ARBUSCULAR MYCORRHIZA (AM) FUNGI : *PHYTOPHTHORA* SUPPRESSION, AM COMMUNITY IN RICE ROOT AND DEVELOPMENT OF T-RFLP TECHNIQUE FOR AM MORNITORING

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เชื้อราไมคอร์ไรซ่า : การยับยั้งเชื้อราก่อโรค ชุมชนของเชื้อราไมคอร์ไรซ่าในระบบการปลูกข้าว และการพัฒนาเทคนิคในการติดตาม

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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เชื้อราไมคอร์ไรซ่าเป็นเชื้อราชนิดหนึ่งที่อาศัยอยู่ในรากพืช เส้นใยของราที่รากพืชส่วนหนึ่งจะงอกสู่ดิน ทำหน้าที่ดูดน้ำ และธาตุอาหาร เช่น ฟอสฟอรัส จากสารละลายดินให้ก่อรากพืช เส้นใยของราชนิดนี้ ช่วยเพิ่มพื้นที่ผิวในการดูดกินธาตุอาหาร จึงทำให้พืชได้รับธาตุอาหารเพิ่มขึ้น นอกจากนี้ ราชนิดนี้ยังมีคุณสมบัติในการยับยั้งโรคพืชในดิน โดยเฉพาะ เชื้อราก่อโรค *Phytophthora* เชื้อราไมคอร์ไรซ่านี้ พบในรากพืชหลากหลายชนิดแบบพึ่งพาอาศัยกัน และได้ถูกจำแนกอยู่ในไฟลัม Glomeromycota โดยอาศัยข้อมูลจากลำดับเบสของดีเอ็นเอ การศึกษาเกี่ยวกับเชื้อราชนิดนี้เป็นสิ่งสำคัญที่ทำให้เกิดการนำไปใช้ในทางเทคโนโลยีชีวภาพ ดังนั้นงานวิจัยนี้ได้แบ่งการศึกษาออกเป็น 3 เรื่อง ซึ่งมีทั้งการใช้เชื้อราไมคอร์ไรซ่า และการศึกษาชุมชนของเชื้อราชนิดนี้ บริเวณรอบรากพืช นอกจากนี้ยังได้วิเคราะห์หาความลำเอียงในการใช้เทคนิคทางชีวโมเลกุล terminal restriction fragment length polymorphism (T-RFLP) เพื่อการหาชุมชนไมคอร์ไรซ่าอีกด้วย

ในหัวข้อแรกนั้น มีจุดมุ่งหมายเพื่อศึกษาเกี่ยวกับการคัดเลือกเชื้อราไมคอร์ไรซ่าที่มีประสิทธิภาพในการเพิ่มการเจริญเติบโต และการดูดกินธาตุฟอสฟอรัสในส้ม นอกจากนี้ยังมีการศึกษาการใช้สายพันธุ์ที่มีประสิทธิภาพในการยับยั้งเชื้อก่อโรครากเน่าโคนเน่า *Phytophthora* ในรากส้ม ผลการศึกษาพบว่า เชื้อราสายพันธุ์ *Glomus etunicatum* สามารถเพิ่มค่า mycorrhizal efficiency index ในส้มสายพันธุ์โชกุน และเขียวหวานได้ดีที่สุด ในขณะที่ส้มสายพันธุ์เขียวหวาน และ C-35 citrange ที่มีการใส่เชื้อ *G. etunicatum* ร่วมกับการใส่ปุ๋ย ได้ให้ผลปริมาณฟอสฟอรัสในใบมากที่สุด จากผลที่ได้ บ่งบอกว่า *G. etunicatum* เป็นสายพันธุ์ที่มีประสิทธิภาพในการเพิ่มการเจริญเติบโต และการดูดกินฟอสฟอรัสในส้ม ในส่วนของการยับยั้งเชื้อก่อโรครากเน่า พบว่า การใส่เชื้อราไมคอร์ไรซ่าร่วมกับการใส่เชื้อราก่อโรคนั้น ทำให้อาการของโรครากเน่าที่รากส้มลดลง ผลการทดลองได้บ่งบอกว่า ชนิดของพืช และชนิดของเชื้อราไมคอร์ไรซ่าที่แตกต่างกันนั้น ทำให้ได้ผลการเจริญเติบโตของพืช และผลของการต้านทานโรคที่แตกต่างกัน ดังนั้นการใช้เชื้อราไมคอร์ไรซ่านี้ พบว่า มีศักยภาพที่จะนำไปผลิตหัวเชื้อไมคอร์ไรซ่าสำหรับการใช้ในสวนส้มได้

ในการศึกษาที่สองนั้นมุ่งเน้นเพื่อหาว่า ระบบการปลูกข้าวแบบประณีต (system of rice intensification, SRI) และระบบดั้งเดิม(conventional system, CS) และการใส่ปุ๋ยอินทรีย์ร่วมกับการใส่เชื้อแบคทีเรียพีจีพีอาร์ (*Azosprillum largimobile* และ *Azotobacter vinelandii*) นั้น มีผลต่อโครงสร้างชุมชนของเชื้อราไมคอร์ไรซ่าในแต่ละช่วงอายุของการปลูกข้าวหรือไม่ ผลการหาลำดับเบสของดีเอ็นเอ พบว่า เชื้อราส่วนใหญ่อยู่ในจีนัส *Glomus* และ *Acaulospora* ในการวิเคราะห์กลุ่มชุมชนเชื้อราไมคอร์ไรซ่าโดยการใช้การวิเคราะห์ principle component analysis (PCA) พบว่า ในอายุข้าวที่ 60 วัน, 90 วัน และ 120 วันนั้น มีการแยกกลุ่มชุมชนเชื้อราระหว่างระบบSRI และ CS ในส่วนของการกระจายตัวของกลุ่มชุมชนเชื้อราโดยอาศัยข้อมูลจากT-RFLP นั้น พบว่า มีการแยกกลุ่มกันระหว่างระบบ SRI และ CS อย่างชัดเจน นอกจากนี้ ผลการทดลองยังพบว่า ข้าวที่ปลูกภายใต้ระบบ SRI นั้น มีความหลากหลายของชุมชนเชื้อราในรากมากกว่าระบบCS โดยอาศัยการประเมินจาก Shannon-Weaver index of diversity (H')

ในหัวข้อสุดท้าย ได้ทำการศึกษาเกี่ยวกับการหาความลำเอียงของเทคนิคT-RFLP ในการหากลุ่มชุมชนไมคอร์ไรซ่า ในการทดลองนี้ ได้ทำการเลียนแบบธรรมชาติ โดยการสร้างชุมชนเชื้อราไมคอร์ไรซ่าขึ้นมาเพื่อเป็นตัวแทนของกลุ่มชุมชนที่แท้จริง ในการทดลองนี้ ได้ทำการผสมพลาสมิดดีเอ็นเอของเชื้อราไมคอร์ไรซ่าสองสายพันธุ์กับดีเอ็นเอของรากแครอท เทคนิค PCR-TRFLP ได้ถูกนำมาใช้เพื่อประเมินปริมาณของชิ้นส่วนT-RF ที่ได้ จุดประสงค์ของการทดลองนี้เพื่อหาว่า จำนวนรอบของ PCR มีผลต่อปริมาณชิ้นส่วนของ T-RF อย่างไร และเพื่อหาว่า ผลของวิธีการทำดีเอ็นเอของรากแครอทให้บริสุทธิ์ และผลของการใส่พลาสมิดดีเอ็นเอของเชื้อราไมคอร์ไรซ่าสองสายพันธุ์ในอัตราส่วนต่างๆ นั้น จะมีผลต่อความลำเอียงของอัตราส่วนของผลผลิตที่ได้หรือไม่ ผลการทดลองที่ได้ พบว่า การใช้จำนวนรอบของPCR ที่มากกว่า 18 รอบ นั้นจะทำให้เกิดปรากฏการณ์ plateau และพบว่า วิธีการทำดีเอ็นเอให้บริสุทธิ์ด้วยวิธีphenol-chloroform นั้น จะให้ปริมาณผลิตภัณฑ์ของT-RF ใกล้เคียงกับปริมาณในทางทฤษฎีมากที่สุด ในขณะที่ดีเอ็นเอที่ไม่มีการทำให้บริสุทธิ์นั้น ทำให้เกิดความลำเอียงของปริมาณT-RF ที่ได้ ซึ่งอาจมีปริมาณมากกว่า หรือน้อยกว่าปริมาณในทางทฤษฎี

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2553 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

NANTIDA WATANAROJANAPORN : ARBUSCULAR MYCORRHIZA (AM) FUNGI : *PHYTOPHTHORA* SUPPRESSION, AM COMMUNITY IN RICE ROOT AND DEVELOPMENT OF T-RFLP TECHNIQUE FOR AM MORNITORING. THESIS ADVISOR : ASSOC. PROF. NEUNG TEAUMROONG, Ph.D., 242 PP.

Arbuscular mycorrhiza (AM) fungi/*Glomus etunicatum*/ *Acaulospora tuberculat/Phytophthora/*CITRUS/*Oryza sativa*/ COMMUNITY STRUCTURE/PGPR/SYSTEM OF RICE INTENSIFICATION (SRI)/T-RFLP/PCR CYCLE NUMBER/PURIFICATION METHOD

Arbuscular mycorrhiza (AM) fungi are symbionts in roots of higher plants. There are extraradical hypae originated from root fragment in the soil. The extraradical mycelium has several functions, the most important of which is the uptake and translocation of water and mineral nutrients such as phosphorus from the soil solution to roots. The highly branched nature of the absorbing hyphae increases the surface area for nutrient uptake, leading to the enhanced nutrient accumulation in plant tissues. Additionally, AM fungi inhibit the root rot caused by soilborne fungal pathogens especially *Phytophthora*. They are formed in an enormously wide variety of host plants by obligately symbiotic fungi which have recently been reclassified on the basis of DNA sequences into a separate fungal phylum, the Glomeromycota. Mycorrhizal fungi have become an important object of investigations to create new opportunities for utilizing these fungi in biotechnology. This thesis, which was divided into three topics, presents both biotechnological applications of mycorrhiza and the AM community structure in rhizosphere. Additionally, the biases in detecting

AM community by terminal restriction fragment length polymorphism (T-RFLP) method were also elucidated in the last chapter.

In the first topic, the aims were to: (i) select efficient indigenous mycorrhizal fungi and investigate the influence of effective indigenous mycorrhizal species on citrus growth and phosphorus uptake, and (ii) determine the influence of AM on *Phytophthora* root rot in citrus (*Citrus* sp.). The results of citrus growth revealed that Shogun *(C. reticulata* Blanco cv. Shogun) and Tangerine (*C. reticulate*) inoculated with *Glomus etunicatum* produced the highest mycorrhizal efficiency index (MEI). Tangerine and C-35 citrange amended with fertilizers and *G. etunicatum* demonstrated the highest P content in leaves. This indicated that *G. etunicatum* had an influence on citrus growth and P uptake, suggesting it to be a highly effective strain. Shogun scion/C-35 citrange rootstock combinations that were inoculated by both *P. nicotianae* and different AM fungi (*G. etunicatum* or *Acaulospora tuberculata*) showed minor root rot symptom. Our results indicated the facts that different host plants and different AM species produced different outcomes in terms of growth and pathogen resistance. The application of both AM isolates, therefore, has an enormous potential to produce the inoculum for citrus orchards.

In the next topic, we explored whether rice root AM community structures (based on T-RFLP derived from 18S rDNA) were affected by rice cultivation systems (the system of rice intensification (SRI) and the conventional rice cultivation system (CS)), and by application of compost and plant-growth-promoting rhizobacteria (PGPR) (*Azospirillium largimobile* and *Azotobacter vinelandii*) during different growth stages in field experiment. The Blast results showed that AM fungal sequences belonged to members of the phylum *Glomeromycota*, the genera *Glomus*

and *Acaulospora*. AM community structure was compared between the different cultivation types (CS and SRI) and different PGPR applications by principle component analysis (PCA). In the 2nd (60 days), the 3rd (90 days) and the 4th (120 days) sampling times, the AM assemblages of conventionally managed system (CS) were separated from those of SRI management. The distribution of AM community composition based on T-RFLP data demonstrated that the AM fungal community structures associated with the different cultivation plots (CS and SRI) were clearly separated. Our output also indicated that rice plants grown under SRI had more diverse AM fungal communities than those grown under CS condition when Shannon-Weaver index of diversity (*H'*) estimation was employed.

For the last topic, the microbial communities profiling method, T-RFLP, was focused in order to detect biases of real community structures in environment. In order to investigate T-RFLP community profiles in terms of relative abundance representing real community structures, artificial pairwise mixtures of 18S ribosomal RNA gene clones derived from two arbuscular mycorrhiza (AM) fungi sequences were designed to construct simple "pairwise mixes". PCR T-RFLP was used for estimation of T-RFs product to answer the questions regarding how the number of PCR cycle could affect T-RFs product in pairwise mixes and how effect of plant genomic DNA purification method and varied ratio of AM plasmid DNA template could affect the bias of template-to-product ratio in relative T-RFs peak area. The result of varying numbers of PCR cycle indicated that T-RFs products were still in the exponential phase at the 18 cycles or less before approaching the plateau. The best T-RFs product quantities based on T-RF peak areas were obtained by using phenol-chloroform purification. Non-purified plant genomic DNA might create T-RFLP

pattern that was not consistent with theoretical yield. Therefore, this generated a bias towards underestimation or overestimation of relative abundance in this study.

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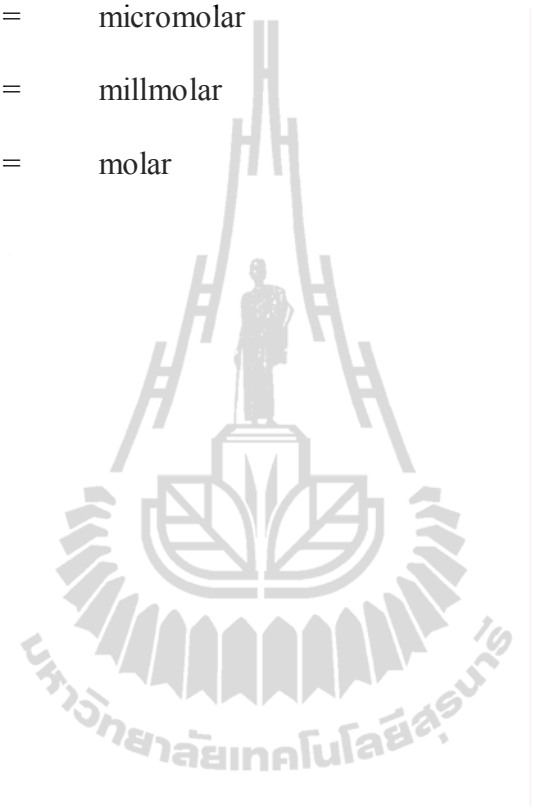
LIST OF ABBREVIATIONS

PCR	=	polymerase chain reaction
DNA	=	deoxynucleic acid
rRNA	=	ribosomal ribonucleic acid
SRI	=	system of rice intensification
T-RFLP	=	terminal restriction fragment length polymorphism
T-RFs	=	terminal restriction fragments
PCA	=	principal components analysis
cm	=	centimeter
^{o}C	=	degree Celsius
ng	=	nanogram
µg	=	microgram
mg	=	milligram
g	=	gram
kg	=	kilogram
m^2	=	square meter
s	=	second
min	=	minute
h	=	hour
d	=	day
µl	=	microliter
ml	=	milliliter

LIST OF ABBREVIATIONS (Continued)

nM	=	nanomolar
μM	=	micromolar
mM	=	millmolar
M	=	molar



CHAPTER I

INTRODUCTION

Mycorrhizal fungi are symbionts in roots of higher plants. There are extraradical hypae originated from root fragment in the soil. The ERM has several functions, the most important of which is the uptake and translocation of mineral nutrients such as phosphorus from the soil solution to roots. The highly branched nature of the absorbing hyphae increases the surface area for nutrient uptake. These associations vary widely in structure and functions, but the most common interaction is the arbuscular mycorrhizal (AM) association. It is estimated that more than 80% of all terrestrial plants form this type of association. These include of many agriculturally and horticulturally important crop species (Smith and Read 1997). The AM symbiosis represents an ancient symbiosis (Pirozynski and Malloch 1975; Pirozynski and Dalpe 1989). AM fungi are probably the most ubiquitous fungi in agricultural soils, accounting for 5–36% of the total biomass in soil and 9–55% of the biomass of soil microorganisms (Olson et al. 1999). These fungi are a critical component in agricultural systems because these organisms can increase plant growth (Smith and Read 1997), plant reproductive capacity (Lu and Koide 1994), plant water stress tolerance (Gupta and Kumar 2000), and plant health through antagonistic and competitive effects on pests and pathogens (Gange and West 1994). This colonization may also enhance the plant's resistance to biotic and abiotic stresses (Newsham et al. 1994; Subramanian et al. 1995; von Reichenbach and Schonbeck 1995; Ricken and Hofner 1996). AM fungi develop an extensive hyphal (extraradical hyphae)network

with the plant root system which makes a significant contribution to the improvement of soil texture and water relations (Bethlenfalvay and Shuepp 1994). The main benefit to the host plant in the mycorrhizae symbiosis is the enhanced uptake of immobile soil nutrients, in particular phosphorus (Jakobsen 1999). Arbuscular mycorrhizal associations increase nitrogen accumulation in plant tissues as a result of the hyphae competing for mineralized organic soil nitrogen (Ibijbijen et al. 1996). Also, these fungi interact with other soil organisms involved in important nutrient cycles.Ecological roles are of special importance in low-input farm management systems because these systems rely on natural nutrient cycles to provide the nutrients required for plant production. Therefore, these fungi constitute an integral and important component of ecosystems and may have significant applications in sustainable agricultural systems (Schreiner and Bethlenfalvay 1995).

Recent developments in the study of mycorrhizas have encouraged us to present this work. A vast expansion of interest in mycorrhiza, resulting in public awareness that the productivity of plants and the quality of leaves, flowers, fruits, and seeds are determined by the activities of root systems and their associated physical, chemical, and biological environment, is manifest worldwide. During its life cycle, a plant root is associated with a myriad of soil microorganisms, especially mycorrhizal fungi. These associations are principally dynamic. Mycorrhizal fungi have become an important object of tests to evaluate some of the new opportunities being developed in biotechnology. While these fungi have been used to stabilize forests since the turn of century, the novelty in recent years has increased recognition that biological processes can be manipulated genetically, opening numerous opportunities for the optimization of plant productivity in both managed and natural ecosystems, while minimizing the risks of environmental damage. It has become increasingly clear that

the vast, expanding field of molecular biology will have a major impact on mycorrhizal studies. The work studied here presents both biotechnological applications of mycorrhizas and study of the AM composition and community structure. We also studied the biases for detecting AM community by terminal restriction fragment length polymorphism (T-RFLP).

1.1 Objectives

The aims of the work reported in this thesis were separated into 3 parts

1.1.1 To investigate the influence of effective indigenous mycorrhizal species on growth and *Phytophthora* root rot in citrus plants

1.1.2 To determine the effect of rice cultivation system on indigenous AM community structure

1.1.3 To estimate biases for detecting AM community by terminal restriction fragment length polymorphism (T-RFLP).

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CHAPTER II

LITERATURE REVIEW

2.1 Definition

Arbuscular mycorrhizas, originally referred toas vesicular – arbuscular mycorrhizas, a namestill used by some authors (Smith and Read1997), are mutualistic symbiotic associationsbetween the roots of most vascular plants and asmall group of fungi in the new phylumGlomeromycota (Schüβler et al. 2001). Althoughsome structural variation exists in this category,most arbuscular mycorrhizas are characterizedby the presence of intraradical hyphae (Figure 2.1a) (intercellularor intracellular in location), arbuscules (Figure 2.1b) (finely branched hyphae involved in nutrientexchange), extraradical mycelium (Figure 2.1c) (hyphae thatconnect the root to the soil), and spores formedin the extraradical mycelium.Some fungal species also form intraradical structuresreferred to as vesicles (Figure 2.1d) (enlarged portions ofhyphae that become filled with lipid bodies, giving this group its originalname vesicular–arbuscular mycorrhiza. Speciesin the genera *Gigaspora* and *Scutellospora* produceauxiliary vesicles (Figure 2.1e) (sometimes called auxiliarybodies or cells) in the extraradical mycelium.Two major types of arbuscular mycorrhizashave been described: the *Arum*-type (Figure 2.1f) and the *Paris*-type (Figure 2.1g) named after theplant genera in the families *Araceae* and*Liliaceae*, respectively, in which they were firstdescribed (Smith and Smith1997). Since some ofthe fungal structures of both types are formedwithin root cells, arbuscular

mycorrhizas areclassified under the broader term, endomycorrhizas.

