AMF inoculants in agriculture.

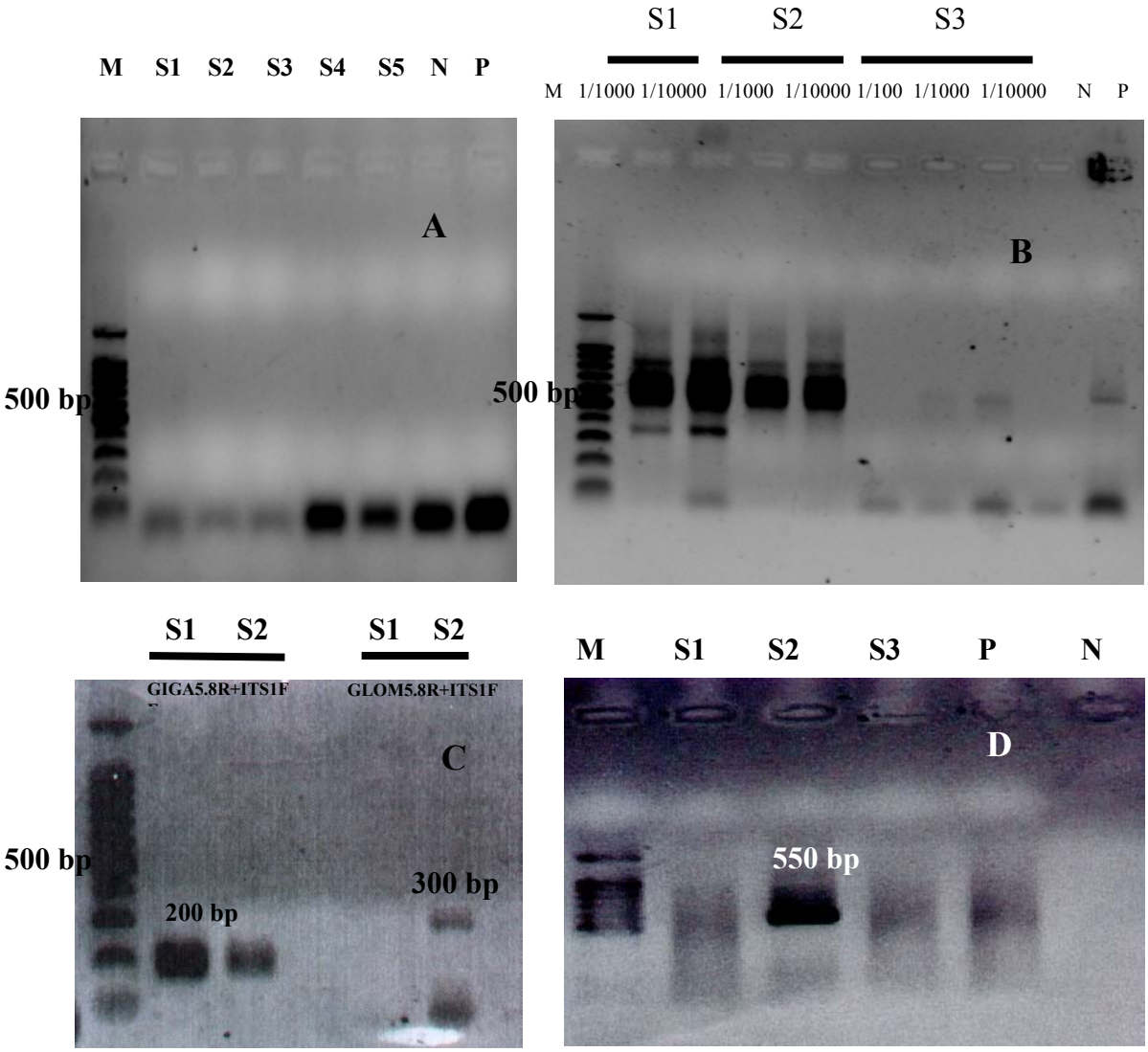


**Figure 37** PCR-pattern of DNA from *A. mangium* root amplified by Nested PCR techniques. After the first amplification by NS5-ITS4 primers (A), dilution PCR product for verify PCR product by ITS1-ITS4 primers (B), amplified by specific primer (second amplification) (C) size of PCR product approximately 200 bp. (primer GIGA 5.8 R+ITS1F) size 300 bp. (primer GLOM 5.8R+ITS1F); (D) same example amplified with VANS1-NS21 primer (PCR product size = 550 bp.)