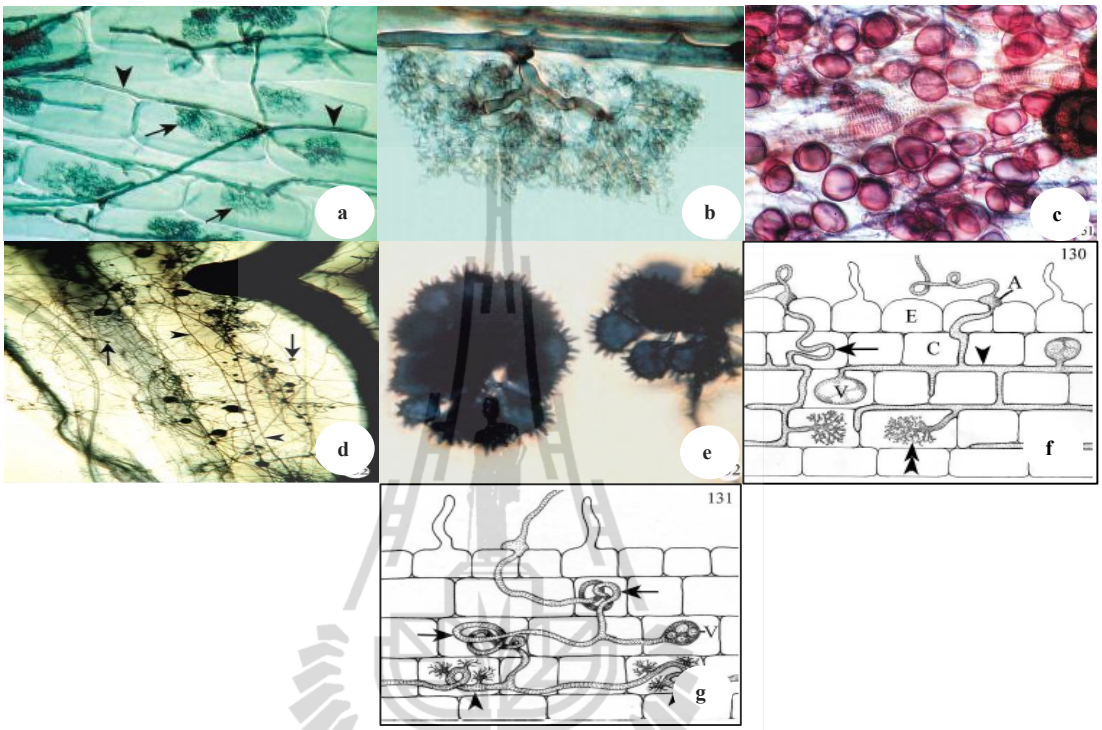

Figure 2.1 Arbuscular mycorrhizal structure: (a) Intercellular hypha (arrowheads) and arbuscules (arrows) of *Glomus mosseae* in a cleared root of *Allium porrum*; (b) Fully developed arbuscule of *G. mosseae* in cortical cell of an *A. porrum* root; (c) Cleared *A. porrum* roots colonized by *G. versiforme* showing extraradical mycelium (arrowheads) and developing spores (arrows); (d) Large number of *G. versiforme* vesicles in an *A. porrum* root; (e) Auxiliary vesicles of *Gigaspora* sp. formed in the extraradical mycelium; (f) *Arum*-type arbuscular mycorrhizal association. Infection hyphae from appressoria (A) penetrate epidermal cells (E) before entering the cortex (C). Frequently, a hypha forms a coil (arrow) before entering the intercellular space system (arrowhead) of the cortex. Arbuscules (double arrowheads) form within inner cortical cells and,

depending on the fungal species, vesicles (V) may form; (g) *Paris*-type arbuscular mycorrhizal association. Early events are similar to the *Arum*-type but extensive hyphal coiling occurs in the cortex (arrows) and small branches form from some of these to form arbusculate coils (arrowheads). Again, depending on the fungal species, vesicles (V) may form. Photos provided by Dr. Mark Brundrett.

2.2 Plant species involved

It has been estimated that over 80% of all vascular plants form arbuscular mycorrhizas. A detailed list for the United Kingdom has been published (Harley and Harley 1987) but such lists generally do not exist for other regions of the world and, in fact, many species of vascular plants have not been assessed for the presence of mycorrhizas. Arbuscular mycorrhizas have been identified in a broad spectrum of plants, including some non-vascular plants, ferns and other seedless vascular plants, groups within the gymnosperms including conifers, *Ginkgo biloba*, the cycads, and the majority of angiosperm families. Fossil records from the Devonian of the first vascular plants show that arbuscular mycorrhizal associations were present in underground rhizomatous structures, confirming the presence of this mutualistic association as far back as 400 million years or more (Taylor et al. 1995). Evidence that the first land plants already possessed this association suggests that their aquatic ancestors also harboured these fungi. Redecker et al. (2000) have documented spores from the Ordovician period (460 Ma) similar to present day spores of Glomalean fungi, indicating that earlier associations with non-vascular plants were likely. Arbuscular mycorrhizas have been studied intensively; they occur in most ecosystems

of the world and are found in many important crop species (e.g., wheat, maize, rice, soybeans, forage crops, grapes, fruit trees, cotton) and horticultural species (e.g., roses, petunias, lilies, carnations) (Figure 2.2). Attempts have been made to increase productivity by adding arbuscular mycorrhiza inoculum during planting or by manipulation of soil conditions to enhance the performance of indigenous fungal species. The inability to axenically culture arbuscular mycorrhizal fungi has made it difficult to produce inoculum on an industrial scale and consequently has limited its use in large agricultural operations. The few angiosperm families that do not have arbuscular mycorrhizas either form other categories of mycorrhizas or lack mycorrhizas. Among the latter families are the *Brassicaceae* (this family includes canola, mustards, cabbages, etc.) and the *Chenopodiaceae* (this family includes garden and sugar beets, spinach and the large genus *Chenopodium*), although even here arbuscular mycorrhizal associations have been reported for a few species. A few aquatic plant families and the sedges may have low levels of colonization by arbuscular mycorrhizal fungi, however, these can be overlooked if roots are collected at the wrong time of the year or if the sample size is too small. The relative abundance and the seasonality of arbuscular mycorrhizas in many plant species remains undetermined.

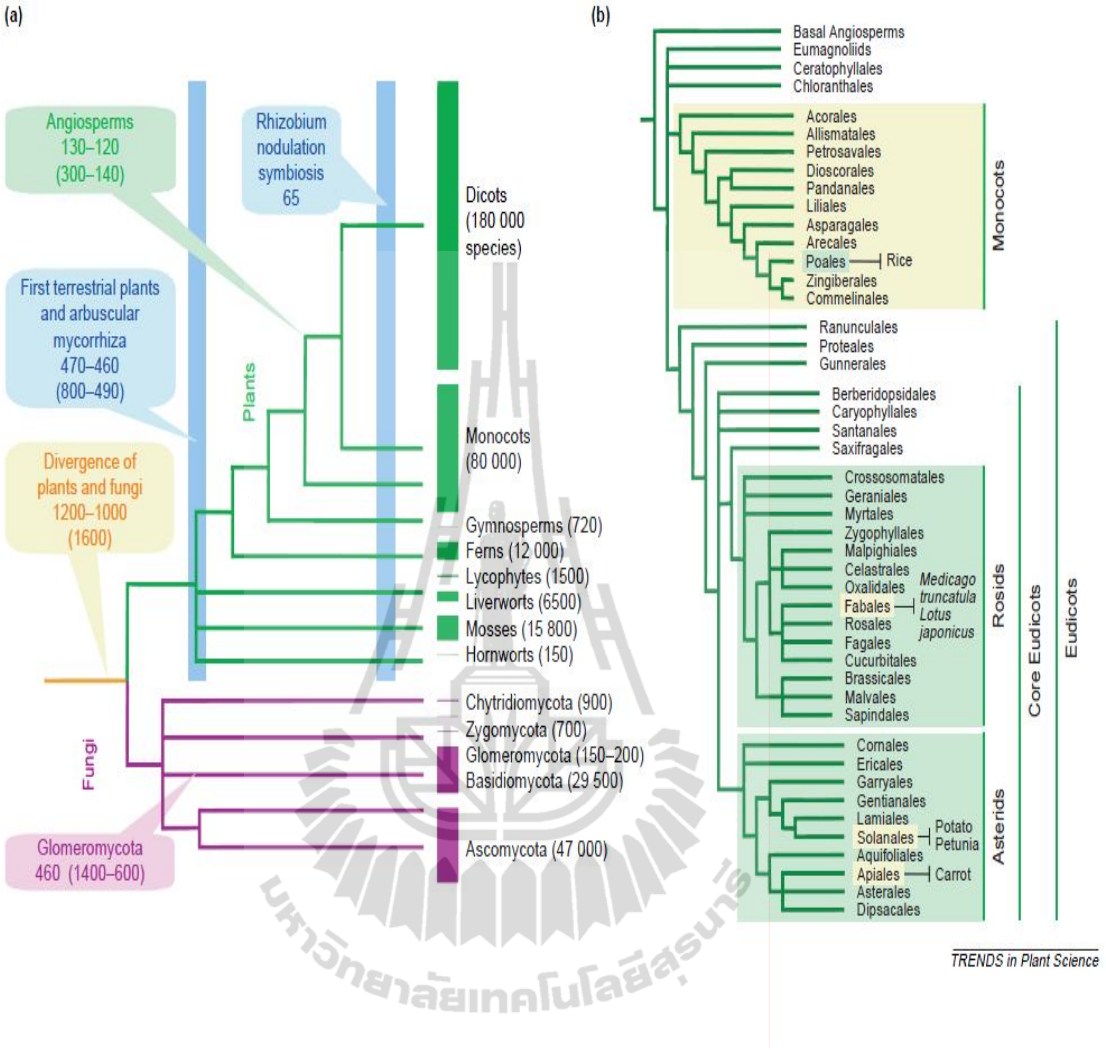

Figure 2.2 Evolution and systematics of fungi and land plants. (a) Simplified phylogenetic tree of major fungal and land plant lineages. Callouts show evolutionary events involved in the appearance of arbuscular mycorrhizal symbiosis (divergence between fungi and plants, origin of AM of the order Glomeromycota, first terrestrial plants and AM, and angiosperm plants). Numbers show possible ages in millions of years, calculated from fossils and molecular (in brackets) data. The tree is not scaled and does not reflect actual evolutionary distances. Bars to the right reflect the approximate numbers of modern species described for each lineage. The suggested number of fungal species is ~1.5 million

(Hawksworth 2001). (b) Simplified phylogenetic tree of the angiosperms. Marked species were used in an experiment to estimate how conserved the signaling pathway triggering mycorrhiza-specific expression of corresponding phosphate transporter genes is in plant species of different taxonomic positions (Karandashov et al. 2004). Promoters of mycorrhiza-inducible phosphate transporter genes from potato (*StPT3*) and *Medicago truncatula* (*MtPT4*) could drive the expression of the GUS reporter gene in mycorrhizal roots of eudicot species, whereas the corresponding rice (*ORYsa;Pht1;11*) promoter failed to do so, suggesting that the pathway is conserved among the dicots, whereas the evolutionary distance between the dicots and the monocots did not allow conservation (Karandashov et al. 2004) .

2.3 Fungal species involved

The fungi involved are ubiquitous soil-borne organisms (Phylum *Glomeromycota*; Class *Glomeromycetes*) belonging to four orders: *Archaeosporales*, *Paraglomerales*, *Diversisporales* and *Glomerales* (Schüβler et al. 2001). Eight genera of arbuscular mycorrhizal fungi have been recognized based mainly on morphological characteristics of asexual spores, although molecular methods and various biochemical parameters are now being used in systematic studies. These genera, *Glomus, Paraglomus, Sclerocystis, Scutellospora, Gigaspora, Acaulospora, Archaeospora,* and *Entrophospora,* include approximately 150 species; however, species delineation remains uncertain and continues to change as more isolates are examined and as the use of molecular techniques increases (Morton and Redecker

2001) (Figure 2.3). A major challenge in the systematics of this group of fungi is the considerable variation within isolates of defined species and the lack of sexual reproductive structures. All arbuscular mycorrhizal fungal species are obligate biotrophs, depending entirely on host plants for carbon compounds. This means that, unlike many ectomycorrhizal fungi these fungi cannot be cultured in the absence of plants. As a result, researchers have used root organ cultures and genetically transformed root cultures to maintain arbuscular mycorrhizal fungi and for use in experimental studies (Fortin et al. 2002).

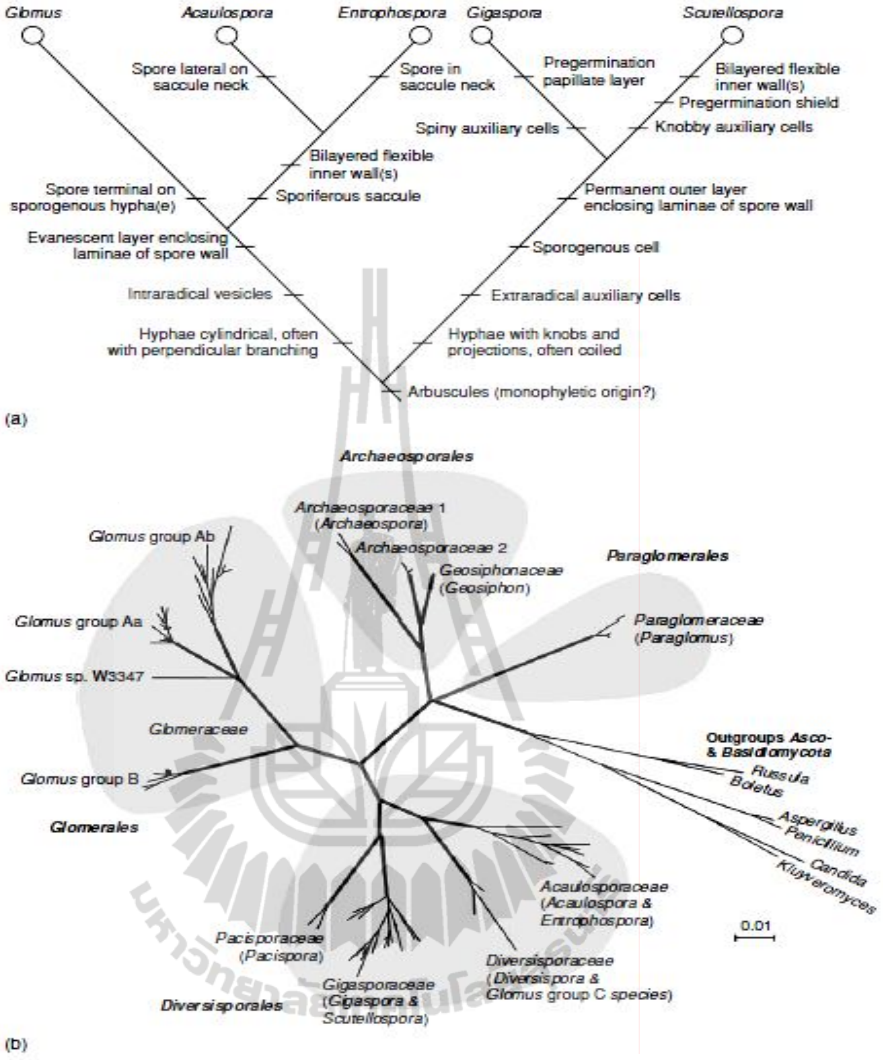

Figure 2.3 Phylogenetic trees for AM fungi, derived from different types of information. (a) A cladogram showing taxonomic and phylogenetic divergence among genera of AM fungi, based on comparative developmental sequences of the spores. Courtesy Joe Morton. (b) A maximum likelihood tree based on molecular data from near full-length SSU rRNA gene sequences, which reflects the topology of different neighbour joining and parsimony bootstrap analyses, showing the phylogeny of the AM fungi (Glomeromycota). Courtesy Arthur Schuβler, modified from Schuβler (2002).

2.4 Structures

2.4.1 Intraradical hyphae

2.4.1.1 Development

The formation of arbuscular mycorrhizas involves a series of steps from the recognition of the root surface by the fungus to the formation of an appressorium, epidermal cell penetration, intraradical hyphal and arbuscular development, and, in some cases, the formation of vesicles. All of these steps are undoubtedly under genetic control, but until recently, there has been little research in this area. Plant mutants that show blocks to various steps in the colonization process are now being used in such studies.

In order for the development of all internal structures to occur, fungal hyphae must contact either the surface of root epidermal cells or root hairs, form specialized attachment structures called appressoria (Figure 2.4) and then penetrate the epidermal layer before reaching the cortex. The regions along the root at which appressoria form and where hyphae enter the epidermis are referred to as entry points. An hypha, on contacting the root surface, may branch and form more than one entry point. Sources of hyphae that attach to the surface of epidermal cells or root hairs are either germinating spores, the existing hyphal network in the soil attached to living roots, or hyphae that grow from colonized root pieces left in the soil as plants die. Sectional views of appressoria show that they are multi-nucleate and possess many small vacuoles. Often an appressorium forms between epidermal cells and hyphae formed from the appressorium penetrate adjacent epidermal cells.

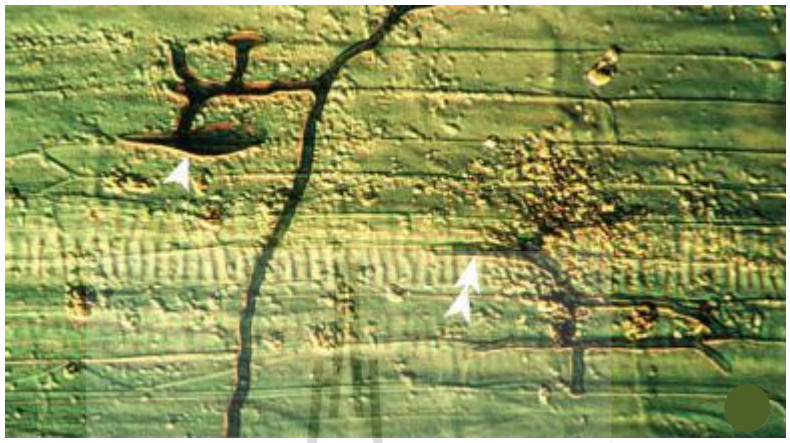

Figure 2.4 An appressorium (arrowhead) and branching hyphae (double arrowhead) of *Glomus versiforme* on the surface of an *Allium porrum* root.

In the *Arum*-type arbuscular mycorrhiza, the hypha that penetrates into the epidermis generally forms a coil either in the epidermal cell or first cortical cell layer before it enters the intercellular spaces of the cortex. In this type, roots can become rapidly colonized along the root axis due to the free movement of hyphae in the intercellular spaces.

In the *Paris*-type mycorrhiza, the intraradical hyphae pass from cell to cell, forming complex coils in both epidermal and cortical cells. Species of plants developing *Paris*-type mycorrhizas lack conspicuous intercellular spaces in the root cortex so that the growth of hyphae in the longitudinal direction of the root is slower than in the *Arum*-type.

Many angiosperm roots develop a modified outer cortical cell layer, the hypodermis (known as an exodermis if it has Casparian bands, wall modifications consisting primarily of the hydrophobic substance, suberin), which has cells that differ in their length in reference to the longitudinal axis of the root. This type of hypodermis or exodermis is referred to as dimorphic, consisting of short and

long cells. Both cell types become modified by the deposition of suberin in their walls, a process that is slower in the short cells compared to the long cells. The short cells, therefore, become the entry points for arbuscular mycorrhizal fungal hyphae into the remaining cortex.

2.4.1.2 Functions

Intraradical hyphae are able to initiate other fungal structures within the root. For example, in the *Arum*-type mycorrhiza, hyphae penetrate through the walls of cortical cells, branch repeatedly, and form arbuscules. In the *Paris*-type, small branches develop laterally from the hyphal coils to form arbuscules (referred to as arbusculate coils). Intraradical hyphae may also initiate intracellular or intercellular vesicles. Intraradical hyphae that persist in decaying root pieces in the soil are important sources of inoculum for subsequent colonization of new roots. By withstanding soil freezing and drying, these hyphae can survive in the soil until conditions are favourable for their re-growth.

2.4.2 Arbuscules

2.4.2.1 Development

***Arum*-type**

Usually a single branch from either an intercellular or intracellular hypha narrows and penetrates the wall of a cortical cell. It forms a trunk hypha which branches repeatedly to form a complex tree-like structure, the arbuscule. Occasionally two trunk hyphae form, leading to the development of two arbuscules within one cortical cell. Arbuscules may form in any region of the cortex but most often they develop in the inner cortex adjacent to the endodermis and the vascular tissues. The trunk hypha, and branches from it, become enveloped by host-derived plasma membrane (the periarbuscular / perifungal membrane) and a host-derived

interfacial matrix consisting of cell wall components. This results in the separation of the arbuscule from the host cell cytoplasm by an apoplastic compartment which must play a role in the transfer and temporary storage of mineral nutrients and sugars.

***Paris*-type**

Plant species that have this type of mycorrhiza lack conspicuous intercellular spaces in their roots and, as a result, only intracellular hyphae are present. These develop complex coils from which fine lateral branches are initiated. The coils, including these fine branches, are the arbuscules or arbusculate coils. The hyphal coils and the fine branches have a perifungal membrane and an interfacial matrix of host-derived cell wall components. Appressoria, hyphae (both extraradical and intraradical), and arbuscules may all have endosymbiotic bacteria within the cytoplasm.

The development of intracellular hyphae and arbuscules leads to dramatic changes in the organization of the cytoplasm in host cells. Cortical cells that were vacuolated prior to fungal penetration have an increased number of organelles such as mitochondria. One of the most striking changes, however, is in the cytoskeleton.

2.4.2.2 Functions

The highly branched nature of arbuscules and the presence of a periarbuscular membrane enveloping all of the fine branches provide a large surface area; this has led to the conclusion that it is through these structures that most of the nutrient exchange occurs. This conclusion is strengthened by the demonstration that both sucrose (Figure 2.5) and phosphate transporters (Figure 2.6) occur in the periarbuscular membrane. It is known that each arbuscule formed in a root has a finite

life span of a few days; therefore, it is possible that nutrients might also be released from the fungus into the root cell at the time of arbuscule degeneration.

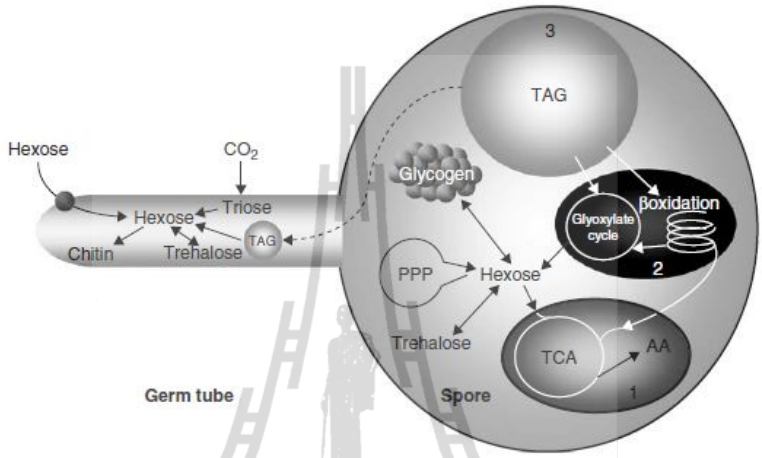

Figure 2.5 Proposed scheme for pathways of carbon (C) metabolism and trafficking in asymbiotic spores and germ tubes of an arbuscular mycorrhizal fungus. Broken arrows indicate transport between different C pools. (1) Mitochondrion, (2) glyoxysome and (3) lipid body. Reproduced from Bago *et al*. *Plant Physiology*, 124, 949–957 (2000), with permission of the American Society of Plant Biologists.

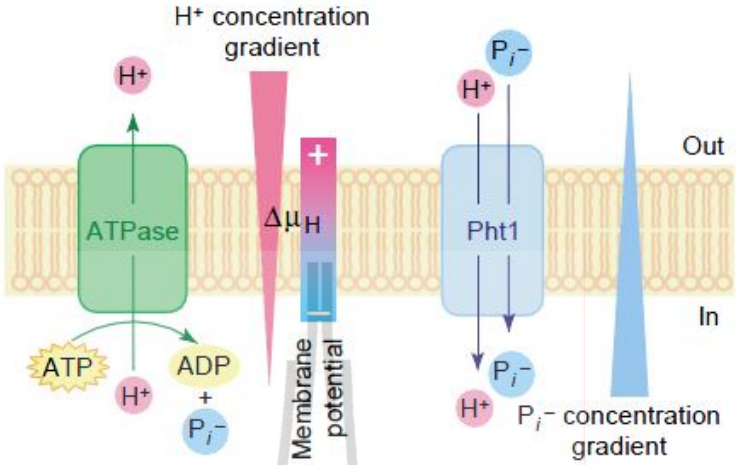

Figure 2.6 The mechanism of phosphate transport across the plant plasma membrane. A membrane-integral proton ATPase unidirectionally extrudes protons (H^+) at the expense of ATP (primary transport). The generated proton concentration gradient and membrane potential constitute a proton electrochemical potential ($\Delta\mu_H$) across the membrane. Proton movement along concentration and electrical gradients facilitates phosphate (Pi^-) allocation through phosphate transporters against a steep concentration gradient (secondary transport) (Karandashov 2005).

2.4.3 Intraradical vesicles

2.4.3.1 Development

As noted above, the genera *Gigaspora* and *Scutellospora* do not form intraradical vesicles. For those genera that do, there has been considerable interest in determining their role in the life cycle of the fungus. To address this, information related to their development and structure is important. Vesicles can develop from the tips of hyphae or from lateral branches, either within cells or in intercellular spaces of the root. When vesicles form within root cells, they frequently

enlarge to occupy the entire volume of the cell; they may assume the shape of the cell and even stretch the walls of the cells in which they occur. Little is known with respect to the early stages of vesicle development or the factors that trigger their formation. It is known that large numbers of vesicles may form in roots towards the end of the growing season, or in roots of pot cultures that have been maintained for long periods of time. Vesicle formation usually follows that of arbuscules, indicating that the fungus may require arbuscular-derived sugars from the host prior to their formation. Vesicle development involves the swelling of a hyphal tip or lateral branch followed by modifications to the cytoplasm and cell wall. The most characteristic cytological features of mature vesicles are the presence of lipid bodies and many nuclei. Bacteria also occur in vesicles of some species. The wall generally becomes thickened and this enhances the ability of vesicles to withstand soil drying and perhaps parasitism by soil organisms.

2.4.3.2 Functions

The storage of large amounts of lipid (as much as 58% of the dry mass of vesicles) indicates that vesicles are important storage structures and that they may act as propagules for the fungus. Declerck et al. (1998) isolated intact vesicles of *Glomus intraradices* (a species known to form a large number of intraradical vesicles) from genetically transformed roots of carrot, placed them on an agar medium, and allowed them to germinate. Germ tubes grew from the subtending hypha and these were able to contact and initiate colonization on adjacent roots. The use of vital stains has shown that vesicles can retain their viability even after three weeks of low temperature (5°C) storage, suggesting that these structures might be important over-wintering propagules. Three classes of lipids (neutral lipids, glycolipids and

sphingolipids, phospholipids), glycolipids, and sphingolipids were the most abundant followed by neutral lipids and phospholipids found in vesicles.

2.4.4 Extraradical vesicles (auxiliary vesicles)

2.4.4.1 Development

Instead of intraradical vesicles, two genera, *Gigaspora* and *Scutellospora*, form auxiliary vesicles in the extraradical mycelium (ERM). These are initiated as lateral branches that rapidly expand into globose structures of varying colour and with ornamented walls. Auxiliary vesicles can occur singly or in clusters. Wall ornamentation patterns are used as taxonomic characters. Each auxiliary vesicle stores large amounts of lipid, has vacuolar inclusions, and is multi-nucleate. Bacteria may be present within the cytoplasm.

2.4.4.2 Functions

Very little is known about the role that auxiliary vesicles play in the life cycle of the fungus, although new hyphae have been observed growing from the subtending hyphae of these structures in *Gigaspora margarita*, suggesting that they may function in propagation.

2.4.5 Extraradical mycelium

2.4.5.1 Development

The ERM of arbuscular mycorrhizas, like that of other mycorrhizas, has been difficult to study *in situ* because of the heterogeneous nature of soil and the ability to locate the hyphae forming the mycelium network. Early studies involved extracting hyphae from the soil and examining these using light microscopy; extraradical hyphae comprising the mycelium network were described as dimorphic, having coarse hyphae with angular branchings as well as fine hyphae. Hyphae that

originated from germinating spores had a narrow diameter and, after contact with a root, repeatedly branched and developed several entry points. Other wide diameter hyphae originated from root fragments in the soil; these also often branched to form several entry points. Some hyphae (runner hyphae) had a wide diameter and usually did not branch as they contacted the root surface; these appeared to function mainly for the rapid spread of the fungus from adjacent living roots. Hyphae that exited the root were of two types. Wide diameter runner hyphae were identified that either grew along the surface of the root and established new entry points, or grew toward other roots and established hyphal bridges between adjacent roots of the same or different species. Other highly branched hyphae functioned presumably as the absorbing hyphal network. ERM development has also been studied in detail *in vitro* using genetically transformed roots inoculated with various fungal species (St. Arnaud et al. 1996; Bago et al. 1998a, b; Fortin et al. 2002). In these studies, hyphae originating from roots grew rapidly in a straight line across the agar surface and were mostly unbranched. Later, regions along the runner hyphae formed short, narrow diameter, lateral branches that in turn branched repeatedly to form 'tree-like' structures. Patches of branched hyphae, branched absorbing structures (BAS), formed at intervals as the runner hypha continued to grow across the agar surface (Figure 2.7) (Bago et al. 1998*a*). Each patch lived 2–3 months under the study conditions. *In vitro* spore formation has occurred after the production of patches of absorbing hyphae and often in close proximity to these hyphae. Spore formation may rely on nutrients obtained via these hyphae. It is known from experiments by Giovannetti et al. (1994, 1996) that substances produced by host roots trigger branching of hyphae. In both soil-based and *in vitro* systems, these highly branched hyphae are likely the most important portion of the mycelium network in terms of nutrient uptake.

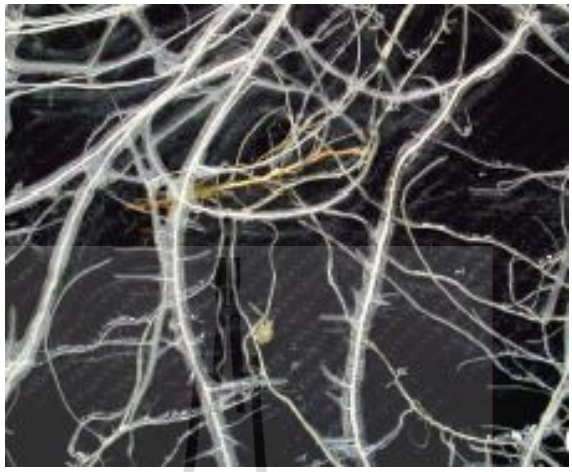

Figure 2.7 The development and branching patterns of extraradical mycelium on axenic agar of transformed *Daucus carota* roots.

2.4.5.2 Functions

The ERM has several functions, the most important of which is the uptake and translocation of mineral nutrients such as phosphorus from the soil solution to roots. The highly branched nature of the absorbing hyphae increases the surface area for nutrient uptake. Hyphal growth away from the depletion zone at the root surface extends the region from which nutrients and water can be absorbed. Increasing evidence suggests that the fine hyphae alter the pH in the adjacent soil resulting in increased availability of some nutrients. Hyphal bridges, known to occur between roots of adjacent plants, can act as a mechanism for the transfer of nutrients between hosts. The transfer of phosphate and carbon compounds is currently of considerable interest in terms of competition among plants.

An exciting new finding implicates hyphal bridges between some non-photosynthetic plant species and neighbouring photosynthetic species (Bidartondo et al. 2002). These authors used molecular methods to show that

Arachnitis uniflora, five *Voyria* species, and *Voyriella parviflora* are associated with very specific AM fungi which were also present in neighbouring photosynthetic plants. It has yet to be shown, experimentally, that carbon compounds are transferred from the photosynthetic to the non-photosynthetic plants.

The ERM is also a source of inoculum for new roots. When the mycelium network is disturbed, either under laboratory or field conditions, there is a delay in colonization of new roots (Miller et al. 1995). This has important implications in agriculture in the way fields are prepared for seeding of new crops. Reduced tillage before seeding leads to the maintenance of a preexisting mycelium network and ensures that seedlings, once colonized, experience enhanced phosphorus absorption (Miller et al. 1995). Also, in various mining operations that involve considerable soil disturbance, reclamation of this soil often depends on planting pre-colonized plants since the mycelium network does not exist. The ERM also may be destroyed by soil organisms that use it as a food source. The ERM aids in soil stabilization; soil particles adhere to the surface of hyphae because of the production of a glycoprotein, glomalin. This process is important in the stabilization of sand dunes, soil in disturbed sites, and is likely important in maintaining soil texture generally. The ERM plays an important role in the formation of spores that are important sources of inoculum for dispersal of the fungus.

2.4.6 Spores

2.4.6.1 Development

Spore characteristics including size, colour, wall layers, and features of the subtending hypha are used to classify species of arbuscular mycorrhizal fungi. This has resulted in considerable information on the structure of mature spores, particularly features revealed by light microscopy. Spores usually

develop either singly as enlargements of the apex of extraradical hyphae or in groups within special hyphal aggregates (sporocarps) occurring in the ERM (Figure 2.8 a, b, c). In the genus *Paraglomus*, spores are initiated from the subtending hypha of an existing spore. Mature spores vary in the number of wall layers, the outer of which is often pigmented and impermeable. Wall layers are also of varying composition including polysaccharides, lipids, proteins and chitin. Chitin is present in one or more layers and often exists in a complex arrangement of fibrils. Characteristic cytological features include numerous storage lipid bodies, nuclei, and bacteria (Figure 2.8 d, e). Spores contain the usual organelles such as mitochondria, endoplasmic reticulum and vacuoles that are found in living fungal cells. As propagules of arbuscular mycorrhizal fungi, spores need to germinate and produce hyphae that can colonize roots. The initial hyphae formed from resting spores are termed germ tubes. In many genera, germ tubes emerge from the subtending hyphal attachment; in others, they may emerge directly through the spore wall or from special regions that form within the spore. In those species in which germ tubes exit directly through the multi-layered wall it appears that enzymes digest the wall, thus enabling the germ tube to pass through.

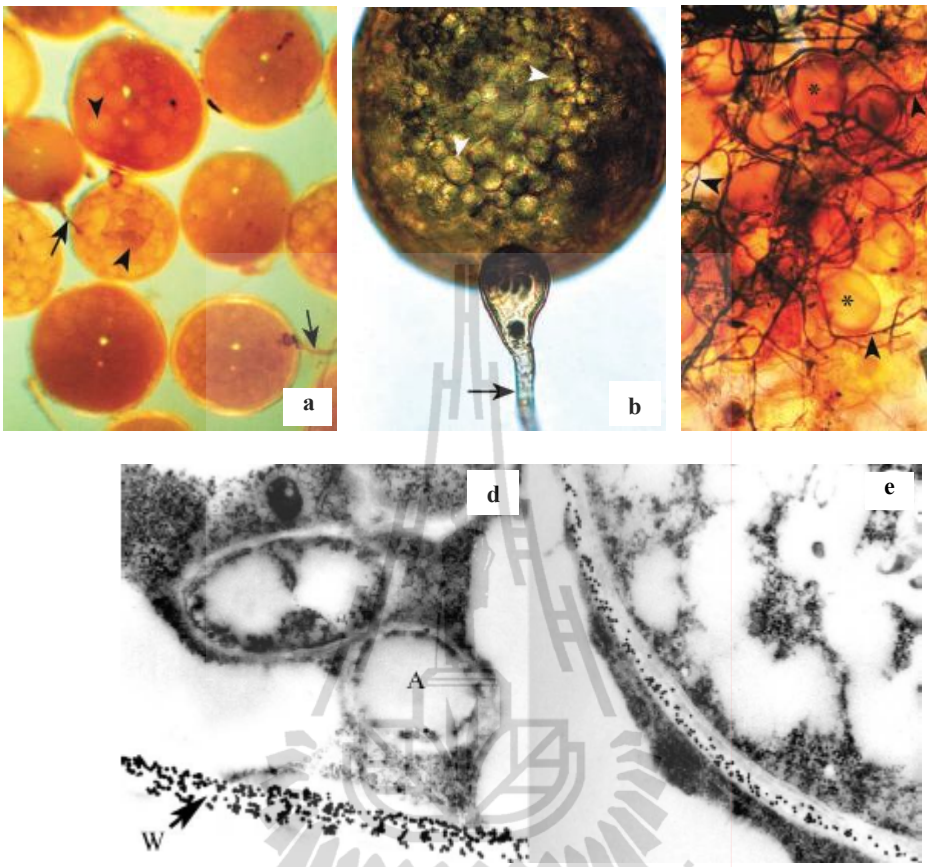

Figure 2.8 Arbuscular mycorrhiza spore: (a)-(b) Spores of *Gigaspora calospora* showing large lipid bodies (arrowheads) and the subtending hypha (arrow); (c) Sporocarp of a *Glomus* sp. from a maple – beech forest. Spores (*) and hyphae (arrowheads) are evident. Photo courtesy of Dr. Mark Brundrett; (d) and (e) Arbuscular mycorrhizas of *Zea mays* colonized by *Glomus versiforme* and labelled for cellulose using a cellobiohydrolase-gold complex. (d) Localization of cellulose in host cell wall (W). Only a few gold particles are localized around the small arbuscule branches (A). (e) Localization of cellulose in the interfacial matrix material around a large arbuscule branch. Both from Balestrini et al. Planta 195**:** 201–209 (1994).

2.4.6.2 Functions

The formation of spores of arbuscular mycorrhizal fungi is affected by factors such as plant growth, light levels, and nutrients. Generally, more spores are formed in the extraradical mycelium towards the end of the growing season presumably after an extensive mycelium network has developed. A decline in spore production often occurs when photosynthesis of the host plant is reduced thereby limiting resource allocation to the root system and to the mycelium network. Spore production in some fungal species is influenced by the particular host species/fungal species combination. The layered resistant wall of spores of most arbuscular mycorrhizal fungi enables them to persist in the soil, sometimes for many years. As new plant roots come into proximity with spores, they are induced to germinate and to form hyphae that colonize these roots. Although the spore wall is complex in structure, mycoparasitic fungi and bacteria frequently occur within spores and may render them non-viable. Spore dispersal by air, water, and small animals is important in the distribution of arbuscular mycorrhizal fungi, especially with respect to severely degraded and disturbed soils, and agricultural soils that have been left either without crop cover or planted to non-mycorrhizal plant species for periods of time. These areas usually have very low spore numbers and the import of spores from adjacent areas becomes critical for the re-vegetation of degraded sites and for crop production on agricultural sites.

2.5 Beneficial Effects of AM Fungi

2.5.1 Nutrient Transport

In the majority of mycorrhizal types, carbohydrates produced by photosynthesis move from the autotroph (host plant) to the heterotroph (fungal

symbiont); while nutrients acquired from the soil solution pass in the opposite direction (Smith and Read 1997; Jakobsen 1999). The contribution of AM to plant nutrient uptake is mainly through the acquisition of nutrients (especially P) from the soil by the extraradical fungal hyphae, especially from root-distant soil not depleted of nutrients by the root (George 2000). Mechanism of nutrient translocation to the host and the carbon drain (photosynthates) of the fungus on the host has significant affects on plant growth (Schellenbaum et al. 1998). Fungal hyphae are functionally analogous to fine root hairs as both are nutrient uptake organs. Diameters of fine root hairs, 5–20 μm (Wulfsohn and Nyengaard 1999), and hyphae, 3–7 μm (Bago 2000; Dodd et al. 2000), are comparable, but hyphal length densities of AM in soil of chamber and field experiments range from 10- to 100-fold greater than root length densities in the corresponding studies (Miller et al. 1995; Ravnskov et al. 1999; Schweiger et al. 1999; Dodd et al. 2000). Fungal hyphae extend the plant's effective absorption surfaces beyond the nutrient depleted zone that develops around the root caused by direct root uptake processes. However, greater hyphal density is not of equal significance for uptake of all ions in soil (Jakobsen 1999; George 2000). It is of importance for ions with small effective diffusion coefficients (De) in soil, such as $H_2PO_4^-$ (10^{-8} to 10^{-11} CM^2 S^{-1}) (Barber 1984). Evidence suggests AM produce extracellular phosphatase that mineralize organic P for uptake (Joner et al. 2000; Koide and Kabir 2000), but the activity of AM phosphatase is relatively small in comparison to the activity of other soil microbes and autolysis (Joner et al. 2000).

2.5.2 Micronutrient Uptake

Micronutrients are needed by the plant in small quantities but are very important for proper growth and development, as they are parts of various enzymes, pigments and other biological molecules essential for plant life. These elements are

copper, zinc, magnesium, manganese and cobalt. AM fungi help the plant in two ways: firstly, they help in the uptake of these elements which are considered to be relatively immobile, and secondly, they take up these elements and store them so as to prevent their concentrations to reach toxic levels. AM fungi could act as a sink for copper, cobalt and zinc (Bowen et al. 1980; Cooper and Tinker 1978). Most benefits of mycorrhiza have been traced to phosphorus uptake. Other elements like zinc also probably play a key role in increase of growth and yield of the plant. Uptake of micronutrients is usually limited by the rate of diffusion, if these elements move through soil to the plant root leading to quick formation of depletion zones around actively growing plant roots. Hyphae of AM fungi extend beyond these zones and help in acquisition and mobilization of these elements (Tinker 1978). Increased uptake of iron by mycorrhizal fungi may be in part due to production of siderophores that specifically chelate iron. Cress et al. (1979) found siderophore activity associated with four species of AM fungi. A point to be noted here is that iron has also been implicated in ion uptake and metabolism in the *Rhizobium* legume symbiosis. Elements like potassium nitrate and sulphate in soil solutions have higher mobilities (Nye and Tinker 1977), and it is unlikely that depletion zones would be formed around plant roots and that these nutrients would move more rapidly through hyphae than soil. AM fungi can increase the sulphate-absorbing power of roots, but this appears to be a secondary effect brought about by improved phosphorus nutrition (Rhodes and Gerdemann 1978).

2.5.3 AM in Natural Ecosystems

Natural ecosystems are disturbed habitats. Here, the diversity of the species (AM) is maintained on the basis of the natural phenomenon of ecosystem survival of the fittest. Mycorrhizal fungi can, to an extent, regulate the communities

in which they occur. Today, the biosphere is in danger in two senses: directly through outright destruction for productive use, and indirectly through the burden of waste disposal. Therefore, there is a need to protect ecosystems. Mycorrhizae are believed to protect the environmental quality by enhancing beneficial biological interactions. A matured ecosystem is characterized by a nutrient conservative system in which nutrient is rapidly cycled between the biotic parts and is not available to leach out of the system. Mycorrhizae are critical in that these fungi permeate the soil, picking up nutrients and channeling them to the host plant. Mycorrhizae regulate the composition and functioning of plant communities by regulating the resource allocation and growth characteristics of interacting plants (Allen 1991). Nicolson (1960) examined the role of mycorrhizal fungi in sand dune succession and found that the highest colonization of the host plant occurred within open communities, particularly within fixed, non-mobile dunes, and the colonization increased as the communities became closed. Dodd et al. (1990) studied the management of populations of AM fungi in acidinfertile soils of Savannah ecosystem and concluded that different plant hosts can caused the build-up of different populations of AM fungi in the soil around the root system. This indicates that, although species of AM fungi may be effective on a wide range of plants when introduced individually into sterile soil (Howeler et al. 1987), under natural soil conditions, different plants are likely to become infected by several different AM fungi.

2.6 Mechanisms of Nutrient Transport

In the symbiotic stage of the AM fungus, plant-derived carbon is taken up by the intraradical mycelium (IRM) and translocated to the ERM, whereas mineral nutrients, especially phosphate, are taken up by the ERM, distributed over the ERM

and translocated towards the IRM. This functional specialization implies that the AM fungus possesses a system for long-distance bidirectional transfer of large quantities of nutrients (Smith et al. 2001). The mechanisms that could be responsible for this transfer are summarized in Table 2.1 and discussed below. Nutrient translocation through the ERM and IRM may take place via mass flow, which is a slow process, or via cytoplasmic streaming (Cooper and Tinker 1978, 1981). In the ERM of monoxenic root organ cultures, it is possible to see bidirectional cytoplasmic streaming, especially in runner hyphae. It is known that in ectomycorrhizal fungi translocation of nutrients occurs at high rates in this way (Finlay and Read 1986), and it might play an important role in AM fungi as well. It has also been shown that the cytoplasm in ERM of *Gigaspora margarita* contained acidic vesicles. These acidic vesicles moved bidirectionally as a component of cytoplasmic streaming, but their net rate of movement could not be determined (Saito et al. 2004). Translocation of nutrients, such as phosphate (P), could also take place via transport in a motile pleiomorphic system of interlinked tubular vacuoles. These tubules form a reticulate system, and probably enable bidirectional translocation of different contents (Ashford and Allaway 2002). The main orientation of translocation is longitudinal, suggesting its role in longitudinal translocation along hyphae (Cole et al. 1998). Such systems are observed in representatives of all major fungal groups including the ectomycorrhizal fungus *Pisolithus tinctorius* (Ashford et al. 1994; Rees et al. 1994; Allaway and Ashford 2001). It has been shown for this ectomycorrhizal fungus that the tubular vacuole system contained substantial amounts of phosphorus, which indicates a role in P translocation (Cole et al. 1998; Ashford et al. 1999; Ashford and Allaway 2002). The existence of a motile tubular vacuole system has also been shown to exist in germ tubes, in ERM and IRM of *Gigaspora margarita* (Uetake et al. 2002) and in ERM of

Glomus intraradices (Olsson et al. 2002). The functioning of the tubular vacuole system of *Glomus intraradices* appeared to be inhibited by high P availability in the growth substrate (Olsson et al. 2002), indicating that the vacuolar system had a higher activity under conditions of nutrient limitation. The bidirectional cytoplasmic streaming of acidic vesicles and lipid bodies seemed to influence the tubular vacuole system of *Gigaspora margarita* (Saito et al. 2004).