## 4. Comparison of AMF DNA sequencing and morphological analysis

Part of rRNA regions of spore from AMF have been widely amplified and sequenced using universal primers (NS1-NS2) (White et al, 1990) and non-coding internal transcribed spacers (ITS) of the DNA from plant root that used to identified species. The results of directly DNA extraction from the single spore and sequencing in this study indicated the infection of *Gigaspora gigantea* (97% homology), *Glomus mosseae* (94% homology), *G. claroideum* (90% homology), *G. albida* (95% homology), *Scutellospora persica* (97% homology) and *Glomus* spp, uncultured ascomycete fungi, uncultured basidiomycete fungi, uncultured soil fungi, and unidentified species (90% homology) in the rhizosphere of *A. mangium* Willd. Whereas, the results of DNA indirect sequencing of root were indicated the infection of *Gi. gigantea,* (98% homology), *Sc. heterogama* (99% homology), *Gi. decipiens* (100% homology), *Gi. margarita* (100% homology), *G. Intraradices* (100% homology) *Acaulospora laevi* (92% homology), uncultured asocomycete fungi (98% homology) and unidentified sp. (100% homology). In the part of morphological analysis showed the infection of *A. laevi, A. koskei, Gi. decipiens*, *Gi. margarita*, *Gi. gigantea*, *Gi. margarita, Sc. heterogama*, *Sc. persica, Sc. pellucida, Sclerocystis coremioides, G. mosseae, G. claroideum,* and 1 unidentified species.

The comparative results from three techniques are summarized in Table 5. . The single spores of AMF contain divergent rDNA sequences (Sanders et al., 1995; Lloyd MacGilp et al., 1996; Redecker et al., 1997), which makes it difficult to distinguish closely related AMF by sequence comparisons. However, the developed DNA extraction gave accuracy identification of reference strain (*Glomus fasciculatum*

98% homology) when compared with morphological identification. Whereas, the fragment of DNA extract from root gave highest species, due to the application of nested-PCR using taxon-specific primers. This technique is a highly sensitive method which allows detection of fungal hyphae present in roots as well as from soil (Van Tuinen, et al., 1998; Jacquot et al., 2000). The result also indicated *G. intraradices*, the fungus spores not found in any of the soil samples but could be detected in the root by this technique. It consistently studied the numerous that the absence of detectable spores does not necessarily indicate the absence of the fungus from roots (Turnau, et al., 2001).

**Table 7** Comparison analysis of DNA sequence both from single spore, AMF colonize root and morphological identification

| **Morphological identification** | **18s rDNA of single spore** | **5.8s rDNA of the plant root** |
|---|---|---|
| *Acaulospora laevi* | ND | *Acaulospora laevi* |
| *Acaulospora koskei* | ND | ND |
| *Scutellospora pellucida* | ND | *Scutellospora pellucida* |
| *Scutellospora persica* | *Scutellospora persica* | ND |
| *Scutellospora heterogama* | ND | *Scutellospora heterogama* |
| *Sclerocystis coremioides* | ND | ND |
| *Gigaspora gigantea* | *Gigaspora gigantea* | ND |
| *Gigaspora decipiens* | ND | *Gigaspora decipiens* |
| *Gigaspora margarita* | ND | *Gigaspora margarita* |
| *Gigaspora albida* | *Gigaspora albida* | ND |
| ND | ND | *Glomus intraradices* |
| *Glomus mosseae* | *Glomus mosseae* | ND |
| *Glomus claroideum* | *Glomus claroideum* | ND |
| *Glomus fasciculatum* | *Glomus fasciculatum* | ND |
| ND | Uncultured *Glomus* | Uncultured *Glomus* |
| ND | *Glomus* spp. | ND |
| *Glomus* spp. | Unidentified sp. | Unidentified sp. |
| ND | Uncultured ascomycete fungi | Uncultured asocomycete fungi |
| ND | Uncultured basidiomycete fungi | ND |
| ND | Uncultural AMF fungus | ND |
| ND | Uncultured soil fungus | ND |