Table 2.1 Mechanisms that can transfer nutrients within the AM fungal mycelium. A + indicates that the mechanism has been demonstrated and a that it is not yet demonstrated/determined.

Transfer mechanism	ERM	IRM	Direction
Cytoplasmic streaming	+	+	2-Way
Acidic vesicles in cytoplasm	+	?	2-Way
Tubular vacuoles	+	+	2-Way
Lipid body movement	+	?	2-Way, net direction towards ERM

An important part of the plant-derived carbon is transported towards the ERM, probably mainly in lipid bodies. This transport mechanism may prevent osmotic stress that otherwise could have been generated by the presence of large quantities of sugars (Smith et al. 2001). The movement of lipid bodies in the ERM of *Glomus intraradices* was highly variable but their maximum speed was not influenced by external temperature (Gavito et al. 2005). Still, carbon transfer to the fungus was reduced at low temperatures resulting in a reduced fungal growth. On the other hand, the phosphorus uptake and transfer to the plant was similar at 10° and 25°C (Gavito et al. 2005). The lipid bodies mainly moved with the cytoplasmic streaming, thus moving bidirectionally, and this resulted in a bidirectional translocation between IRM and ERM. However, it was suggested that their net movement is toward the growing

tips of the ERM and that they are slowly consumed on this track (Bago et al. 2002b). Thus, translocation of C and mineral nutrients (such as P) might occur in different systems. The major part of C is most likely translocated in lipid bodies and possibly by acid vesicles, whereas P is largely translocated in a system of tubular vacuoles. Another part of the transport of both nutrients can occur through cytoplasmic streaming and/or mass flow. These different mechanisms for translocation of nutrients are also indicators of hyphal vitality, and they respond to nutrient availability or external factors. They are important mechanisms for nutrient partitioning between the ERM and IRM in arbuscular mycorrhizal fungi.

2.6.1 Carbon Transport

2.6.1.1 Lipid Translocation

A prerequisite for effective partitioning of the ERM is a fast and precise lipid transport from the IRM. Just as plants may alter their shoot/root ratio, a two-phase organism such as an AM fungus can be expected to have evolved similar strategies for optimising the partitioning between the two phases. These strategies should include a lipid transport system since lipids are major carbon compounds, and since the ERM probably completely depends on the C transferred from the plants (Nakano et al. 1999). Olsson et al. (2002) calculated that 32% of labelled C in ERM of monoxenic *Glomus intraradices* cultures was present in triacylglycerols. Also, glycogen is transported from IRM to ERM (Bago et al. 2003). The studies of lipids have, however, the advantage that the lipids have a specific fatty acid profile that differ AM fungal lipids from plant lipids. The most common fatty acids in Glominae as well as *Scutellospora* is 16:1ω5. *Gigaspora*, however, seems mainly to have a fatty acid profile more similar to plants – dominated by 18-C fatty acids (Graham et al. 1995) and it could therefore be difficult to apply the fatty acid technique in order to

track these fungi. It is most probably so, that in most type of samples the signal to noise ratio for *Gigaspora* fatty acids is very low.

Indeed, the lipid movement in AM fungal hyphae is fast. By filming lipid body movement in living hyphae it is possible to estimate the speed of lipid movement. For *Glomus intraradices* in monoxenic cultures the speed was 4 μm/s and for *Gigaspora margarita* 8–11 μm/s (Gavito et al. 2005). This serves for a rapid translocation from IRM to ERM; within 24 h the lipids in *Glomus intraradices* can potentially have moved 35 cm (Bago et al. 2002b). Accordingly, the whole mycelium seems to act as one system for lipid distribution in which newly transferred plant assimilates are rapidly distributed throughout the mycelium. In pot systems where *P. lanceolata* was colonized by *Glomus intraradices* the carbon flow was studied using ^{13}C labelled CO_2. By estimating the labeling in the specific AM fungal fatty acid 16:1ω5 in neutral lipids (including triacylglycerols) the timing of flow from IRM to ERM was estimated (Olsson and Johnson 2005). Large amounts of labeling was already detected in the ERM 2 days after labelling. At all time points sampled the labeling in IRM was almost exactly the same as in ERM. It does seem likely that lipid transport can support AM fungal mycelia at large distance from host roots. Only the hyphal tips seem to have a lower lipid content than the rest of the mycelium (Bago et al. 2002b).

When it comes to lipid content, the whole system of IRM and ERM seems to be like a system with free distribution of lipids within it. However, when it comes to uptake and synthesis the IRM is much more important. It is not only so that it is the IRM that is in contact with the C source, it is also so that hexoses can be taken up by the IRM, but not by the ERM (Pfeffer et al. 1999; Bago et al. 2002a). It may be that the enzymes needed for lipid synthesis are specifically expressed in the

intraradical AM structures (Trepanier et al. 2005). This fact makes the translocation of lipids even more important for the AM fungal growth.

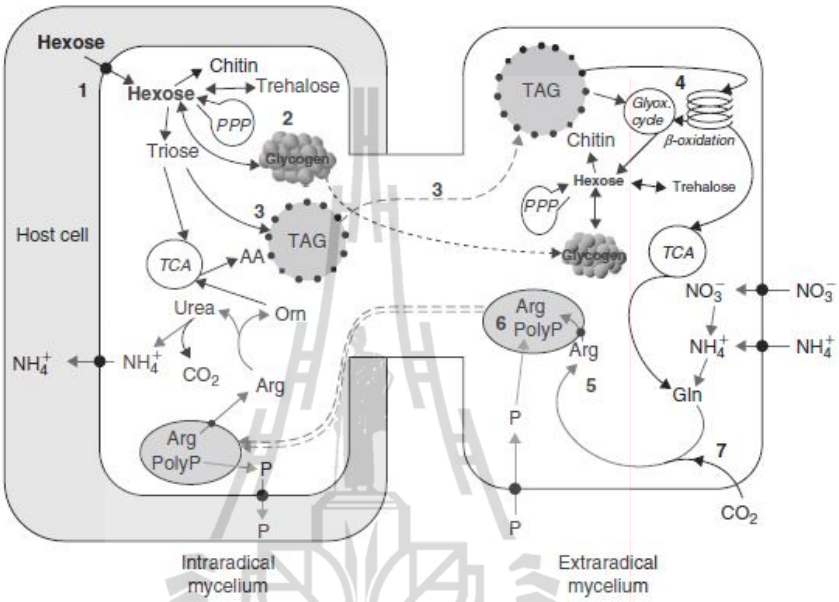

Figure 2.9 A proposed scheme describing metabolism of organic carbon (C), N and P in AM symbioses. Pathways and directions of fluxes are based on data from NMR findings. Names of metabolic pathways are in italics. The numbers refer to results of NMR experiments: (1) the uptake of hexose by intraradical parts of the fungus; (2) the formation of glycogen and trehalose from hexose within the host root; (3) the site of synthesis for storage lipids (TAG) is within the intraradical fungal tissue; this lipid is then exported to the extraradical mycelium; (4) the glyoxylate cycle is active in transforming exported lipid into gluconeogenic precursors for hexose synthesis in the extraradical mycelium; (5) arginine is actively synthesized by AM fungi by the usual metabolic pathway; (6) arginine may be associated with polyP in mycorrhizal vacuoles; (7) dark fixation of CO_2 is active in AM fungi. Reproduced from Pfeffer *et al*. *New Phytologist*, 150, 543–553 (2001).

2.6.2 Phosphate Transport and Partitioning

2.6.2.1 Phosphatase Enzymes

Phosphatases are important for most intracellular P transformations and they are also a part of the nutrition strategies. They can be exuded and thereby release phosphate from organic molecules, making it available for uptake. Plants usually exude phosphatases, and it is believed that this strategy is an important part of P nutrition in plants. Plants do, however, only have phosphatases active at low pH values, and these acid phosphatases have probably little activity in more alkaline environments. AM fungi on the other hand contain both acid and alkaline phosphatases, just as do other fungi (Nahas et al. 1982; Haas et al. 1992). High phosphatase activity has been detected both in intraradical (Gianinazzi et al. 1979; Van Aarle et al. 2002b) and extraradical (Van Aarle et al. 2001, 2002b; Olsson et al. 2002) AM fungal mycelium, but so far it seems that AM fungi do not excrete phosphatases very much (Joner et al. 2000). We will consider AM fungal phosphatases both in the aspect of intracellular P transport and transformations, and the fungal nutrition.

The intracellular phosphatase activity was examined in a number of studies, for example with the AM fungi *Glomus intraradices* and *Gigaspora margarita*. One interesting observation was that the alkaline phosphatase activity was higher than the acid phosphatase activity in the AM fungal ERM, while the opposite was true for the IRM (Van Aarle et al. 2002a, 2002b, 2005; Olsson et al. 2002). On the other hand, both alkaline and acid phosphatase activity were higher in the IRM than in the ERM of *Gigaspora margarita* (Van Aarle et al. 2002b).

The phosphatases in AM fungal ERM seem to be very little involved in exudation. A next question is then where they are located in the ERM.

Joner and Johansen (2000) showed that, for the ERM of two *Glomus* isolates, a large part of the phosphatase activity was associated with the hyphal wall, whereas a smaller part was associated with internal structures. However, they did not work in sterile systems and it was not possible to separate AM fungal phosphatases from the phosphatase activity originating from bacteria associated with the hyphae. Enzymatic activity staining with the fluorescent ELF substrate indeed showed that some phosphatase activity was associated with the vacuoles (Olsson et al. 2002) whereas another part was associated with the hyphal wall (Van Aarle 2002). It has been hypothesized that plant cell wall-associated phosphatases may aid in the uptake of P from the soil or of P-esters that have leaked from the plant roots (Barrett- Lennard et al. 1993), and we believe that hyphal wall-associated phosphatases might have a similar recovery function. Because of their location, organic P compounds can be degraded close to the mycelium and orthophosphate could be taken up before physical immobilization or use by other microorganisms.

The phosphatase activity in AM fungi is influenced by the external environment. Alkaline phosphatase activity associated with the ERM was, for example, inhibited by application of fungicides (Kjoller and Rosendahl 2000), increased pH (Van Aarle et al. 2002a) or simulated acid rain (Vosatka and Dodd 1998; Malcova et al. 1999). Furthermore, it was shown that the external nutrient availability may influence the phosphatase activity of the ERM (Olsson et al. 2002), and it could thus possibly also influence the P uptake of the AM fungus.

The location of phosphatases in the IRM indicates their involvement in the P transfer to the plant. The phosphatase activities have been localized in most structures (Gianinazzi et al. 1979; Ezawa et al. 1995; Van Aarle et al. 2002b, 2005) and these activities seem primarily located in the vacuoles

(Gianinazzi et al. 1979; Gianinazzi-Pearson and Gianinazzi 1995; Saito 1995). However, the activities are highest in arbuscules and arbusculate coils (Van Aarle et al. 2005). These results support the role of these structures in the P transfer to the plant. Since alkaline phosphatase is mainly of microbial origin, it only appears in roots upon the development of an intraradical AM fungal mycelium. Alkaline phosphatase is considered as an important enzyme in metabolic processes that lead to P transfer to the host plant. Kojima and Saito (2004) related the phosphate efflux from IRM to fungal alkaline phosphatase activity, and their results suggested that fungal phosphatase may indeed be involved in the fungal P transfer to the plant. It was, furthermore, shown that alkaline phosphatase genes from *Glomus intraradices* and *Gigaspora margarita* were constitutively expressed in mycorrhizal roots and their levels of transcripts were higher than those in ERM (Aono et al. 2004). Probably, alkaline phosphatase in the IRM has a role in the fungal P transfer to the plant, whereas in the ERM it may be involved in P uptake from the rhizosphere.

2.6.2.2 Polyphosphate and Phosphate Partitioning

Inorganic phosphate that has been taken up by the AM fungal ERM is often accumulated before being translocated. Like in other fungi, accumulation of phosphate in the form of polyphosphate partly takes place in acidic, presumably vacuolar, compartments (Viereck et al. 2004). Polyphosphate is a linear polymer of phosphate residues that are linked by high energy bonds (Ogawa et al. 2000). Formation of polyphosphate maintains a low orthophosphate content in the mycelium, and it has a role in the promotion of long-term uptake and accumulation of phosphate (Ogawa et al. 2000). It is most likely that at least part of the fungal phosphate content is translocated in this form (Callow et al. 1978; Rasmussen et al. 2000). Viereck et al. (2004) characterized polyphosphate in the ERM of *Glomus*

intraradices with ^{31}P NMR spectroscopy, and their data seemed to support the previous postulated hypothesis (Ashford and Allaway 2002; Ezawa et al. 2002; Van Aarle 2002) that polyphosphate is an important P metabolite which is translocated in a network of tubular vacuoles. Even though this polyphosphate is normally transferred towards the plant, it can be transferred bidirectionally, and it can be translocated within the ERM (Olsson et al. 2002) or to spores. Olsson et al. (2002) showed an accumulation of polyphosphate in ERM of *G. intraradices* which was obtained from medium without phosphate, when the fungus was grown in monoxenic root organ cultures. They visualized the polyphosphate content in the hyphae after precipitation with ethanol and subsequent staining with Toluidine blue O.

The average chain length of polyphosphate in the ERM is 13–15 phosphate residues (Rasmussen et al. 2000; Viereck et al. 2004), but the polyphosphate chain is shorter in IRM (Solaiman et al. 1999; Viereck et al. 2004). This is consistent with hydrolysis of polyphosphate in the IRM upon which the resulting orthophosphate is released into the apoplast of the plant-fungus interface (Ezawa et al. 2001). The net hydrolysis of polyphosphate in the ERM is however much weaker; there is more polyphosphate than there is degradation. There are strong indications that acid phosphatase is partly responsible for the hydrolysis of polyphosphate in the vacuoles of the IRM (see discussion in previous section). In the IRM of *Gigaspora margarita*, between 5 and 8% of the total P was present in the form of polyphosphate, whereas in the ERM between 8 and 17% was present in this form (Solaiman et al. 1999). This is equivalent to what was earlier reported for other AM fungi. *Gigaspora margarita* contained about 75% of its phosphate in the IRM, whereas about 25% was located in the ERM (Solaiman and Saito 2001).

Considerable amounts of P are taken up and transferred towards the plant, but AM fungi may differ in their strategy. Several indications are found that AM fungi from the Gigasporaceae can retain a large proportion of P in the mycelium and thus control the P transfer to the plant, whereas this is much less the case with fungi from the Glomeraceae. For *Scutellospora calospora* it has been shown that P accumulated in hyphae, but this was less for an *Acaulospora* and a *Glomus* isolate (Jakobsen et al. 1992b). The same authors observed in another study (Jakobsen et al. 1992a) that *Acaulospora* had the most extensive hyphal spread whereas that *Scutellospora* had most of its ERM close to the plant roots. Furthermore *Acaulospora* produced the largest increase in P uptake and plant growth (Jakobsen et al. 1992a). In the ERM of *Gigaspora rosea* an accumulation of polyphosphate was found, whereas this accumulation was not found for *Glomus manihotis* (Boddington and Dodd 1999). Furthermore, Solaiman and Saito (2001) observed a high P content in the mycelium of *Gigaspora margarita*, especially in the IRM. These observations indicate that either the P translocation or the P transfer to the host plant was delayed. Besides the amount of P that is translocated, the distance of translocation from the ERM to the host plant is dependent on the fungal isolate. Translocation distances of up to 7 cm for *Acaulospora* have been shown using ^{32}P tracers, whereas a *Glomus* species could translocate only up to 4.5 cm and *Scutellospora* already had difficulties in reaching 2.5 cm (Jakobsen et al. 1992b). A high specific radioactivity in hyphae of *Scutellospora calospora* in comparison to that found in the hyphae of a *Glomus* and *Acaulospora* isolate indicates that the mycorrhizal fungal P transport from the soil to the plant is probably more limited by fungal translocation and transfer processes than by the fungal P uptake from the soil (Jakobsen et al. 1992b). This suggestion was confirmed in a study performed by Pearson and Jakobsen (1993). It thus seems very

important to consider the fungal isolate or strategy before making general conclusions on AM fungal phosphate partitioning and transfer to the host plant.

2.7 The Biocontrol Effect of Mycorrhization on Soilborne Fungal Pathogens

Most data about bioprotection of mycorrhization are available for soilborne fungal pathogens. Numerous studies show a clear localized protective effect (reviewed by Singh et al. 2000; Azcon-Aguilar et al. 2002; Xavier and Boyetchko 2004; St- Arnaud and Vujanovic 2007) while recently a systemic protective effect with different soilborne fungal pathogens has also been reported (Cordier et al. 1998a;Pozo et al. 2002; Khaosaad et al. 2007).

2.7.1 Factors Affecting Bioprotection Through Mycorrhization

The Host Genotype

In a number of studies, it has been demonstrated that depending on the host genotype the degree of AM root colonization and the plant growth effect of mycorrhization can vary, and it has been suggested that the AM development and its effect on the host plant are at least partially under the genetic control of the host (Lackie et al. 1987; Hetrick et al. 1993; Vierheilig and Ocampo 1990, 1991). The host genome also seems to affect the protective effect provided by the AM, as the host genotype seems to result in a differing bioprotective response by the mycorrhizal association. Depending on the genotype, mycorrhizal strawberry showed a different susceptibility to *Phytophthora fragariae* (Mark and Cassels 1996).

The AM

In an overview on studies on the bioprotective effect of mycorrhization listing the host plant, the pathogen, the AM, and the effect of mycorrhization (Singh

et al. 2000; Whipps 2004), we can see that more than 80% of the studies on the bioprotective effect of mycorrhization have been performed with the genus *Glomus*, whereas only about 14% were performed with the genus *Gigaspora,* while there are almost no studies available using other AM genera. Moreover, within the genus *Glomus,* nearly half of the studies were performed with *G. mosseae* (25%) and *G. fasciculatum* (23%) and 8% each with *G. etunicatum* and *G. intraradices*. This means that data on a bioprotective effect of mycorrhization originate mostly from the genus *Glomus,* and within this genus from two species. From a few studies comparing the bioprotective effect of AM species, we can conclude that depending on the AM species the bioprotective effect differs, e.g., *G. intraradices* has been reported not to protect clover against nematode infection (Habte et al. 1999) and was not effective in reducing disease symptoms produced by *P. parasitica* infection in tomato (Pozo et al. 2002). In contrast, root colonization by *G. mosseae* resulted in a clear protective effect against nematodes and *P. parasitica* infection (Habte et al. 1999; Pozo et al. 2002). As some AM exhibit a clear bioprotective effect, whereas other AM do not affect the plant–pathogen interaction in terms of bioprotection, more comparative studies are definitely needed involving a greater variety of AM.

The Degree of Mycorrhization

After plant inoculation with an AM, the first signs of root colonization are visible after a few days. Thereafter, AM root colonization can increase drastically until it reaches a final plateau (e.g., for tomato and soybean around 60%; Vierheilig et al. 1994; Wyss et al. 1991). In several studies, a local bioprotective effect has been linked with a high degree of AM root colonization, whereas intermediate and low levels of AM root colonization showed no bioprotective effect. Apparently a critical level of AM root colonization is needed to provide bioprotection for mycorrhizal

plants. In mycorrhizal tomato plants a bioprotective effect against *P. parasitica* (Cordier et al. 1998b) and *Fusarium oxysporum* (Caron et al. 1986a,b), and in wheat plants against *Gaeumannomyces graminis* (Graham and Menge 1982), could only be observed when roots were heavily colonized by the AM; low mycorrhization levels resulted in no bioprotection. The sequence of inoculation has also been suggested as an important factor in mycorrhizal bioprotection. In general, it has been postulated that the inoculation has to be prior to inoculation with the soilborne pathogen (Singh et al. 2000; Azcon-Aguilar et al. 2002; Xavier and Boyetchko 2004). However, this aspect is probably closely linked with the degree of mycorrhization. The earlier the AM colonizes the root, the higher the mycorrhization level will be before a pathogen infection.

2.7.2 Mechanisms of Mycorrhization-mediated Bioprotection

A number of mechanisms have been suggested to be involved in the bioprotective effect of mycorrhization against soilborne fungal pathogens, but hard data are not yet available for all of them. Classically, four major groups of mycorrhizal mode of action mechanisms that mediated bioprotection have been considered: (1) direct competition, (2) mechanism mediated by alteration in plant growth, nutrition and morphology, (3) biochemical and molecular changes in mycorrhizal plants that induce pathogen resistance, and (4) alterations in the soil microbiota and development of pathogen antagonism. Although some of the suggested mechanisms might play no role, it is generally agreed that bioprotection through mycorrhization is the result of a combination of several of mechanisms and not of a single mechanism.

2.7.2.1 Direct Competition

Competition for Infection Sites

A competition effect at the infectional level has also been suggested. The AM might occupy infection sites on the root surface needed by the pathogen to penetrate the root, or cells in the root already occupied by the AM cannot be colonized any further by the pathogen (Cordier et al. 1998a, b).

Direct Competition/Inhibition in Soil

Some experimental evidence has been reported about possible mechanisms of direct action of AM fungi against pathogens in soil (St-Arnaud et al. 1995; Filion et al. 1999; Garcia-Garrido and Ocampo 1989). Nevertheless, at the present time, the production of antibiotics or inhibitory compounds by AM fungi has not been proven.

2.7.2.2 Mechanism Mediated by Alteration in Plant Growth, Nutrition

Improved Nutrient Status/Root Damage Compensation and Morphology

It has been suggested that the improved nutrient status of mycorrhizal plants makes them more tolerant to damage caused by pathogens and carbon drain from the plant to the pathogen. However, there is strong evidence that the nutritional effect of the AM symbiosis is only one among several aspects of the mycorrhizal effect on pathogens (Trotta et al. 1996). Moreover, it has been suggested that the nutrient uptake by the fine extraradical mycelium of the AM could compensate for a pathogen-reduced root system (Singh et al. 2000).