ND = Not detected; *18s rDNA and 5.8s rDNA partial sequence

**Table 8** Sequence results compared with AMF strains from data based

| DNA analysis | Compared with strains from data based | % Homology |
|---|---|---|
| *Gigaspora albida* | Small subunit rRNA (Z14009) | 99 |
| *Gigaspora gigantea* | Isolate E30, 18s rRNA gene partial sequence (GGI410736) | 97 |
| *Glomus mosseae* | Isolate BEG185-11 ISU rRNA gene, partial sequence (AY541917) | 94 |
| *Glomus clarodeum* | Isolate BEG185-12 ISU rRNA gene, partial sequence (AY541918) | 90 |
| Uncultrued soil fungi | Clones-Canopy-750-03-15 18r rRNA gene (AY382468) | 100 |
| *Acaulospora laevi* | Clones 3-18s 18s rRNA gene, partial sequence (AF320636) | 92 |
| *Scutellospora pellucida* | Strain JJ57 18s rRNA gene partial sequence (AY035662.1) | 98 |
| *Scutellospora heterogama* | 18s rRNA gene, partial sequence (AF004691) | 95 |
| *Scutellospora persica* | Isolate E28, 18s rRNA gene partial sequence (AJ410729) | 96 |
| *Gigaspora decipiens* | Clone 12 ITS 1, partial sequence (AY442361.1) | 100 |
| *Gigaspora margarita* | Clone p pTR28-1a 18S rRNA gene, partial, isolate BEG34, (Y17646.1) | 100 |
| *Glomus intraradices* | 18s rRNA gene, partial sequence (AJ517460) | 100 |
| Uncultrued *Glomus* | Clone Pa040 18s rRNA gene, partial sequence (AY236250.1) | 100 |
| *Glomus* sp. | 18s rRNA gene, partial sequence (AF082581) | 96 |
| Uncultured AMF fungi | 5.8s RNA gene, partial sequence (AY267221) | 100 |
| Uncultured soil fungus | Clone f95-2 small subunit rRNA gene, partial sequence (AF515414.1) | 96 |
| Uncultured soil ascomycete | 18S rRNA gene, clone s20-72, partial sequence (AJ515949.1) | 95 |
| Uncultured soil Basidiomycete | 18S rRNA gene, clone s20-72, partial sequence (AJ515169.1) | 92 |

However, identification of AMF with several methods (table 5) indicated the different results. Differences of the AMF preference both in root and rhizosphere were due to the different geographical regions or the selectivity of these primers with respect to 5 phylogenetic groups in the root and the primer for spore with respect to universal fungi. In many cases sequences from different habitat types and different times are identical in other groups the sequences are more variable. The morphological identification should be used for identification in genera level which, accurate with the other data. From our results found that the morphological and molecular identification method were consistent with the using primer both 18s rDNA and 5.8s rDNA which, cover the ITS region of this fungi. The accuracy of AMF identification should be combined with the molecular studies and similarities in spore

morphology. On the other hand, the polyphasic or called Molecular systematic studies (Simon et al., 1993) should be the important role for AMF biodiversity investigation. The sequence species recognized by these molecular methods cannot be equated with the formal species that are identified on the basis of spore morphology, but many of the species have been recovered repeatedly in different studies and appear to represent entities as widespread and stable as those defined by morphology. Whether the classification is molecular or morphological, there are likely to be important functional differences between the species, but there many also significant hidden variations within them. Nevertheless, the molecular typing allows addressing questions of AM community composition and distribution in plant roots, which is the ecologically significant niche and is inaccessible to methods based on spore morphology. The information that presented in this study is of potential importance for future studies of AM population in the field.

## 3.5. Effect of AMF inoculation on the plant growth of *Acacia mangium* Willd.

After *A. mangium* was inoculated with AMF collection strains, the result showed that the inoculation of AMF was more significantly effected to the plant root length and plant root weight, whereas, the shoot heights, fresh and dry shoot weight were not significantly effected (Table 6). The colonized root was trend to promote the root length when compared with control treatment. Eventhough some AMF strains have highly percentage of colonization, this event did not confirm that the plant root will be developed. On the other hand, it related with plant age and fungal growth, life cycle of each fungal strain. Another reason is the delayed spore formation in different AMF species were affect to compatible to the plant or not. Bever et al, 2001 found that the AMF spore formation or sporulation variable depending on AMF species (some species may not form spores at all), host plants and the other environmental factors. These factors affect to the colonization percentage in each species. Even AMF are very non-specific in their ability to associate with plants. However, this study demonstrated that there are preferences, in that host plant species may select different mycorrhizal partners from the mix of fungi available in the soil. This results also suggested that the mainly Genera infection of AMF in *Acacia mangium* Willd. were *Acaulospora, Glomus, Gigaspora and Scutellospora*, respectively. However, the preliminary study found that genus *Gigaspora* less appeared in the rhizosphere even use single spore analysis, and the plant root. Eventhough, this genus is the major population particularly in the forest area. These results support the results from Table 9 which showed that only five of the ten strains of *Gigaspora* sp. Could not colonized the root of host plant (50% colonization) and three of the five

strains performed low root colonization. Therefore, genus Gigaspora may incompatible with the *A. Mangium*.