Morphological Alterations of the Root

Due to mycorrhization, the morphology of the root changes (Berta et al. 1995; Copetta et al. 2006), but no clear correlation with a bioprotective effect of mycorrhization has yet been found. In basil, the root fresh weight, the total root length, the number of root tips, and the degree of branching was altered differently depending on the root-colonizing AM (Copetta et al. 2006). Linking these alterations of the root parameters with data on a bioprotective effect of the different AM could show whether morphological alterations of the root due to mycorrhization are really involved in the bioprotective effect of mycorrhization.

2.7.2.3 Biochemical and Molecular Changes in Mycorrhizal Plants that Induce Resistance to Pathogens

Several physiological and biochemical alterations of the host after mycorrhization have been reported. Some are possibly linked with a protective effect of the mycorrhizal plant against pathogens, e.g. the induction of hydrolytic enzymes (Pozo et al. 1999), enhanced levels of PR proteins, the accumulation of phytoalexins and callose (Harrison and Dixon 1993; Morandi 1996; Larose et al. 2002; Cordier et al. 1998b), the accumulation of salicylic acid (Blilou et al. 2000a, 2000b; Medina et al. 2003), and reactive oxygen species (Salzer et al. 1999). During AM development, there is evidence that these defensive responses occur (Garcia-Garrido and Ocampo 2002) and that they are strongly stimulated when a subsequent challenge with a pathogen takes place. Possibly the mechanisms of plant defence are activated faster and to a greater extent in mycorrhizal plants when challenged by a pathogen compared to nonmycorrhizal plants, and it has been suggested that AM colonization acts as a priming system for the process of pathogen resistance (Azcon-

Aguilar et al. 2002; Pozo and Azcon-Aguilar 2007). In this respect, elevated jasmonic acid (JA) levels occurring upon mycorrhization, likely associated with a fully established mycorrhiza, may mediate the enhance defence status of the mycorrhizal plant (Vierheilig and Piche 2002; Hause et al. 2007).

However, the importance of each component of the plant resistance response in the bioprotective effect of AM against soil pathogens and the signaling pathway that control these responses are unknown. As the whole metabolism of the plant is altered by mycorrhization, alterations of the root exudation pattern are no surprise. These alterations could act on the pathogen indirectly, through an altered pH in the rhizosphere and/or directly through an altered composition of the exudates with reduced levels of stimulatory compounds and/or the presence of inhibitory compounds. Changes of the pH in the rhizosphere of the mycorrhizal plant have been reported before, however, no data are availble yet how these pH changes of the rhizosphere affect root pathogens (Bago et al. 1996; Villegas et al. 1996).

2.8 Diversity Patterns of Arbuscular Mycorrhizal Fungi–Community Composition

AM are ubiquitous plant root symbionts that have been considered as 'keystone mutualists' in terrestrial ecosystems. They form a link between biotic and abiotic components of ecosystems via carbon and nutrient fluxes that pass between plants and fungi in the soil (O'Neill et al. 1991). The global carbon flux from plants to AM fungi has been estimated as 5 billion tons per year (Bago et al. 2000). The global biomass of the *Glomeromycota* has been estimated as 1.4 Pg (Treseder and Cross 2006), compared with the estimation of global soil microbial biomass of 13.9 Pg (Wardle 1992). Thus, *Glomeromycota* is a considerable but largely overlooked

sink in the carbon cycle, whilst these fungi possess ecologically important functions in plant nutrition, pathogen resistance and water relations (Smith and Read 1997).

AM fungal communities have been shown to affect plant diversity and productivity (van der Heijden et al. 1998; van der Heijden and Sanders 2002). Large differences in functional complementarities between plant and AM fungal species coexisting in the same ecosystem have been demonstrated with plant species from temperate forest (Helgason et al. 2002) and temperate grassland (Klironomos 2003). There is further evidence that plant–AM fungal coupling is of importance for the performance of not only the symbiotic partners but also for ecosystem function (van der Heijden et al. 2006). However, information on spatiotemporal organization of AM fungal assemblages is still rather limited at all scales. What is the structure of an AM fungal assemblage within a root system? Within a patch of soil? Within a patch of a grassland/forest/desert etc.? Such biogeographical data exist for most of the larger organisms, at least to a certain degree, but they are lacking for microorganisms. It is important to gain a better understanding of AM fungal diversity at a range of scales in order to be able to extrapolate from research with individual isolates and species to functioning of communities and ecosystems. However, knowledge of taxon diversity without a concomitant understanding of the biology of taxa would restrict any understanding of natural patterns.

2.8.1 Taxon Diversity of AM Fungal Communities

2.8.1.1 Taxon Delimitation

In order to estimate the number of taxa in a sample, one needs first to delimit them. Principles of species delimitation of *Glomeromycota* are far from solved, though traditionally a morphological species concept has been used. The

suitability and possible pitfalls of using rDNA sequences for molecular identification purposes are similarly disputed.

Groups (of mostly rDNA sequences) were used to phylogenetically defined as operational taxonomic units (OTU) if fungi were identified by molecular means. These groups have been intended to correspond approximately to species level. These groups were referred as 'taxa', because the relationship between morphologically defined AM fungal species and phylogenetically defined OTUs of sequences of environmental origin is unclear due, primarily, to the scarce information about intraspecific genetic variability of the *Glomeromycota*. In the literature the terms 'sequence group' and 'sequence type' are commonly used. The level of sequence difference between these taxa can be 1–3% depending on the researcher's choice (99–97% similarity; e.g., Helgason et al. 1999). Still, a combination of roughly fixed similarity level with good support of phylogenetic clades might be preferred as a basis for OTU definition over the arbitrary sequence similarity level. The sequence divergence in the SSU rDNA region in particular might be insufficient in order to separate some taxa, as argued for example in the *Glomus intraradices* group, where other genes might be more appropriate (Raab et al. 2005). Overall, in order to be more able to efficiently detect and identify naturally occurring *Glomeromycota*, there is a clear need to clarify *Glomeromycota* taxonomy linking molecular and morphological diversity. A concerted understanding of intra- and interspecific variation in morphology, genetics, development, and physiology of these fungi are needed. Developments in this area would greatly enhance ability to interpret ecological data, but also to learn about economically important AM fungi and provide grounds for extrapolating from individual studies. At the global scale, the number of described *Glomeromycota*

species is over 150 (Walker and Trappe 1993). The number of SSU rDNA sequence-based taxa of *Glomeromycota* was ca. 100 at the end of the year 2005 (Opik et al. 2006b). A minority of these taxa are related to morphotaxa, whilst many others are known only as sequences from a single location. The 'sequence-only' taxa are increasingly becoming known from several locations due to the increasing number of molecular surveys of natural *Glomeromycota*. However, the proportion of matches with 'known' species is likely to increase as more isolates of culture collections are sequenced; presently only a small number are represented in sequence databases. Therefore, the total number of *Glomeromycota* is probably much greater than the abovementioned 150 species, as previously proposed by Helgason et al. (2002).

2.8.1.2 Diversity of AM Fungi in Soil as Spores

As with other organisms, the taxon richness of AM fungi has been shown to vary from location to location. There is a wealth of spore-based AM fungal surveys from a range of locations and ecosystems. Traditional methods to identify AM fungi have for a long time been based on spores captured from soil, either by direct extraction or via so-called 'trap-culturing' with plants (e.g., Stutz and Morton 1996). The value of spore surveys has been questioned and re-valued (Sanders 2004). Most importantly, it is still unknown what triggers the onset of sporulation, what determines sporulation intensity, and which factors influence observed variation in spore numbers of AM fungi in natural environments. Furthermore, the communities of spores in the soil and fungi colonizing roots are not necessarily identical (Clapp et al. 1995); neither is there a direct relationship between sporulation and root colonization levels (Dodd et al. 2000). Therefore, while spores identified from soils constitute important information regarding the species pool with a potential to colonize plant roots in a given location, as well as revealing some

biological and functional properties of morphospecies, spore communities should not be equated with the total AM fungal community at a site without further information about root-colonizing fungi. Probably the highest known AM fungal richness at a single site, 37 taxa, was detected by extensive trap-culturing of spores over many years from an old field (Bever et al. 2001; Youpensuk et al. 2005).

2.8.1.3 Diversity of AM Fungi in Roots

Pioneering publications of Simon et al. (1992) and Helgason et al. (1998), who first described a molecular method to detect and identify *Glomeromycota* in plant roots, and who designed the present most commonly used AM-specific primer, respectively, were followed by a large increase in information about natural root-colonizing AM fungal communities. Described natural diversity patterns include community composition differences in space and time (Helgason et al. 1999; Daniell et al. 2001), between hosts (Helgason et al. 2002; Vandenkoornhuyse et al. 2002), and between management practices, within a root system (Helgason et al. 1998; Hijri et al. 2006; Opik et al. 2006b; Scheublin et al. 2004).

Richness

The number of AM fungi inhabiting a single root system has been shown to reach ca. 10 taxa (e.g., Helgason et al. 2007) and can possibly be greater. Evidence relating to plant individual-level AM fungal richness is rather limited. During the life of an individual plant some changes probably occur in terms of number of AM taxa inhabiting the roots. There are some indications that seedlings may be colonized by more and different set of AM fungi as compared to adult plants (Husband et al. 2002b; Opik et al. 2003).

The number of AM fungal taxa per host plant species at any location may differ between habitat types (Opik et al. 2006b). Currently the highest AM fungal richness has been observed in tropical forests (18 SSU rDNA taxa of AM/plant species), followed by grasslands, temperate forests, and habitats under anthropogenic influence (arable fields and polluted sites; Opik et al. 2006b). Furthermore, ecosystems vary in the number of fungal species identified from them: 23 AM fungal sequence types have been identified from a single temperate broad-leaved forest location, 10–24 from different temperate grassland locations, 7–22 from dry or wet tropical forests, 2–7 from temperate arable fields, 5–7 in few studied polluted sites, and 14–20 from temperate wetlands (references in Opik et al. 2006b; Franke et al. 2006; Helgason et al. 2007; Hijri et al. 2006; Santos et al. 2006; Vallino et al. 2006; Wubet et al. 2006). Care need to be taken in the interpretation of such data as sampling intensity has not been even across different biomes and studies. However, related evidence suggests that there are trends of different AM fungal species richness in different ecosystems. Again, little is known about the number of species in the *Glomeromycota* flora of different regions. Species lists of AM fungi have been compiled for only a few locations (Błaszkowski 1994; Tadych and Błaszkowski 2000). An important resource to establish regional (and ecological) information about *Glomeromycota* flora are the databases of culture collections such as the International Bank of the *Glomeromycota* (BEG, http://www.kent.ac.uk/bio/beg) and the International Culture Collection of (Vesicular) Arbuscular Mycorrrhizal Fungi (INVAM, http://invam.caf.wvu.edu/cultures/accessions) which provide information on location, habitat type, edaphic characteristics, host plant species, etc. of the fungal isolates maintained in these collections. For more thoroughly

investigated regions this data would provide species lists and distribution data which, though limited to cultured *Glomeromycota*, give an estimate of richness patterns within these regions. The number of AM taxa discovered in a location might be an indicator of ecosystem status in terms of disturbance intensity versus stability. However, in terms of ecosystem function the phylogenetic taxon distribution might be of greater importance because larger families of *Glomeromycota* exhibit some conserved functional trait diversity (Maherali and Klironomos 2007). Therefore, effects of AM diversity (including richness) on ecosystem function may be more obvious when fungi from different genera/families with different strategies are present, as argued by van der Heijden et al. (2006). Thus, the composition of the AM fungal community can be more important than number of taxa.

Composition

Glomeromycota taxon distribution patterns within a community, expressed as diversity indices, vary remarkably between sites/ecosystems and across seasons, demonstrating different levels of taxon dominance. For example, the Shannon- Wiener diversity index (*H*) of AM fungal communities may vary from <1 in some arable fields (Daniell et al. 2001; Hijri et al. 2006) to >2 in tropical forests (Husband et al. 2002a; Wubet et al. 2004) and temperate wetlands (Wirsel 2004).

At the level of individual plants, root-colonization data suggest that several different AM fungi can colonize a root fragment and that they occupy different spatial niches within a root system (e.g., Merryweather and Fitter 1998a). *Viola mirabilis* and *V. elatior* individuals, from different locations, hosted different AM fungi in replicate 1-cm root samples from the same plant (Opik et al. 2006a). However, when the entire root system was considered, the fungal assemblages

showed plant species-related differences regardless of the location of origin (Opik et al. 2006a). A pot experiment with *Medicago truncatula* co-inoculated with *Gigaspora rosea*, *Glomus mosseae* and *Glomus intraradices* demonstrated different frequencies (occurrence) of each constituent species in 1-cm-long root fragments (Jacquot-Plumey et al. 2001). Furthermore, there is evidence that the bulk roots and nodules of legumes may be inhabited by distinct AM fungal assemblages (Scheublin and van der Heijden 2006).

A (molecular) quantitative approach based on real-time PCR was taken by Alkan et al. (2006) who reported different spatial patterns of root colonization by *Glomus mosseae* and *G. intraradices* in various experimental settings including phosphorus availability gradients and salinity stress. These observations were based on one plant individual per treatment, and one isolate per fungal species. Whilst quantitative information regarding the AM fungal colonization is badly needed, this report highlights the requirement of the quantitative PCR methodology to generate sample throughput high enough to allow robust testing of hypotheses. The lack of replication of experimental units provides no confirmation on consistency or significance of the reported differences between experimental treatments, including different isolates and the studied species.

Small-scale spatial arrangement of AM fungal taxa has rarely been described. Soil spore data suggests, however, that at a local scale the AM fungi can occur in a highly spatially structured, i.e. patchy way (Abbott and Robson 1991; Allen 1996; Klironomos et al. 1999). This was recently corroborated by Wolfe et al. (2007) who sampled a single 2 × 2 m plot and discovered considerable variation in T-RF richness of AM fungi in roots. In addition, plant species affects the sporulation

intensity (total spore number) and spore diversity in a grassland (Johnson et al. 1992). Seasonal colonization patterns and variability between different years in terms of *Glomus* and *Scutellospora* morphotypes colonizing bluebell roots. (Merryweather and Fitter (1998b). Temporal changes in root-colonising community structure were reported in arable field rotation (Daniell et al. 2001) and in broad-leaved forest understorey (Helgason et al. 1999).

A fine-scale spatial description of root-colonizing AM fungal communities demonstrated variable relative abundance of 5 RFLP types in adjacent 2 × 2 m plots (Yamato and Iwase 2005). Similarly fine-scale survey of AM fungi was performed using bait-plants grown on soil cores from a single 2 × 2 m plot in a calcareous fen. High spatial variation in AM fungal T-RF richness was revealed, ranging from 2 to 14 T-RFs per soil core, but no significant spatial patterns or association with the aboveground plant community pattern were detected (Wolfe et al. 2007). Clearly more descriptive data are needed about small-scale spatial and temporal arrangement of communities of AM fungi, considering both intra- and extraradical fungi. At the ecosystem or biome level there are indications that specific AM fungal communities, of differing compositions, occur in broadly defined habitat types such as tropical forests, temperate forests, disturbed habitats or grasslands (Opik et al. 2006b).

2.8.1.4 Development of an AM Symbiosis (see review: Parniske 2004)

Plant genes required for symbiotic development

Plant mutants are the key tools for the genetic dissection of AM development. AM mutants have been identified in non-legume species, most prominently in tomato (David-Schwartz et al. 2001, 2003). By far the largest number

of AM-mutants, however, have been isolated from legumes by examining the ability of mutants that have defects in the nitrogen-fixing root nodule symbiosis (RNS) to develop AM (Duc et al. 1989; Bradbury and Bowley 1991; Table 2.2). The mutants isolated this way are defective in both bacterial and fungal symbiosis. The impaired genes are thus collectively referred to as the 'common' *SYM* genes (Kistner and Parniske 2002). These genes define a partial overlap between the genetic programmes for fungal and bacterial endosymbiosis (Marsh and Schultze 2001; Oldroyd and Downie 2004). The evolutionary implication is that the younger RNS has recruited functions from the ancient AM symbiosis (Kistner and Parniske 2002; LaRue and Weeden 1994). The common *SYM* genes are therefore in the centre of interest from both a mechanistic and an evolutionary viewpoint. The bacterial symbionts of legumes, the rhizobia that inhabit root nodules, produce lipochitin-oligosaccharide signals that induce symbiotic responses in the host root. Most of the more thoroughly analysed common *SYM* genes play a role in the transduction of the Nod-factor signal (Catoira et al. 2000; Stracke et al. 2002). However, two nodulation-specific Nod factor receptor (NFR) kinases, NFR1 and NFR5, have been positioned upstream of these common pathway components. These kinases contain LysM motifs in their extracellular domains, which are implicated in binding N acetyl-glucosamine-containing molecules. The presence of LysM domains makes NFR1 and NFR5 prime candidates to be Nod-factor receptors (Madsen et al. 2003; Limpens et al. 2003; Parniske and Downie 2003). Mutations in *NFR1* or *NFR5* affect the earliest Nod-factor responses (Radutoiu et al. 2003) but not the AM symbiosis (Wegel et al. 1998), suggesting that the essential fungal signalling factors (Kosuta et al. 2003) are dissimilar to the Nod-factor. The Nod-factor perception event that involves NFR1 and NFR5 is likely to be transmitted through SYMRK (Stracke et al. 2002; Endre et al.

2002). Whether this occurs directly through the formation of SYMRK–NFR heterocomplexes or indirectly via secondary signals that are released from a NFR1/5 complex, as suggested by Radutoiu et al. (Radutoiu et al. 2003), remains to be established. Within seconds, Nod-factors induce ion fluxes across the plasma membrane of the root hair cell, as evidenced by an extracellular alkalinisation and membrane depolarisation. *NFR5* is absolutely required for this response, whereas the different responses observed in single and double mutants suggest that the contribution of *SYMRK* and *NFR1* might be synergistic (Radutoiu et al. 2003). Localised changes of ion concentration, similar to those induced by Nod factors, are likely to occur in the interaction with AM fungi but have not yet been experimentally verified.

Table 2.2 Orthologous relationship between some legume symbiosis genes.

	L. japonicus	*Pisum sativum*	*M. truncatula*	Predicted function	Reference(s)
(a) Cloned nodulation genes that are required for early stages of RNS					
Cloned gene	Initial designation				
NFR1	*LjSYM1*	*(PsSYM2?)*	*LYK3*	Nod-factor receptor kinase	[38••,39••]
NFR5	*LjSYM5*	*PsSYM10*	*(NFP?)*		
NIN		*PsSYM35*	?	Membrane protein with DNA-binding domain	[76,77]
(b) Common *SYM* genes required for AM and RNS					
	?	*(PsSYM8?)*	*DMI1*	Ion channel	[44••]
SYMRK	*LjSYM2*	*PsSYM19*	*DMI2 (MsNORK)*	Symbiosis receptor kinase	[37••,43••]
	?	*PsSYM9/30*	*DMI3*	Calcium- and calmodulin-dependent kinase	[45••,46••]
	LjSYM3	?	?		[26]
	LjSYM4	?	?		[26]
	LjSYM15	?	?		[26]
	LjSYM23	?	?		[27]
	LjSYM30	?	?		[27]
	?	*PsSYM36*	?		[57]

Symbiotic mutants that are affected in bacterial and fungal symbiosis have also been isolated in *Melilotus alba*, *Vicia faba* and *Phaseolus vulgaris* (reviewed in [34]) but their relationships to the listed genes are unclear.

Only a few markers are available that can differentiate between the phenotypes of common *SYM* mutants, and that provide a framework to position the genes that are affected in these mutants in a conceptual signaling cascade. One such marker is the calcium-spiking response of root hair cells of various legume species that occurs 10–

30 min after Nod-factor treatment (Oldroyd and Downie 2004). These rhythmic oscillations in cytoplasmic calcium concentration are probably a signal, but to prove this experimentally is a key challenge for the future. The common *SYM* gene *DOES NOT MAKE INFECTIONS1 (DMI1)* from *Medicago truncatula* encodes a predicted ion channel (Ane et al. 2004) that acts upstream of calcium spiking. The receptor-kinase structure of SYMRK (or its orthologue DMI2 in *M. truncatula*) suggested that it acted mechanistically upstream of the ion channel. In a hypothetical scenario, SYMRK is involved in the direct or indirect perception of a fungal signal, which is then transduced through the intracellular kinase to activate the ion channel (Figure 2.10). It will be interesting to establish which of the early-symbiosis-induced ion fluxes DMI1 is involved in. DMI3 is a calcium- and calmodulin-dependent protein kinase (CCaMK), which contains three EF hands implicated in calcium-binding and a calmodulin-binding site (Levy et al. 2004; Mitra et al. 2004). These sequence features open the exciting possibility that the CCaMK is able to interpret the calcium-spiking signal, resulting in a phosphorylation event (Levy et al. 2004; Mitra et al. 2004). It appears likely that this phosphorylation event is not the only signal that is required for the onset of symbiotic development, and that additional components will be revealed through genetics and biochemistry.