**Table 9** Effect of AMF colonization on growth promotion of *Acacia mangium*

| ISOLATES | Genera | Site | Total Height (cm.) | Root Legnth (cm.) | Shoot Height (cm.) | Total Weight (g.) | Root Weight (g.) | Shoot Weight (g.) | Shoot Dry Weight (g.) | % I |
|---|---|---|---|---|---|---|---|---|---|---|
| control | - | NM | 11 | 6 | 5 | 0.5 | 0.1 | 0.4 | 0.15 | 0 |
| DA 5051 | *Glomus* | BK | 18.02 | 9.45** | 8.57** | 1.33 ** | 0.54 ** | 0.79 ** | 0.45 ** | 80 |
| DA 5023 | *Aculospora* | PB | 12.05 | 7.50NS | 5.00NS | 0.55 * | 0.3 NS | 0.25 NS | 0.14 NS | 40 |
| DA 5083 | *Glomus* | CM | 15.3 | 8.30NS | 7 ** | 1.33 ** | 0.57 ** | 0.76 ** | 0.58 ** | 50 |
| DA 5022 | *Glomus* | PB | 10.9 | 5.30** | 5.6 NS | 1.32 ** | 0.95 ** | 0.37 NS | 0.15 NS | 0 |
| DA 5170 | *Gigaspora* | PB | 11 | 6.30** | 4.7 NS | 1.52 ** | 0.88 ** | 0.64 * | 0.24 NS | 0 |
| DA 5114 | *Aculospora* | CM | 17.13 | 8.40NS | 8.73 ** | 0.95 ** | 0.13 NS | 0.82 ** | 0.71 ** | 95 |
| DA 5117 | *Aculospora* | CM | 12.4 | 7.20NS | 5.2 NS | 0.67 ** | 0.42 ** | 0.25 NS | 0.1 NS | 70 |
| DA 5010 | *Gigaspora* | NA | 10.1 | 5.20** | 4.9 ** | 1.43 ** | 0.89 ** | 0.54 * | 0.35 ** | 0 |
| DA 5023 | *Aculospora* | PB | 12.78 | 7.75NS | 5.03 NS | 1.23 ** | 0.58 ** | 0.65 ** | 0.61 ** | 60 |
| DA 5100 | *Aculospora* | CM | 18.23 | 10.98** | 7.25 ** | 4.64 ** | 1.52 ** | 3.12 ** | 2.95 ** | 70 |
| DA 5054 | *Gigaspora* | CM | 15.7 | 8.40NS | 7.3 ** | 2.96 ** | 1.97 ** | 0.99 ** | 0.98 ** | 70 |
| DA 5090 | *Aculospora* | CM | 15.8 | 9.30** | 6.5 ** | 1.61 ** | 0.76 ** | 0.85 ** | 0.72 ** | 80 |
| DA 5069 | *Glomus* | CM | 9.6 | 6.40NS | 5.2 NS | 1.11 ** | 0.64 ** | 0.47 NS | 0.17 NS | 0 |
| DA 5226 | *Glomus* | CM | 8.50* | 5.80NS | 3.9 ** | 0.56 NS | 0.21 NS | 0.35 NS | 0.31 ** | 0 |
| DA 5013 | *Glomus* | NA | 11 | 7.30** | 4.5 ** | 1.05 ** | 0.25 NS | 0.80 * | 0.44 ** | 90 |
| DA 5198 | *Aculospora* | LP | 21.6 | 13.30** | 8.3 ** | 0.84 ** | 0.37 ** | 0.47 NS | 0.45 ** | 20 |
| DA 5096 | *Glomus* | CM | 17.1 | 10.80** | 6.3 ** | 0.82 ** | 0.45 ** | 0.27 NS | 0.24 NS | 98 |
| DA 5113 | *Aculospora* | CM | 12.6 | 8.40NS | 4.2 NS | 1.01 ** | 0.78 ** | 0.23 NS | 0.12 NS | 95 |
| DA 5184 | *Glomus* | PR | 14 | 7.60NS | 5.4 NS | 4.78 ** | 1.94 * | 2.84 ** | 2.73 ** | 65 |
| DA 5154 | *Glomus* | BK | 11 | 5.30NS | 5.7 NS | 0.69 ** | 0.33 * | 0.36 NS | 0.24 NS | 0 |
| DA 5075 | *Aculospora* | CM | 11.9 | 6.70NS | 5.2 NS | 0.65 ** | 0.42 ** | 0.23 NS | 0.15 NS | 0 |
| DA 5222 | *Glomus* | NA | 11 | 6.00NS | 5 NS | 0.44 NS | 0.26 NS | 0.18 NS | 0.13 NS | 0 |
| DA 5109 | *Glomus* | CM | 13 | 7.00NS | 6 ** | 1.02 ** | 0.57 ** | 0.45 NS | 0.3 ** | 70 |
| DA 5120 | *Glomus* | CM | 11.7 | 6.30NS | 5.4 NS | 0.38 NS | 0.18 ** | 0.20 NS | 0.14 NS | 0 |
| DA 5123 | *Aculospora* | CM | 11.00* | 6.30NS | 4.7 ** | 0.92 ** | 0.51 ** | 0.41 NS | 0.32 ** | 0 |
| DA 5176 | *Aculospora* | CB | 10 | 5.70NS | 4.3 ** | 0.89 ** | 0.54 ** | 0.35 NS | 0.26 * | 0 |
| SUTSC | *Sclerocystis* | NM | 12.5 | 6.50NS | 5.7 NS | 1.21 ** | 0.53 ** | 0.68 * | 0.65 ** | 85 |
| SUTsmY | *Glomus* | NM | 14.7 | 7.70NS | 7 ** | 4.01 ** | 1.12 ** | 2.89 ** | 2.87 ** | 75 |
| SUTRB | *Gigaspora* | NM | 10.6 | 6.50NS | 4.1 ** | 0.53 NS | 0.32 NS | 0.21 NS | 0.17 NS | 0 |
| SUTR | *Scutellospora* | NM | 12.3 | 5.20** | 7.1 ** | 0.35 NS | 0.13 NS | 0.22 NS | 0.1NS | 10 |
| SUTW | *Scutellospora* | NM | 13.7 | 7.40NS | 6.3 ** | 0.43 NS | 0.15 NS | 0.28 NS | 0.25 * | 40 |
| SUTY | *Gigaspora* | NM | 12.5 | 4.20** | 8.3 ** | 1.07 ** | 0.42 ** | 0.65 * | 0.56 ** | 30 |
| SUTsmR | *Glomus* | NM | 15.8 | 8.70* | 7.1 ** | 1 ** | 0.37 * | 0.63 * | 0.45 ** | 95 |
| SUTB | *Gigaspora* | NM | 9.9 | 6.00NS | 3.9 ** | 0.35 NS | 0.23 NS | 0.12 NS | 0.1 NS | 0 |

Footnote: AMF almost strains obtained from DA:Department of Agriculture, Bangkok; SUT: Suranaree Unversity of Technology, Nakhon ratchasima Province, AMF strains from this study; Site: NM (Nakhon ratchasima P.), CB (Chonburee P.), CM (chaingmai P.), NA (Nan P.), PR (Prae P.), BK (Bangkok), LP (Lumprang P.), PB (Phetchabun P.), % I: % infection, Statistical analysis by LSD, NS = non significantly *=significant; (p,0.05); **=Highly significant (p<0.01)

**Table 9.** (continued)

| ISOLATES | Genera | Site | Total Height (cm.) | Root Length (cm.) | Shoot Height (cm.) | Total Weight (g.) | Root Weight (g.) | Shoot Weight (g.) | Shoot Dry Weight (g.) | % I |
|---|---|---|---|---|---|---|---|---|---|---|
| control | - | NM | 11 | 6 | 5 | 0.5 | 0.1 | 0.4 | 0.15 | 0 |
| DA 5151 | *Glomus* | BK | 16.5 | 9.25** | 7.25** | 0.48 NS | 0.18 NS | 0.3 NS | 0.28 * | 70 |
| DA 5154 | *Glomus* | BK | 9.75 | 5** | 4.75 NS | 0.44 NS | 0.14 NS | 0.5 NS | 0.25 NS | 0 |
| DA 5169 | *Glomus* | CB | 18 | 12** | 6** | 0.67 * | 0.43 ** | 0.29 NS | 0.24 NS | 10 |
| DA 5170 | *Gigaspora* | PB | 23.5* | 15.5** | 8** | 1.15 ** | 0.65 ** | 0.45 NS | 0.415 ** | 15 |
| DA 5175 | *Glomus* | PC | 13.75 | 9.5** | 4.25** | 0.19 NS | 0.09 NS | 0.1 NS | 0.1 NS | 35 |
| DA 5007 | *Glomus* | NA | 10 | 6.00** | 4.00** | 0.4 NS | 0.1 NS | 0.3 NS | 0.2 NS | 0 |
| DA 5118 | *Glomus* | CM | 12 | 5.70** | 6.30** | 1.05 ** | 0.25 NS | 0.8 * | 0.44 * | 10 |
| DA 5017 | *Aculoapora* | LP | 15.25 | 10.00** | 5.25NS | 0.75 ** | 0.11 NS | 0.64 NS | 0.07 NS | 30 |
| DA 5150 | *Glomus* | BK | 18 | 11.00** | 7.00** | 1.12 ** | 0.13 NS | 0.99 ** | 0.55 ** | 70 |
| DA 5122 | *Glomus* | CM | 12.5 | 7.50NS | 5.00 NS | 1.3 ** | 0.38 ** | 0.92 ** | 0.72 ** | 40 |
| DA 5019 | *Aculoapora* | PN | 9.00* | 4.25** | 4.75 NS | 1.18 ** | 0.34 * | 0.84 * | 0.71 ** | 95 |
| DA 5119 | *Aculoapora* | CM | 11.35 | 7.30NS | 4.05** | 1.05 ** | 0.3 NS | 0.75 * | 0.4 * | 20 |
| DA 5013 | *Glomus* | NA | 18 | 10.00** | 8.00** | 1.62 ** | 0.5 ** | 1.12 ** | 0.98 ** | 70 |
| DA 5178 | *Glomus* | PB | 12.54 | 5.00NS | 7.54** | 0.93 ** | 0.18 NS | 0.75 * | 0.7 ** | 90 |
| DA 5123 | *Acualoapora* | CM | 9.00* | 4.00NS | 5.00NS | 0.89 ** | 0.35 * | 0.54 NS | 0.44 * | 0 |
| DA 5215 | *Gigaspora* | LP | 14.4 | 9.25** | 5.15NS | 1.12 ** | 0.57 ** | 0.45 * | 0.12 NS | 30 |
| DA 5184 | *Glomus* | NA | 8.50* | 4.20NS | 4.30** | 0.56 * | 0.21 NS | 0.35 NS | 0.25 NS | 0 |
| DA 5157 | *Glomus* | CB | 19.3 | 12.00** | 7.30** | 5.03 ** | 1.91 ** | 3.12 ** | 2.93 ** | 60 |
| DA 5009 | *Glomus* | NA | 9.8 | 6.00NS | 3.80** | 0.54 ** | 0.09 NS | 0.45 * | 0.27 * | 0 |
| DA 5028 | *Glomus* | PJ | 10.2 | 6.10NS | 4.10** | 3.14 ** | 0.91 ** | 2.23 ** | 1.87 ** | 0 |
| DA 5148 | *Acaulospora* | BK | 10.3 | 5.0NS | 5.30NS | 1.86 ** | 0.46 ** | 1.4 ** | 1.13 ** | 0 |
| DA 5051 | *Glomus* | BK | 13 | 8.70** | 4.30** | 0.83 ** | 0.43 ** | 0.4 NS | 0.2 NS | 35 |
| DA 5152 | *Gigaspora* | BK | 10.15 | 4.90NS | 5.25** | 0.67 ** | 0.13 NS | 0.54 NS | 0.24 NS | 0 |
| DA 5052 | *Acaulospora* | BK | 11.03 | 6.23NS | 4.80NS | 0.89 ** | 0.11 NS | 0.78 * | 0.53 * | 0 |
| DA 5088 | *Gigaspora* | CM | 10 | 5.33NS | 4.67** | 6.39 ** | 2.89 ** | 3.5 ** | 3.36 ** | 95 |
| DA 5016 | *Glomus* | NA | 11.85 | 4.40NS | 7.45** | 1.21 ** | 0.26 NS | 0.95 * | 0.8 ** | 95 |
| DA 5014 | *Glomus* | NA | 13.42 | 9.12** | 4.30** | 2.23 ** | 0.78 ** | 1.45 ** | 1.15 ** | 95 |