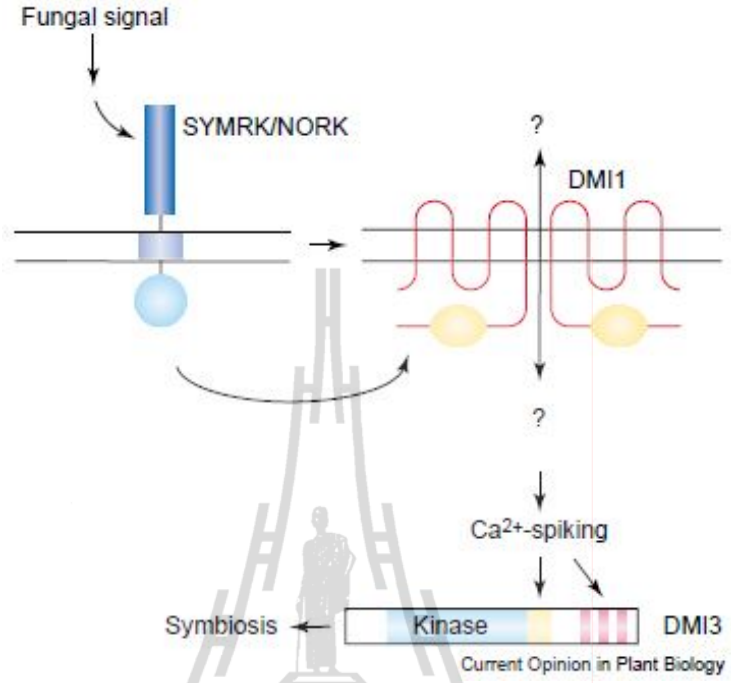

Figure 2.10 A model of events that are mediated by the predicted protein products of cloned common *SYM* genes. Among the identified components, the SYMRK/ NORK/DMI2 receptor kinase may be the earliest to act in the AM signalling pathway (Stracke et al. 2002; Endre et al. 2002). It perceives signals emanating from the fungal microsymbiont either directly or indirectly, and transduces the event through its intracellular kinase domain. This, in turn, activates the predicted ion channel, DMI1 (Ane et al. 2004). The availability of purified bacterial signalling compounds and experimental difficulties arising from the obligate biotrophic nature of the fungus have contributed to a situation in which we know more about early signalling events in root nodule symbiosis than in AM. In particular, we do not know whether the calcium-spiking response that is characteristic of the rhizobial symbiosis also occurs in the mycorrhizal interaction. The DMI3 kinase potentially responds

directly to oscillations in calcium-concentration, however, implying that Ca^{2+} is also a messenger in mycorrhizal signalling (Levy et al. 2004; Mitra et al. 2004).

Signal exchange before infection

How do fungal and plant partners find each other within the soil? One possibility is that root-derived signals redirect fungal growth. At least on Petri-dishes, however, the negative geotropism of *Gigaspora* germtubes does not appear to be influenced by the presence of roots (Chabaud et al. 2002). An alternative strategy would be to ramify hyphae in the vicinity of roots to increase the chance of an encounter with the host. A root-derived signal induces the branching of (free-living) fungal hyphae (Giovannetti et al. 1993) (Figure 2.11a).

Evidence for a fungal signalling molecule that induces plant gene activation was gained from experiments by Kosuta et al. (Kosuta et al. 2003) in which fungal hyphae and host roots were grown in close proximity but separated from each other by membranes that were impenetrable for roots or fungal hyphae. In this set-up, a *Medicago EARLY NODULATION11 (ENOD11)*-promoter::β-glucuronidase (*GUS*) fusion, which was responsive to both arbuscular mycorrhiza fungi and rhizobial Nod-factor (Journet et al. 2001), was activated at a distance from fungal hyphae (Kosuta et al. 2003). This activation was the first experimental evidence of a long-postulated fungus-derived diffusible signalling molecule. *GUS*-staining was limited to roots sectors located in the vicinity of ramified hyphae, suggesting that signal production by individual hyphae either was too low to elicit a response or was induced concomitantly with the hyphal-branching response (Kosuta et al. 2003). Whether a single plant molecule induces hyphal branching and production of the

fungal factor remains to be shown. In further work using the *ENOD11*-promoter::*GUS* transgenic line carried out in the same laboratory, Chabaud et al. (2002) and Kosuta et al. (2003) found that gene expression patterns differed dramatically depending on whether fungal contact with the root was permitted or not. In the absence of fungal contact, large root sections of several centimetres in length responded, and *GUS* staining was observed in most epidermal and cortical cell layers of these sections (Kosuta et al. 2003). In contrast, once fungal contact with the root was established, *ENOD11* expression was confined to infected cells (Chabaud et al. 2002). This striking difference provides a conundrum that can be resolved by postulating a negative regulatory mechanism that, only upon fungal contact, suppresses *ENOD11* in those cells that are not infected by the fungus (Figure 2.11c).

Recent evidence suggests that AM fungi produce diffusible symbiotic signals (Maillet et al. 2011). They showed that *Glomus intraradices* secretes symbiotic signals that are a mixture of sulphated and non-sulphated simple lipochitooligosaccharides (LCOs), which stimulate formation of AM in plant species of diverse families (Fabaceae, Asteraceae and Umbelliferae). In the legume *Medicago truncatula* these signals stimulate root growth and branching by the symbiotic DMI signalling pathway. These findings provide a better understanding of the evolution of signalling mechanisms involved in plant root endosymbioses and will greatly facilitate their molecular dissection. They also open the way to using these natural and very active molecules in agriculture.

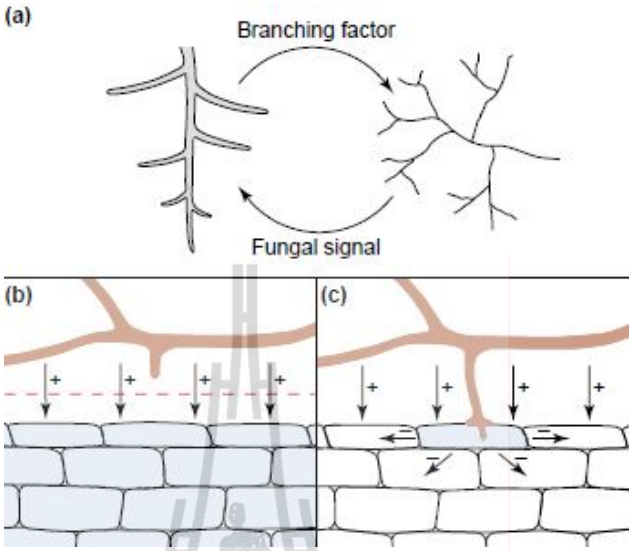

Figure 2.11 Signal exchange between the plant root and the hyphae of AM fungi before infection. (a) Roots (left) release a branching factor that induces alterations in the growth pattern of the fungus. In turn, the fungus (right) releases a diffusible signal that is recognised by the plant and that leads to symbiosis-related gene activation. (b) The Kosuta experiment (Kosuta et al. 2003). Fungal hyphae (brown) growing in the vicinity of the host root but separated from the host root by a cellophane membrane release a diffusible signal (+) that induces the widespread expression of a *ENOD11*-promoter::*GUS* fusion in *M. truncatula* roots. This gene induction also occurs in the roots of *dmi1, dmi2* and *dmi3* mutants. (c) The Chabaud experiment (Chabaud et al. 2002). When fungal contact with the root is established *ENOD11*-promoter::*GUS* expression is limited to cells that are contacted by the fungus. The different root responses in (b) and (c) can be explained by postulating a negative regulatory signal (-) that limits the symbiotic response to those cells that are actually engaged. This negative regulatory circuit also appears to be

active in *dmi2* mutants, as no *ENOD11*- promoter::*GUS* staining was observed when fungal contact with the roots of this mutant was permitted.

Cell-type-specific symbiotic programmes

The most frequently described AM phenotype of common *SYM* mutants in Medicago and pea is appressorium formation at the root surface and a block of all subsequent infection steps (reviewed in Marsh and Schultze 2001). More recently, a series of mutant alleles in *L. japonicus*, and the relative vigour with which the AM fungi *G. intraradices* or *Gigaspora margarita* attack plant roots have enabled studies of the role of common *SYM* genes in cell types other than the epidermis.

Epidermal opening

After colonization of the root surface, fungal hyphae typically enter the root through a cleft that opens between the anticlinal walls of two adjacent epidermal cells. Genetic evidence indicates that the opening of this cleft is, to a large extent, an activity of the host plant (Demchenko et al. 2004). These clefts are not fully formed in plant mutants that are affected in the *LjSYM15* gene, and fungal hyphae continue to grow on the root surface of these mutants precisely along the boundaries of epidermal cells, as if in search of such opening clefts. This epidermal opening occurs along the middle lamella and is probably mediated through the production and localised release of pectinolytic enzymes by epidermal cells. *LjSYM15* is presumably involved in the regulation of this process. Surprisingly, neither LjSYM4 (Novero et al. 2002) nor the receptor kinase SYMRK are required for the epidermal-opening response, which must therefore rely on alternative signal perception and transduction components (Demchenko et al. 2004).

Intracellular passage to the inner cortex

Fungal hyphae form appressoria to gain entry into root epidermal or exodermal cells. Appressorium formation by an AM fungus was induced by purified epidermal cell wall fragments, suggesting that the physical properties of these fragments are sufficient to elicit this response (Nagahashi and Douds 1997). Fungal hyphae grow through the initially infected cell and are guided to the underlying cell layer. The hyphae then pass through the outer layers of living cells, where they are surrounded by a plant-derived accommodation structure that comprises a perifungal membrane that is continuous with the plasma membrane. Additional symptoms of the induction of symbiotic programmes in these cells are the activation of a *M. truncatula serine carboxypeptidase II* promoter::*green fluorescent protein* (*GFP*) (Liu et al. 2003) and an *ENOD11*-promoter::*GUS* fusion (Chabaud et al. 2002), both of which are also induced in arbuscule-containing cells. The activation of the host's intracellular accommodation programme in the outer cell layers is dependent on the *LjSYM4* and *LjSYMRK* genes (Demchenko et al. 2004; Novero et al. 2002). When these genes are defective, fungal entry into the first cell occurs but the interaction aborts. In the *Ljsym4* mutant at least, this aborted interaction is associated with the death of both the plant cell and the hyphal tip that is physically involved in the penetration event (Bonfante et al. 2000).

The initial intracellular passage through the outer cell layers constitutes a bottleneck of AM establishment, after which the fungus is released into the apoplast between cortical cells. This extracellular phase allows for the rapid proliferation of fungal hyphae along the longitudinal axis of the root, and relatively large root sectors can be colonised as the result of a single successful initial infection event (Figure 2.12).

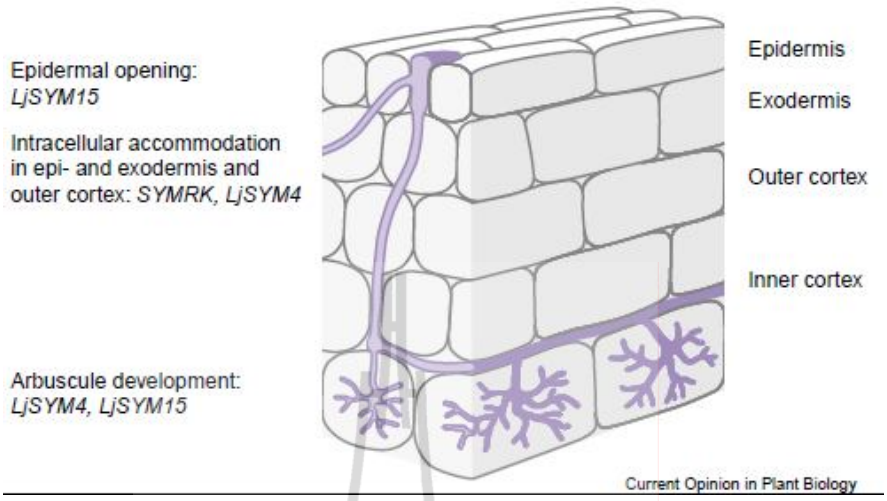

Figure 2.12 Cell-layer-specific responses that are mediated by distinct subsets of common *SYM* genes. A schematic overview of AM development, as compiled from cytological analysis of the interaction of L. japonicus roots with *G. intraradices* or *G. margarita* (Demchenko et al. 2004; Novero et al. 2002; Bonfante et al. 2000). Fungal hyphae (blue) grow through epidermis and exodermis of the root to form arbuscules in the inner cortex. Plant genes and cell types that are involved in different steps are depicted. Directly below a fungal hypha, the walls of two adjacent epidermal cells separate from each other, a process that the *LjSYM15* gene appears to contribute to. A fungal hypha enters this gap, and subsequently penetrates an epi- or exodermal cell. Successful intracellular accommodation within this and the subsequent cell layer requires the *Lotus SYMRK* and *LjSYM4* genes (Demchenko et al. 2004; Novero et al. 2002). The fungus leaves the plant cell and re-enters the apoplast in the inner cortex. There, hyphae can grow relatively rapidly in the spaces between adjacent cells. They branch to penetrate cells of the two innermost cortical layers, forming arbuscules. The uptake of

hyphae into the inner cortical cells requires the *LjSYM4* and *LjSYM15* genes (Demchenko et al. 2004; Novero et al. 2002). Once inside these cells, proper arbuscule development in pea depends on the *PsSYM36* gene (Gianinazzi-Pearson 1996; Guinel and Geil 2002).

Arbuscule development and function

As the fungus grows through the apoplast, hyphal branches are formed that penetrate inner cortical cells to initiate arbuscule formation. No obvious appressoria are formed before fungal penetration of the plant cortical cell wall. The entry of hyphae into inner cortical cells is impaired in *LjSYM4* and *LjSYM15* mutants, indicating that the plant has an active role in this process (Demchenko et al. 2004; Novero et al. 2002). Lotus plants carrying weak mutant alleles of the *LjSYM15* gene suffer from delayed nodulation and arbuscule development (Demchenko et al. 2004). Once inside inner cortical cells, fungal hyphae branch profusely and form a tree-like structure, the arbuscule. Host genes that are required for arbuscule development have been identified: mutants affected in the pea *PsSYM36* gene are low and late nodulating and form only stumpy branches instead of proper arbuscules (Gianinazzi-Pearson 1996; Guinel and Geil 2002). The periarbuscular membrane in this interaction does not stain for an ATPase activity observed in the wildtype, indicating that these rudimentary arbuscules are probably non-functional (Gianinazzi-Pearson et al. 1995).

2.8.1.5 Methodological Issues, Obstacles

The molecular identification of AM fungi in the natural environment is frequently done by extracting the DNA from a root (or soil) sample followed by PCR (singlestep, nested or several group-specific PCR-s), separation of

multiple products by cloning or electrophoresis (SSCP, DGGE, TGGE, or T-RFLP after digestion), and sequencing clones or individual bands. Size of root samples ranges from 0.5 cm to ca. 20 cm. The samples may be collected following different spatial/temporal sampling designs. The size of soil samples taken depends on the DNA extraction method, but usually does not exceed a 1-g representative sample from a larger initial homogenous sample. It is worthy of contemplation how well these small samples represent AM fungal (or other soil microorganism) communities in natural ecosystems. Can a forest be described with a handful of pieces of root.

It is possible to estimate the plant individual-based fungal community structure by studying several small root samples, instead of one large sample, from the same plant. This has rarely been done, but such an approach reveals the spatial structure of the fungal community within root system (Jacquot-Plumey et al. 2001; Kjoller and Rosendahl 2001; Turnau et al. 2001; Opik et al. 2006a). Commonly in published papers, if the size of a sample is specified, ca. 20 cm length of roots from an individual plant constitutes a sample, thus also retaining the information of the host identity. However, depending on the research question and the spatial scale of interest, different levels of sample pooling down to a single sample may be considered as an alternative to intensive sampling. In order to compare fungal assemblages of locations, but eliminating variation related to host-specificity or preference, random root samples consisting of a mixture of several plant species might be used (e.g. Heinemeyer et al. 2004). This approach would require that plant species are roughly similarly distributed in the study sites in order to avoid hidden effect of host preference due to different representation of plant hosts in samples. However, if host effect to AM fungal diversity is high and host distribution in

samples uneven, this may result in hidden host effect on the recovered fungal diversity.

Some authors have used an approach where individual samples are pooled after collection or after DNA extraction (Jumpponen et al. 2005; Saito et al. 2004), possibly in order to minimize number of samples to be cloned and thus the cloning expense. From such a pooled sample, larger number of clones might be screened and/or sequenced. Is it better to analyse 10 clones from each of 10 samples or 25 clones from each of 4 samples? Assuming that fungal taxa are patchily distributed in the study area but their relative abundances are more or less even, pooling samples before cloning may enable a description of the real diversity whilst screening fewer clones in total. On the contrary, if the study area is dominated by a few taxa, but there are many infrequent taxa, more clones would need to be screened either from pooled or unpooled samples in order to capture most of the taxon diversity present. Furthermore, pooling in this case would reduce the probability of recording rare taxa present compared to an unpooled strategy.

Depending on the expected diversity and spatial agglomeration of taxa in the study system, a different number of clones (in total and/or per sample) would describe the fungal community present in a sample at a level satisfactory for the research question. Rarefaction analysis implemented by Wirsel (2004) suggests that larger number of samples would have detected higher diversity in autumn (over 20 as opposed to 16 discovered OTU-s), but not in spring and summer. There seems to be a trend that a larger sampling effort (i.e., more samples, ignoring the number of clones screened) reveals a larger number of fungal taxa (Opik et al. 2006b). This suggests that a ‘satisfactory’ level of sampling intensity has mostly not been reached.

Sampling intensity should depend on the study system – how many taxa are in the system and how evenly/unevenly they are distributed? For example, a taxon-poor system like fields in intensive agriculture could be described by a smaller number of samples than a taxon-rich system, e.g. grasslands or tropical forests. However, if a taxon-poor system is heavily dominated by a single taxon, then more samples are needed to recover the other, infrequent taxa. As the recovered diversity might not be a linear function of sampling intensity (Suzuki et al. 2004), the best one can do at the present state of knowledge is to be aware of the effect of sampling and to be careful when drawing conclusions.

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CHAPTER III

SELECTION OF ARBUSCULAR MYCORRHIZAL FUNGI FOR CITRUS GROWTH PROMOTION AND *PHYTOPHTHORA* SUPPRESSION

3.1 Abstract

In order to reduce unnecessary amount of P-fertilizer and severity of *Phytophthora* root rot in citrus orchards, the experiment was set up. Thirteen indigenous arbuscular mycorrhiza (AM) fungi species were isolated from rhizosphere soil of citrus orchards in Thailand and were then propagated into three host plants [sorghum (*Sorghum bicolor*), maize (*Zea mays*), and leek (*Allium cepa*)] by trap culture. We also tested whether indigenous AM species (13 different species) could colonize into three cultivars of citrus scions and rootstocks (Shogun: *Citrus reticulata* Blanco cv. Shogun; Tangerine: *C. reticulata*; and C-35 citrange: *Citrus sinensis* ×*Poncirus trifoliata*). With root colonization rates, the results indicated that *Acaulospora tuberculata* and *Glomus etunicatum* provided the best colonization in all citrus cultivars. We selected, therefore, those AM species to verify their influences on citrus growth and *Phytophthora* root rot resistance. Three cultivars of citrus scions and rootstocks, Shogun, Tangerine and C-35 citrange, were inoculated with two effective indigenous AM species, *G. etunicatum* or *A. tuberculata* in order to determine the influences on citrus growth. The plants were investigated to determine the mycorrhizal efficiency index (MEI), AM colonization, P content, and other

parameters. Co-inoculation of AM species (*G. etunicatum* or *A. tuberculata*) with *Phytophthora nicotianae* was also carried out in Shogun scion/C-35 citrange rootstocks. The results of citrus growth revealed that Shogun and Tangerine inoculated with *G. etunicatum* produced the highest MEI. Tangerine and C-35 citrange amended with fertilizers and *G. etunicatum* showed the highest P content in leaves. This indicated that *G. etunicatum* has an influence on citrus growth and P uptake, suggesting it to be the highly effective strain. Shogun scion/C-35 citrange rootstock combinations that were inoculated by both *P. nicotianae* and different AM fungi (*G. etunicatum* or *A. tuberculata*) showed root injury at low level of root rot symptom. However, the part of Shogun scions grafted on rootstocks showed severe symptom of shoot die back in treatment inoculated with *P. nicotianae* alone, while treatment inoculated with different AM species (*G. etunicatum* or *A. tuberculata*) and *P. nicotianae* rendered lower shoot die back symptoms than that of *Phytophthora* treatment. The low level of shoot die back symptom was shown at first, then healthy young shoot was restored. Our results indicated the facts that different host plants and different AM species produced different outcomes of growth and pathogen resistance. The application of both AM isolates, therefore, has an enormous potential to be produced the inoculum for citrus orchards.

Glomus etunicatum/*Acaulospora tuberculata*/*Phytophthora*/CITRUS

3.2 Introduction

Citrus is the most commonly grown trees worldwide including in Thailand. Many citrus orchards are located in slope land areas where the soil is infertile and strongly acidic, and has low organic matter content. Moreover, citrus producers noted

that temperature and geographic location cause disease problems. The two fungal species of *Phytophthora* most commonly associated with citrus are *Phytophthora parasitica* Dastur (syn. *Phytophthora nicotianae* Breda de Haan) and *Phytophthora citrophthora* Leonian (Klotz, 1978; Timmer and Menge, 1988). Both species are widely distributed in citrus-growing areas and cause root rot, foot and crown rot, brown rot of fruit, and damping-off and blight of seedlings (Timmer and Menge, 1988). The symptoms caused by *Phytophthora* spp. are primary factors in poor growth and death of citrus trees of all ages both in the nursery and in the field (Carpenter et al., 1975; Graham and Timmer, 1992). Researchers reported that *P. citrophthora* is typically the predominant causal agent at lower temperatures while *P. nicotianae* predominates at higher temperatures. In New South Wales, *P. citrophthora* is the predominant species and one of the most destructive pathogens of citrus. In Queensland and Florida, *P. nicotianae* is the predominant species (Broadbent etal., 1971; Grimm and Whidden, 1962; Whiteside, 1971) while in California, both *P. citrophthora* and *P. nicotianae* occur (Klotz et al., 1958; Klotz et al., 1968). *Phytophthora syringae* Kleb. and *Phytophthora hibernalis* Carne have also been found to cause root rot and foot rot of citrus in California (Klotz, 1978). In Thailand, *P. nicotianae* is the predominant species and causes many serious diseases of citrus countrywide (Paradanuwatana et al., 1984).

Based on observations, citrus producers began to manage with one or more fungicide applications to control *Phytophthora* (McCoy and Duncan, 2000). However, *Phytophthora* may reinvade the soil and become even more severe problems on citrus roots because those applications often destroy soil microorganisms which compete with *Phytophthora* (Ferguson and Timmer, 1987; Ridingsetal., 1977). Several studies reported that arbuscular mycorrhizal (AM) fungi increased nutrient

absorption and reduced the severity of disease (Schenck etal., 1975; Roncadori and Hussy, 1977; Herreetal., 2007). Previous studies (Peng et al., 1993; Fidelibus et al., 2000) demonstrated utilization of AM fungi strains collected from citrus orchards that there was a high potential to increase the shoot and a root biomass and the number of leaves including photosynthetic efficiency in citrus, *Citrus volkameriane*, under continually moist soil (soil water tension; approximately−0.01 MPa) and periodically dry soil (soil water tension; approximately −0.06 MPa) conditions in the United States of America. It is clearly shown that AM fungi could increase complete citrus root system even though it was cultured on the limited phosphorus condition. AM strains, *Glomus intraradices* and *Glomus etunicatum*, were applied with citrus *Citrus paradise* and *C. volkameriane* and the results demonstrated that they strongly affected the lateral root of citrus (Espeleta et al., 1999). Nemec et al. (1996) found that AM fungus, *G. intraradices*, inhibited the citrus root rot caused by fungal pathogen *P. nicotianae*. The mycorrhizal sweet orange root (*Citrus sinensis* (L.) Osbek) was able to tolerate the root rot caused by *P. nicotianae* (Davis and Menge, 1981). Another report showed that mycorrhizal (*Glomus fasciculatus*) sweet orange inoculated with *P. nicotianae* had a greater growth rate than that of non-mycorrhizal citrus (Davis and Menge, 1980).

One of the best solutions for control of *Phytophthora* and other diseases or other stress environmental factors is the use of tolerant or resistant rootstocks. The success of a rootstock is determined by its tolerance to overcome conditions of soil, climate and diseases, while still producing high yields of good quality fruit. There are many citrus rootstocks cultivars which provide different tolerance or resistance to diseases, soil factors or environmental factors (Castle etal., 1989; Graham, 1990; Cooper and Gorton, 1952; Grieve and Walker, 1983; Walker and Douglas, 1983).

One of soil problems for citrus is salinity which interacts with diseases including root rot (*Phytophthora* spp.), nematodes and AM fungi. Afek and Sztejnberg (1993) reported that plant defense mechanisms against *Phytophthora* might be inhibited by salinity stress and also decreased root regeneration under pathogen pressure. In green house experiments, irrigation with salinity water not only could have direct effects on root pathogens, but salinity could also predispose citrus rootstock seedlings (Troyer citrange, Carrizo citrange, Volkamer lemon and rough lemon) to attack by a group of root pathogens (*Phytophthora* spp., *Fusarium solani* and citrus nematode) (Combrink et al., 1996). Under saline conditions, the resistance of *Phytophthora*-tolerant rootstocks could significantly be diminished by the ability of *Phytophthora* to tolerate high salanity (Blaker and MacDonald, 1986).

Since Thailand is located in the tropical area, the major factors that constrain tropical soil fertility and sustainable agriculture include low nutrient concentration, moisture stress, erosion, high P fixation, high acidity with aluminum toxicity, and low soil microbial diversity. These problems of tropical soils limit agricultural production, including citrus production, in annual crop production. Moreover, Paradanuwatana et al. (1984) revealed that citrus root rot diseases were typically the predominant problem. For better nutrient management and disease control in the tropical area, utilization of biological potential such as AM fungi and resistant rootstocks are an important alternative approach. Arbuscular mycorrhizal (AM) fungi are probably most abundant microorganisms in natural soil. AM fungi account for 5–50% of the biomass of soil microbes (Olsson et al., 1999). Almost all tropical crops are strongly responsive to arbuscular mycorrhizas. From many studies of tropical AM–host combination, Nemec (1978), Kiers et al. (2000) and Herre et al. (2005) have clearly

shown that different AM fungi species have generated different effects on host growth response.

3.3 Objectives

The aims of this study were to:

3.3.1 select efficient indigenous mycorrhizal fungi and investigate the influence of effective indigenous mycorrhizal species on citrus growth and phosphorus uptake.

3.3.2 determine the influence of AM on *Phytophthora* root rot in citrus.

3.4 Materials and Methods

3.4.1 Field sampling and AM spore isolation

AM spores were collected from the rhizosphere soil of citrus orchards in 5 areas of Thailand (22 sampling sites), including in the Northern region Chiangmai (lat. 98.98◦E and long. 18.80◦N) and Kamphaengphet (lat. 99.52◦E and long. 16.47◦N) provinces, in the Eastern region Chantaburi (lat. 102.11◦E and long. 12.61◦N) and in the Central region Nakhonpathom (lat. 100.06◦E and long. 13.82◦N) provinces, and in the Northeastern region Nakhon Ratchasima (lat. 102.10◦E and long. 14.97◦N) province as shown in Table 3.1. Samples were collected only once in the dry season in December 2005. Within sampling field, five 10×10 m plots were marked out, and in each plot 20 soil cores (0–30 cm depth) were collected with a 5 cm diameter soil auger and pooled to provide a 5 kg sample.

AM spores were extracted from soil by wet sieving and sucrose density gradient centrifugation method adapted from Daniels and Skipper (1982). Briefly, 20

g of soil fresh weight were dispersed in 50 ml distilled H_2O and shaken for 30 min. A 20 ml of the suspension was removed and added into a 50 ml Falcon tube containing 20 ml water sucrose solution (50%, w/v) for 10 min at 2000 rpm. The supernatant was then poured onto a 37 µm sieve and washed until the sucrose was completely removed. The material caught on the sieve was washed into a fresh Falcon tube with distilled H_2O and made up to 50 ml volume. Isolated spores were stored at 4 ◦C for 24 h prior to classification. AM spores were then counted, distinguished and morphologically identified under a stereomicroscope. Permanent slides of all spores were prepared by placing them in polyvinyl alcohol-lactic acid-glycerin (PVLG) mixed with Melzer's reagent (1:1, v/v). Spores were cracked open under the cover slip to allow for observation based on spore color, shape, surface ornamentation, spore content and wall structures (INVAM; http://www.invam.caf.wvu.edu/). A total spores of 13 different morpho-species were identified and summarized in Table 3.1.

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Table 3.1 Arbuscular mycorrhizal fungi species isolated from citrus orchards in Thailand

Species	Spore abundance (spores/20 g soil)				
	North/Ch[1**] (6 sites)	North/Ka[2] (4 sites)	Cent/Ch[3] (6 sites)	Cent/Na[4] (1 site)	N.East/Na[5] (5 sites)
	1 2 3 4 5 6	1 2 3 4	1 2 3 4 5 6	1 1 2 3 4 5	
G.etunicatum	48 46 51 44 42 53	47 38 42 44	53 49 46 58 55 39	67	23 26 31 18 27
G.caledonium	38 20 21 23 14 12	ND	ND	ND	ND
G. aggregatum	21 23 20 15 11 24	ND	ND	ND	ND
G. microaggregatum	19 22 19 22 23 1	ND	ND	ND	ND
G. sinuosum	15 15 21 17 20 18	ND	ND	36	11 10 14 15 17
G. intraradices	ND*	ND	ND	42	ND
G. clarum	ND	ND	ND	ND	12 23 19 12 16
A. tuberculata	45 47 42 39 45 51	41 34 37 35	42 44 38 49 50 40	55	28 22 33 21 25
A. scrobiculata	26 28 29 31 25 22	14 26 36 13	30 39 23 45 51 32	ND	8 13 11 15 20
E. colombiana	20 15 28 14 30 27	ND	ND	ND	ND
S. gregaria	ND	ND	28 30 19 35 42 22	27	ND
Gi.calospora	ND	ND	ND	ND	ND ND 11 6 12
Sclerocystis sp.	ND	ND	ND	ND	8 ND 7 ND10
Total[***]	232/216/231/206/210/218	102/98/115/92	153/162/126/187/198/133	227	90/94/126/87/127
(Total mean, n=5)[****]	$232^{a\dagger}$/216.4^{d}/230.6^{a}/205.6^{f}/210.4^{e}/217.8^{c}	102.4^{n}/98.2^{o}/115.4^{m}/92.2^{q}	153.6^{j}/161.8^{i}/125.6^{l}/187.4^{h}/197.6^{g}/133.2^{k}	227.2^{b}	90.2^{r}/93.8^{p}/126^{l}/ 86.8^{s}/126.6^{l}

* Not detected

** Abbreviation of sampling sites,

1; North/Ch : Northern region/Chiangmai site 1-6

2; North/Ka : Northern region/Kamphaengphet site 1-4

3; Cent/Ch : Central region/Chantaburi site 1-6

4; Cent/Na : Central region/Nakhon-pathom site 1

5; N.East/Na : Northeastern region/Nakhon Ratchasima site 1-5

*** Total spore numbers of each sampling site

**** Mean of total spore numbers calculated by 5 replications

† Means under each parameter followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple-range test

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3.4.2 AM spore propagation and trap culture

In order to increase fungal inoculum, the classified AM spores (13 different morpho-species) were propagated by trap culture method (Brundrett et al., 1996). Seeds of host plants, i.e. sorghum (*Sorghum bicolor*), maize (*Zea mays*) and leek (*Allium cepa*), were surface-sterilized and germinated onto Petri dishes containing humid filter paper for 1 week. The 3–5 germinated seeds were transplanted into each pot containing sterilized sandy soil. Seedlings were watered with Hoagland's solution weekly (Hoagland and Arnon, 1938). Before spore propagation, the classified AM spores were surface-sterilized with streptomycin (Mosse, 1973) to eliminate the mycorrhizosphere microflora, and then 30 spores of each AM isolate were inoculated into each pot. Plants were grown in a growth room under a 16-h-light (25 ◦C)/8-h-dark (25 ◦C) and conducted with 10 pots per host plant. After approximately 12 weeks, newly produced spores were collected according to the method mentioned above. The spores were isolated from 100 g of soil from each trap pot following the same procedure as for the field sampling and AM spore isolation. The spores were observed and identified under a compound microscope as above. Abundances of spores of each AM species were estimated and further used for the next experiment. Each host plant roots were stained with 0.03% trypan blue in lactoglycerol according to the method of Phillips and Hayman (1970), and colonization of AM fungi were observed. Spore abundances of each AM species and AM colonization were noted in Table 3.2.

Table 3.2 Arbuscular mycorrhizal (AM) colonization (%) and AM spore numbers produced in pot culture using *Sorghum bicolor*, *Zea mays* and *Alluim cepa* as host plants (*n*=10)

Species	Number of spore (spores/100 g soil)			AM colonization (%)		
	***S. bicolor*‡**	***Z. mays***	***A. cepa***	***S. bicolor***	***Z. mays***	***A. cepa***
G. etunicatum	86.30^{a†}(0.37*)	93.60^{a} (0.26)	46.30^{c} (0.29)	50.31^{b} (0.23)	60.40^{b} (0.46)	28.46^{b} (0.23)
G.caledonium	74.00^{d} (0.50)	80.70^{c} (0.47)	51.60^{b} (0.22)	47.67^{c} (0.27)	56.23^{c} (0.23)	22.16^{e} (0.52)
G. aggregatum	64.40^{e} (0.34)	62.70^{d} (0.60)	36.80^{f} (0.46)	41.93^{d} (0.45)	45.70^{d} (0.27)	18.90^{h} (0.20)
G. microaggregatum	61.90^{g} (0.47)	59.40^{e} (0.24)	31.70^{i} (0.50)	39.33^{d} (0.30)	35.20^{f} (0.27)	17.27^{i} (0.29)
G. sinuosum	63.60^{f} (0.84)	60.63^{e} (0.36)	35.51^{g} (0.36)	34.67^{ef} (0.45)	38.40^{e} (0.31)	23.31^{d} (0.23)
G. intraradices	61.60^{g} (0.57)	63.00^{d} (0.50)	41.40^{d} (0.46)	36.40^{e} (0.22)	35.00^{f} (0.50)	21.20^{f} (0.52)
G. clarum	64.42^{e} (0.55)	57.30^{f} (0.45)	32.60^{h} (0.62)	35.23^{e} (0.28)	31.51^{g} (0.25)	18.17^{h} (0.26)
A. tuberculata	78.40^{b} (0.58)	81.90^{c} (0.22)	56.20^{a} (0.27)	53.90^{a} (0.47)	62.67^{a} (0.60)	33.23^{a} (0.23)
A. scrobiculata	76.40^{c} (0.55)	86.70^{b} (0.41)	41.80^{d} (0.25)	49.70^{b} (0.61)	60.04^{b} (0.51)	25.93^{c} (0.62)
E. colombiana	46.70^{i} (0.38)	56.30^{f} (0.45)	36.50^{f} (0.40)	31.20^{f} (0.27)	37.40^{e} (0.31)	29.83^{b} (0.61)
S. gregaria	37.60^{j} (0.38)	34.50^{h} (0.35)	37.40^{e} (0.24)	29.50^{g} (0.25)	26.20^{i} (0.27)	23.67^{d} (0.68)
Gi. calospora	47.70^{h} (0.59)	49.10^{g} (0.25)	35.50^{g} (0.79)	32.70^{f} (0.62)	28.31^{h} (0.29)	20.80^{g} (0.65)
Sclerocystis sp.	31.70^{k} (0.47)	34.40^{h} (0.62)	30.70^{j} (0.27)	20.97^{h} (0.56)	24.23^{j} (0.53)	21.30^{f} (0.29)

† Means under each parameter followed by the same letter are not significantly different (P < 0.05) according to Duncan's multiple-range test.

‡ Means under each parameter are calculated by each column (each host plant).

* Values in the parentheses indicate standard error (SE)

3.4.3 Selection of indigenous AM fungi on citrus seedlings

This experiments were carried out to screen the effective AM fungi on three commercial citrus cultivars, C-35citrange (rootstock), Shogun and Tangerine. To test whether isolated AM fungi were able to colonize into citrus roots, three citrus cultivars of citrus seeds, C-35 citrange (*C. sinensis* × *P. trifoliate*), Shogun (*Citrus reticulata* Blanco cv. Shogun) and Tangerine (*C. reticulata*), were surface-sterilized and put onto Petri dishes for germination. After 2 weeks, those citrus cultivars were transplanted and 13 different morpho-species were inoculated by 100 AM spores a pot (100 spores of each AM species). The citrus were then planted and daily watered

with Hoagland's solution (Hoagland and Arnon, 1938). The experiments were carried out by a factorial approach in completely randomized design (two factor CRD) with five replications of citrus plants. Root samples (three sampling roots/plant) for assessment of mycorrhizal colonization were harvested, cleared in 10% (w/v) KOH and stained with 0.03% (v/v) trypan blue in lactoglycerol according to the method of Phillips and Hayman (1970). The assessment of colonization was determined by the magnified line intersect method (McGonigle et al., 1990) after 16 weeks. In this part, we selected the best AM species among 13 AM species that could provide the highest mycorrhizal colonization in citrus roots (see results noted in Table 3.3).

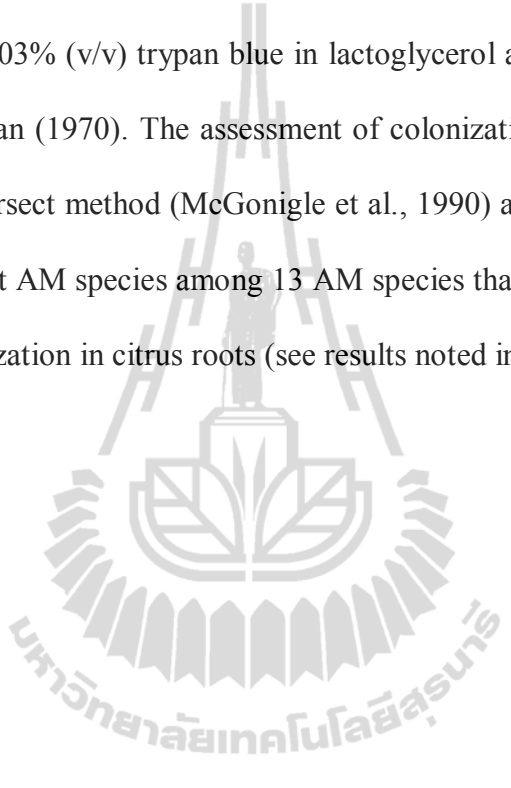

Table 3.3 Means of arbuscular mycorrhizal (AM) colonization (%) assessed in three citrus cultivars (Tangerine, Shogum and C-35 citrange) that were inoculated with different arbuscular mycorrhizal species isolated from pot culture (*N*=5, *n=3*)

Species	**AM colonization (%)**		
	Tangerine‡	**Shogun**	**C-35 citrange**
G. etunicatum	$50.73^{d\dagger}(0.18^{*})$	60.13^{b} (0.39)	42.27^{f} (0.43)
G. caledonium	46.42^{e} (0.26)	57.26^{c} (0.37)	40.23^{g} (0.41)
G. aggregatum	28.04^{j} (0.41)	40.39^{g} (0.19)	30.12^{ij} (0.21)
G. microaggregatum	29.17^{j} (0.40)	30.79^{ij} (0.23)	25.49^{k} (0.29)
G. sinuosum	28.23^{j} (0.22)	30.54^{ij} (0.21)	24.71^{kl} (0.48)
G. intraradices	29.87^{ij} (0.38)	31.20^{hi} (0.19)	23.35^{l} (0.19)
G. clarum	30.67^{ij} (0.30)	32.45^{h} (0.27)	28.22^{j} (0.19)
A. tuberculata	50.17^{d} (0.55)	62.36^{b} (0.18)	70.89^{a} (0.24)
A. scrobiculata	45.02^{e} (0.21)	60.10^{b} (0.37)	43.46^{f} (0.45)
E. colombiana	20.68^{m} (0.20)	29.26^{j} (0.19)	20.13^{m} (0.12)
S. gregaria	17.25^{n} (0.37)	20.74^{m} (0.32)	26.15^{k} (0.18)
Gi. calospora	20.87^{m} (0.24)	19.28^{m} (0.19)	15.49^{o} (0.37)
Sclerocystis sp.	18.61^{mn} (0.20)	17.68^{n} (0.20)	19.31^{m} (0.37)

† Means of AM colonization followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple-range test.

‡ Means are calculated by all column.

* Values in the parentheses indicate standard error (SE).

N Replications of plant were used in this study.

n Root systems were sampled in each plant for AM colonization.

3.4.4 Selection of effective AM fungi on citrus growth

The experiments were carried out on three cultivars of 4-month-old commercial citrus: C-35 citrange, Shogun and Tangerine, in order to screen the effective AM fungi. Citrus rootstocks (C-35 citrange) and the scions (Shogun and Tangerine) were grown in 5 l pots containing sterilized sand. Approximately 500 spores of two effective AM species (*G. etunicatum* and *Acaulospora tuberculata*) were then inoculated to the three citrus cultivars according to the methods mentioned

above. The experiment was designed with treatments that consisted of: (i) control treatment (no fertilizer and no AM inoculation); (ii) chemical fertilizer treatment (added N-P-K: 8-8-8); (iii) and (iv) AM fungi treatment (inoculated *A. tuberculata* or *G. etunicatum*). Control and chemical fertilizers (N–P–K: 8–8–8) treatments were also tested to compare with treatments that inoculated AM fungi. The experiment was done by a factorial approach in completely randomized design (two factor CRD) with five replications and carried out under greenhouse conditions for 4 months. After harvest, P content, mycorrhizal colonization and mycorrhizal efficiency index (MEI) were estimated according to Bagyaraj (1994):

$$MEI = 100 \times \left[\frac{\text{weight of inoculated plant} - \text{weight of uninoculated plant}}{\text{weight of inoculated plant}} \right]$$

Total dry weight, height, root fresh weight (g), stem fresh weight (g), total fresh weight (g), root length (cm), root dry weight (g), stem dry weight (g), total dry weight (g) were analyzed. Data analyses were subjected by one-way ANOVA at 5% confidential value (Gianinazzi et al., 2002).

3.4.5 Disease survey and *Phytophthora* isolation

Isolates of *P. nicotianae* were isolated from citrus orchards in Nakhon Ratchasima, Thailand. Almost all citrus were Shogun grown for more than 3 years. All citrus grown in orchards were citrus scions, not rootstock cultivars. They were therefore more susceptible to *Phytophthora* root rot. *Phytophthora* was isolated from soil and then infected roots of citrus by baiting method (Mitchell and Kannwisher-Mitchell, 1993) followed by culturing on the PARPH selective medium developed by Jeffers and Martin (1986).

3.4.6 Infectivity test of *Phytophthora* in Shogun roots

To test and confirm whether isolated *P. nicotianae* is able to infect the citrus roots, Shogun scion seedlings (12 weeks old), were grown and a wound was made onto the infection sites which are the lower trunk or crown roots. Soil was infested with the isolated *P. nicotianae*, while control treatment was not infested. For inoculation, Zoöspores were produced by the method of Tsao (1971) and mixed with moist autoclaved sand. The inoculum mix was then incubated for 10 days, and propagule densities were determined by plating on the PARPH medium (Jeffers and Martin, 1986) using the methods described by Timmer et al. (1988). The inoculum was mixed with autoclaved sand to achieve a density of 10 propagules/cm^3. A seedling was transplanted to infested soil in a 5-l pot grown under a greenhouse (22–36 °C). The experimental design was carried out in a completely randomized design (CRD) with two treatments (i) control treatment, no *P. nicotianae* infection and (ii) infection treatment, infested with *P. nicotianae*). The experimental pots were conducted on 10 replications and flooded every 5 days to maintain conductive conditions for pathogen activity by placing a dish under the pot and filling it with water. After 4 weeks, *P. nicotianae* infection was examined by culturing on the PARPH medium (Jeffers and Martin, 1986).

3.4.7 Influence of effective AM fungi on *Phytophthora* root rot

In this experiment, the inoculations of both selected AM species and *P. nicotianae* were conducted using citrus scion/roostock combinations. Shogun scions grafted on C-35 citrange rootstocks (4 months old) were grown in an autoclaved sandy soil. Shogun scion/C-35 citrange rootstock combinations were inoculated by 100 spores of selected AM strain. After 4 weeks of AM inoculation, Shogun scion/C-35 citrange rootstock combinations were infested with *P. nicotianae*

inoculum density of 10 propagules/cm^3 following the method described above. The experimental design was done in a completely randomized design (CRD) with six treatments and five replications consisting of control treatment, treatment inoculated with *A. tuberculata*; with *G. etunicatum*; with both *A. tuberculata* and *P. nicotianae*, with both *G. etunicatum* and *P. nicotianae*; and treatment inoculated only *P. nicotianae*. Plants were grown in a greenhouse and weekly watered with Hoagland's solution (Hoagland and Arnon, 1938). The citrus scion / roostock combinations were repeatedly inoculated with *P. nicotianae* inoculum every 4 weeks for 3 months. After 12 weeks of inoculations, plants were harvested and examined for *P. nicotianae* infection and AM colonization. *P. nicotianae* infection was considered by plating on the PARPH medium (Jeffers and Martin, 1986).