Footnote: AMF almost strains obtained from DA: Department of Agriculture, Bangkok SUT: Suranaree Unversity of Technology, Nakhon ratchasima Province, AMF strains from this study Site: NM (Nakhon ratchasima P.), CB (Chonburee P.), CM (chaingmai P.), NA (Nan P.), PR (Prae P.), BK (Bangkok), LP (Lumprang P.), PC (Phetchabun P.), PB (Phetchaburee), PJ (Prajuabcririkun), %I: % infection, Statistical analysis by LSD, NS = non significantly *=significant; (p,0.05); **=Highly significant (p<0.01)

# CHAPTER VI

# CONCLUSION

Since, the high variations of morphological AMF are obstacle for biodiversity study. The DNA extraction from single spore has proposed for solving of investigation AMF. Due to it has a advantage on requirement of the low number of healthy spore, less risk to contamination from the microorganisms. However, it is not cover fungal community especially, lacking of sporulation in some species. In addition, the genetic heterogeneity of thousands of nuclei in a single spore often poses a difficulty for the identification of species levels. So, there was still confusion about the monophyly of the AM fungi. The direct nested PCR using taxon-specific primers for AM fungal species opens up new possibilities for investigateing mycorrhizal community structure, and competition between different fungal species within roots and within soil. It overcomes the limitations linked to simply evaluating the presence of spores in the roots. Furthermore, as shown in this study for *G. intraradices*, the absence of detectable spores does not necessarily indicate the absence of the fungus from roots. This developed method might be hope used to monitor a mixed population of mycorrhizal fungi.

The morphological study can used only to investigate within genera level. Whist the molecular techniques were supposed to be used for elucidating in species levels and between groups of population. When focus on the genetic diversity, the results of DNA sequencing indicate the population of AMF in tree legumes with high biodiversity. Most of strains belong to genera*, Gigaspora, Scutellospora, Glomus* and *Acaulospora,* respectively.

Interestingly, the sequencing analysis also indicates the high contamination in community which mixed in AMF population such as uncultured ascomycete fungi and uncultured basidiomycete fungi which always found in analysis. So it could effect to uninterpretation of DNA sequence. Nevertheless, this approach, in combination with the classic morphological analyses of spores, is highly promising and should provide a workable strategy to better characterize AMF in communities level.

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