3.4.8 Influence of effective AM fungi on nutrient uptake

To test the effectiveness of AM fungi on commercial citrus cultivar (3 months old), Tangerine was used in this experiment. The soil used was sandy loam that was unfertilized and therefore low in nutrients. Soil properties showed a pH of 6.00, organic matter content of 0.72%, and CEC of 5.88 me/100 g. Soil nutrient contents were analyzed as; 3.59 ppm P, 0.18 me/100 g K, 3.18 me/100 g Ca, 0.007 me/100 g Mg, 22.23 ppm Fe, 16.57 ppm Mn, 1.87 ppm Cu, and 0.06 ppm Zn. The citrus was grown in 5 l pot containing sterilized infertile soil. Then, approximately 100 spores of AM fungi were inoculated according to the methods mentioned above. The treatments consisted of: (i) control treatment, no inoculation of both AM spores and fertilizer; (ii) fertilizer treatment (added N, P, and K); (iii) fertilizer with micronutrients treatment (added N, P, K, Fe, Mn, Zn and Cu); (iv) AM treatment (added selected AM); (v) AM and fertilizer treatment (added both AM and N, P, K, Fe, Mn, Zn, and Cu). The fertilizer treatment was added with nutrient solution which

consists of N (10.6 mmol/l No_3), P (1.5 mmol/l H_2PO_4), K (5.5 mmol/l K), Fe (20 µmol/l Fe), Mn (10 µmol/l Mn), Zn (3 µmol/l Zn) and Cu (0.5 µmol/l Cu). The experiment was designed as a completely randomized design (CRD) in five replications, and carried out under greenhouse condition for 3 months. After 3 months, AM colonization and leaf nutrient contents were measured. Citrus leaves were harvested, washed and dried at 65 °C for 48 h. Dried leaves were then ground and digested in acidic mixture solution (HNO_3–H_2SO_4–HClO). Samples were ashed at 220 C and resuspened in 20 ml 0.1 N HCl. Extracted phosphorus was examined by colorimetry (John, 1970). Data analyses were subjected by one-way ANOVA at 5% confidential value (Gianinazzi et al., 2002).

3.4.9 Molecular identification

3.4.9.1 DNA extraction

A single spore of morphologically identified spores was separated and used for molecular identification. The spore was washed with distilled water three times, then transferred into 0.5-ml microcentrifuge tubes containing 200 µl sterile 1% SDS in double distilled water and sonicated at 35 kHz for 30 s in an ultrasonic cleaner (Crest Tru-Sweep 1875T ultrasonic cleaner, USA). Then, the spore was washed again, transferred to a 1.5-ml microcentrifuge tubes on ice containing 1.5 µl sterile double-distilled water and used for DNA extraction within 1 h or stored at −80 °C for later use. The cleaned spore was crushed within the microcentrifuge tubes with a sterile pipette tip under a dissection microscope, and 200 µl Dynabeads solution (Dynabeads DNA Direct System 1, Dynal A.S., Oslo, Norway) was added immediately. The DNA extraction procedure by means of magnetic particles was performed following the instructions of the manufacturer. DNA was resuspended in 30 µl TE buffer, put on ice and then used as template for PCR (Schwarzott and

Schuβler, 2001)

3.4.9.2 PCR amplification of a partial LSU rDNA region

The primers LR1 (5′-GCA TAT CAA TAA GCG GAG GA-3′) and FLR2 (5′-GTC GTT TAA AGC CAT TAC GTC-3′) ([Trouvelot et al., 1999] and [van Tuinen et al., 1998]) were used for the amplification of the 5′ end of LSU rDNA sequences in fungi in general. A 25 µl reaction mix contained 2 µl 10× PCR buffer, 200 mM dNTPs, 500 nM each primer and 0.4 U Taq polymerase. An aliquot (2 µl) of spore DNA extract was added and run as follows: 93 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min (35 cycles), followed by 10 min at 72 °C. One microliter of first PCR products was used as templates for the second PCR with the primers FLR3 (5′-TTG AAA GGG AAA CGA TTG AAG T-3′) and FLR4 (5′-TAC GTC AAC ATC CTT AAC GAA-3′) (Engels, 1993) under the same PCR conditions. FLR3 is situated between the D1 and D2 domains of LSU rDNA while FLR4 is in the D2 domain. Then, PCR products were run on a 1% agarose gel in TAE buffer and visualized under UV light after staining with ethidium bromide.

3.4.9.3 Construction of LSU rDNA libraries and sequencing

The PCR products generated from AM spore using the primers FLR3 and FLR4 were cloned into the pGEM-T Easy Vector (promega) according to the instructions of the manufacturer. This ligation solution was then incubated at 4 °C to be used for cloning. Then, the −70 °C stored competent cells (DH5α) were thawed on ice. Thereafter, 3 µl of the ligation solution were added and gently mixed on ice, and this solution was followed by electroporation under the condition: field strength of 1.8 kV/cm; parallel resistance of 200 Ω; capacitance of 25 µF. Immediately after the pulse delivery, the cells were resuspended in 900 µl of SOC medium and shaken at 37 °C, 200 rpm in an incubator for 1h. After this procedure, the cells were ready to

be spread on SOB plates containing amplicillin and x-gal for blue/white screening. Plates were incubated overnight at 37 °C, and randomly white clones were picked and partly sequenced (ca. 380 bp) by Macrogen Sequence Laboratories, South Korea. Sequences were compared to known sequences using BLASTN and were deposited in the EMBL database (http://www.ncbi.nlm.nih.gov).

3.4.10 Statistical analysis

The experiment was conducted during December 2005–February 2009. The data were analyzed using analyses of variance (ANOVA). Statistical analyses were performed with SPSS software (ver. 13.0 SPSS Inc. 1989–2004). Significant differences between means were analyzed post hoc with Duncan's multiple-range test ($P<0.05$).

3.5 Results

3.5.1 Indigenous AM fungi isolation

After AM spore collection from citrus orchards, the spore abundances were counted as the total of spore numbers/20 g soil as recorded in Table 3.1. The highest spore abundance was found in the Northern region, Chiangmai: orchard 1 and 3 whereas the least one was in the Northeastern region, Nakhon Ratchasima: orchard 4. Spore morphotypes isolated from citrus orchards were identified based on morphological characteristics and counted for each morphotype as shown in Figure3.1 and Table 3.1. A total of 13 morpho-species in 6 genera were identified; 7 species in *Glomus*, 2 species in *Acaulospora*, 1 species in *Entrophospora*, 1 species in *Scutellospora*, 1 species in *Gigaspora*, and 1 species in *Sclerocystis* that could not be matched with species descriptions in INVAM. The most AM species abundances

were found in Chiangmai province; *G. etunicatum*, *Glomus caledonium*, *Glomus aggregatum*, *Glomus microaggregatum*, *Glomus sinuosum*, *A. tuberculata*, *A. scrobiculata* and *Entrophospora colombiana.* There were two species, *G. etunicatum* and *A. tuberculata*, that were commonly observed in all sampling sites while *Gi. calospora* and *Sclerocystis* sp. were so restricted in distribution since they were found in only the Northeastern region.

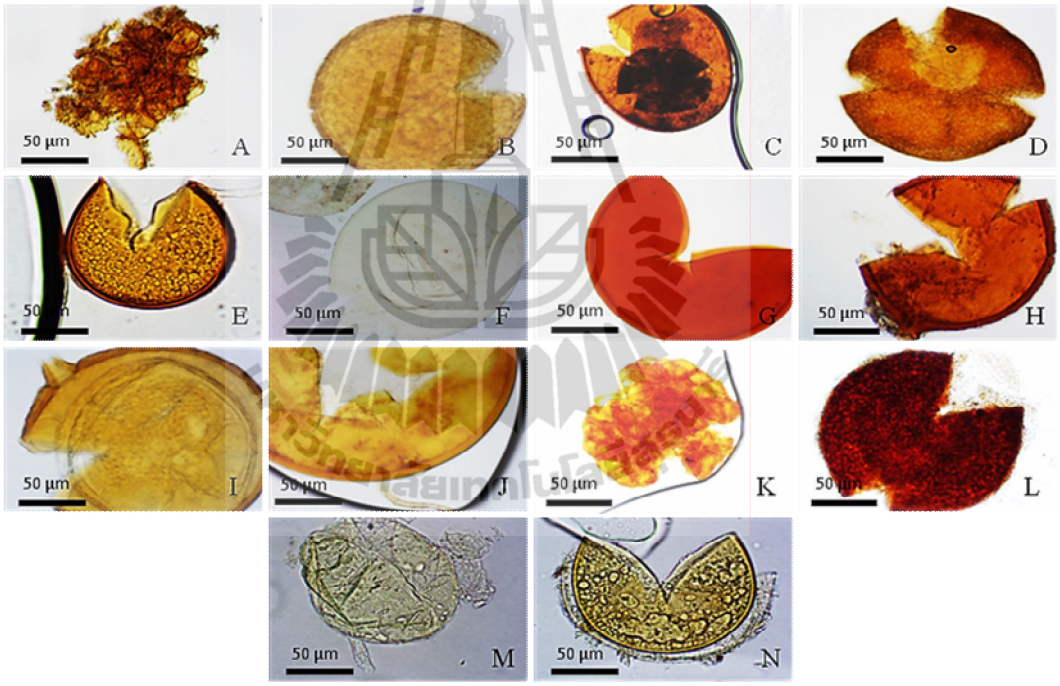

Figure 3.1 Spores of AM fungi cracked open and mounted under a microscope cover slide isolated from citrus orchards. A) *G. microaggregatum* B) *G. aggregatum* C) *Entrophospora colombiana* D) *G. sinuosum* E) *G. intraradices* F) *A. scrobiculata* G) *A. tuberculata* H) *G. etunicatum* I) *G. clarum* J) *Gi. calospora* K) *Sclerocystis* sp. L) *G. aggregatum* M) *A. trappei* and N) *G. intraradices* stained by polyvinyl alcohol-lactic acid-

glycerin (PVLG) mixed with Melzer's reagent (1:1 vol/vol). scale bar = 50 µm

3.5.2 Spore propagation and trap cultures

To increase spore numbers, the pot culture method was used to propagate 13 different AM morpho-species. Thirty spores of each AM isolate were inoculated into each pot. After 12 weeks of trap pot culture, the increment of newly produced spores was revealed in each host plants (sorghum, maize, and leek) as data recorded in Table 3.2. The best host plants for AM spores proliferation seemed to differ from individual species. Host plant was an important determinant of spore abundance of two AM species, *G. etunicatum* and *A. tuberculata*. *G. etunicatum* showed preferences for sorghum and maize with the highest spore numbers (86.30 and 93.60 spores/100 g soil respectively) while the spores of *A. tuberculata* thrived the best under host plant with leek (56.20 spores/100 g soil). The roots from trap pot culture assessed for AM percentage colonization were listed in Table 3.2. Significant differences in mycorrhizal colonization rate between AM species were detected under microscopes. The highest root colonizations were observed in sorghum, maize and leek roots that were inoculated with *A. tuberculata* (53.90%, 62.67%, 33.23% respectively), and the second highest colonizations were detected in three host plants inoculated with *G. etunicatum* (50.31% in sorghum root, 60.40% in maize root and 28.46% in leek root).

3.5.3 Mycorrhizal association on citrus seedlings

The citrus roots of three cultivars (Tangerine, Shogun and C-35 citrange) from pot experiment were assessed for AM association. On the basis of citrus

seedling colonization, significant differences in AM colonization rate between 13 different AM species in each citrus cultivar were observed and shown in Table 3.3. Statistical results indicated that there were interactions in AM colonization rate between citrus cultivars and AM species. For citrus cultivars, shogun was the best citrus cultivar that could be colonized by all AM species. Ranking the colonization rate of AM species in citrus roots indicated that *A. tuberculata* were the best AM species that could colonize all citrus cultivars, followed by *G. etunicatum*, *A. scrobiculata*, *G. caledonium*, *G. aggregatum* and *G. clarum*, while the lowest colonization rates were found in *Sclerocystis* sp. and *Gi. calospora*. Comparing 13 AM species, shogun root inoculated with *G. etunicatum* or *A. tuberculata* or *A. scrobiculata* showed the best colonization rate (60.13%, 62.36%, and 60.10%, respectively) whereas *Sclerocystis* sp. revealed the least colonization rate (17.68%). Similar to shogun, tangerine roots inoculated with *G. etunicatum* or *A. tuberculata* provided significantly the highest percentage of colonization at 50.73 and 50.17, while the lowest colonization rate was apparently observed in the tangerine roots colonized by *S. gregaria* and *Sclerocystis* sp. For C-35 citrange root colonization, the greatest rate was produced in *A. tuberculata* at 70.89%, followed by *G. etunicatum*, *A. scrobiculata*, and *G. caledonium*. Observation made in this experiment indicated the effectiveness of two AM species, *G. etunicatum* and *A. tuberculata*, for colonization in all citrus cultivars. We selected, therefore, those species to use for further experiment.

3.5.4 Influence of AM fungi on citrus growth

In order to screen the effectiveness of AM fungi on citrus growth, two AM species, *G. etunicatum* and *A. tuberculata*, were selected to test with commercial

citrus rootstocks [Shogun, C-35 citrange, and Tangerine]. After 4 months under greenhouse condition, all parameters, i.e. mycorrhizal efficiency index (MEI), mycorrhizal colonization, P content, and growth (height, root fresh weight (g), stem fresh weight (g), total fresh weight (g), root length (cm), root dry weight (g), stem dry weight (g), and total dry weight (g)) of citrus rootstocks inoculating AM fungi, were analyzed and compared between treatments (Table 3.4). Means were calculated by a Duncan's test, and levels of significance ($P<0.05$) were summarized in Table 3.4.

The results revealed that combinations of different hosts (citrus cultivars) and AM species influenced outcomes of growth pattern. Statistical analysis demonstrated that there were interactions in MEI and AM colonization rate between citrus cultivars and two AM species (*A. tuberculata* and *G. etunicatum*). Citrus cultivars, shogun, provided the greatest MEI and colonization rate in both AM species. In case of AM species consideration, we found that AM species, *G. etunicatum*, gave higher MEI and colonization rate than *A. tuberculata* in all citrus cultivars. Tangerine inoculated with *G. etunicatum* significantly showed highest percentage of MEI at 39.88. For AM colonization rate, shogun inoculated with *G. etunicatum* produced the highest colonization rate (59.67%) when compared with others. In case of P contents, Tangerine and C-35 citrange leaves showed the highest P contents in the plants amended with fertilizers and *G. etunicatum* spores.

For the part of citrus growth, statistical results indicated that the parameters, i.e. height, root length, and root dry weight showed interactions between citrus cultivars (shogun, tangerine, C-35 citrange) and treatments (control, fertilizer, *A. tuberculata*, and *G. etunicatum*). For citrus cultivars, C-35 citrange exhibited the highest height, root length, and root dry weight in all treatments. Considering the

treatments, *G. etunicatum* revealed the best height, root length, and root dry weight in all citrus cultivars. The results presented that the height of shogun, Tangerine and C-35 citrange differed between treatments (P<0.05). Ranking the height of citrus demonstrated that C-35 citrange cultivars inoculated with *G. etunicatum* produced the best height, followed by the control treatment, *A. tuberculata* and the fertilizer treatment. There was a significant difference (P<0.05) of root length in all citrus cultivars. The highest root length was measured at 44.66 cm in control treatment of C-35 citrange cultivar and at 42.00 cm in C-35 citrange cultivar inoculated with *A. tuberculata*, followed by *G. etunicatum* (37.33 cm), tangerine inoculated with *G. etunicatum* (38.43 cm), and with *A. tuberculata* (34.23 cm). For the root dry weight, three citrus cultivars showed a significant difference (P<0.05) between the treatments. The highest root dry weight was found in tangerine and shogun roots inoculated with *G. etunicatum* (6.10 and 4.80 g respective), and also detected in C-35 citrange roots inoculated with *A. tuberculata* (5.04 g). The results of root fresh weight showed that there was a significant difference (P<0.05) in all citrus cultivars. The highest root fresh weight was observed at 23.60 g in tangerine roots inoculated with *G. etunicatum*. Considering citrus cultivars, tangerine cultivar rendered the best stem fresh weight, total fresh weight, stem dry weight, and total biomass in all treatments (control, fertilizer, *A. tuberculata*, and *G. etunicatum*). Tangerine inoculated with *G. etunicatum* showed the highest stem fresh weight, total fresh weight, stem dry weight, and total biomass (128.20, 151.80, 85.13, and 91.23 g, respectively).

Table 3.4 Means of mycorrhizal efficiency index (MEI), arbuscular mycorrhizal (AM) colonization, phosphorus (P) content in leave and growth parameters in three citrus cultivars, Shogun (*C. reticulata* Blanco cv. Shogun), Tangerine (*C. reticulata*) and C-35 citrange (*C. sinensis* ×*P. trifoliate*) that were inoculated with arbuscular mycorrhizal (AM) fungi (*n*=5).

Cultivar	Treatments	MEI (%)	AM colonization (%)	P content (g /100 g dry wt)	Height‡ (cm)	Root fresh weight (g)	Stem fresh weight (g)	Total fresh weight (g)	Root length (cm)	Root dry weight (g)	Stem dry weight (g)	Total biomass (g)
Shogun	Control	NA*	ND**	0.075^{abc}(0.02)	2.67^{f}(0.09)	11.90^{f}(0.42)	66.73^{f}(0.55)	78.63^{e}(0.84)	24.00^{e}(0.35)	3.06^{f}(0.08)	30.10^{e}(0.34)	33.16^{e}(0.12)
	Fertilizer	NA	ND	0.076^{abc}(0.02)	3.57^{e}(0.10)	5.50^{ef}(0.16)	122.93^{b}(1.50)	128.43^{b}(1.08)	15.26^{g}(0.21)	3.13^{f}(0.07)	68.56^{b}(0.24)	71.70^{b}(0.40)
	A. tuberculata	30.22^{gf}(0.32***)	48.90^{b}(0.13)	0.075^{abc}(0.01)	4.00^{e}(0.17)	11.23^{e}(0.23)	77.83^{e}(1.00)	89.06^{d}(0.65)	19.86^{f}(0.36)	3.13^{f}(0.20)	39.06^{d}(0.44)	42.20^{d}(0.25)
	G. etunicatum	33.00^{a}(0.55)	59.67^{a}(0.32)	0.074^{abc}(0.01)	5.16^{d}(0.13)	12.13^{d}(0.65)	52.93^{g}(1.05)	64.40^{f}(0.61)	29.80^{d}(0.58)	4.80^{bc}(0.09)	29.93^{e}(0.04)	34.06^{e}(0.36)
Tangerine	Control	NA	ND	0.038^{c}(0.01)	3.74^{e}(0.08)	3.40^{ef}(0.23)	102.10^{c}(0.52)	105.50^{c}(0.50)	15.23^{g}(0.08)	4.23^{de}(0.12)	50.33^{c}(0.12)	54.56^{c}(0.06)
	Fertilizer	NA	ND	0.125^{a}(0.03)	2.97^{f}(0.07)	3.16^{ef}(0.27)	127.46^{a}(0.88)	130.63^{b}(0.99)	12.33^{g}(0.42)	1.06^{g}(0.04)	72.86^{b}(0.20)	73.93^{b}(0.09)
	A. tuberculata	13.93^{c}(0.01)	49.00^{b}(0.35)	0.028^{c}(0.01)	1.42^{h}(0.07)	9.53^{e}(0.33)	91.00^{d}(0.85)	104.43^{c}(0.77)	34.23^{c}(0.24)	5.13^{b}(0.04)	51.73^{c}(0.17)	56.86^{c}(0.12)
	G. etunicatum	39.88^{a}(0.01)	49.16^{b}(0.65)	0.124^{a}(0.03)	2.19^{g}(0.09)	23.60^{a}(0.67)	128.20^{a}(1.20)	151.80^{a}(0.38)	38.43^{b}(0.14)	6.10^{a}(0.22)	85.13^{a}(0.34)	91.23^{a}(0.08)
C-35 citrange	Control	NA	ND	0.035^{c}(0.01)	7.16^{b}(0.06)	18.03^{b}(0.17)	26.92^{h}(0.62)	44.96^{g}(0.41)	44.66^{a}(0.24)	4.65^{cd}(0.08)	8.57^{f}(0.04)	13.23^{g}(0.11)
	Fertilizer	NA	ND	0.121^{a}(0.03)	5.66^{c}(0.19)	10.52^{d}(0.50)	25.54^{hi}(0.64)	36.06^{g}(0.07)	33.33^{c}(0.27)	3.90^{e}(0.13)	13.34^{f}(0.14)	17.25^{f}(0.08)
	A. tuberculata	20.52^{b}(0.06)	49.23^{b}(0.06)	0.049^{bc}(0.01)	6.00^{c}(0.35)	15.79^{b}(0.20)	23.37^{i}(0.90)	39.16^{g}(0.68)	42.00^{a}(0.22)	5.04^{bc}(0.37)	10.96^{f}(0.31)	16.01^{f}(0.17)
	G. etunicatum	17.67^{b}(0.03)	44.93^{b}(0.32)	0.112^{ab}(0.04)	7.83^{a}(0.09)	13.19^{b}(0.23)	19.28^{i}(1.32)	32.48^{g}(0.41)	37.33^{b}(0.12)	4.33^{de}(0.05)	8.83^{f}(0.12)	13.15^{g}(0.06)

† Means under each parameter followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple-range test.

‡ Indicated the height after inoculation [(Height after inoculation) – (Height before inoculation)]

* Not analyzed

** Not detected

***Values in the parentheses indicate standard error (SE)

3.5.5 *Phytophthora* isolation and infection of *P. nicotianae* in Shogun scions

In *Phytophthora* isolation from citrus orchards, the features of sporangium and chlamydospores were identified as *P. nicotianae* by the methods described by Mitchell and Kannwisher-Mitchell (1993). In order to test and confirm infection of *P. nicotianae*, Shogun seedlings were infested with *P. nicotianae*. After 4 weeks of *P. nicotianae* inoculation, the symptoms of *Phytophthora* root rot were observed on all pots that were infested with *P. nicotianae*. Below ground, bark tissue showed a decay around the wound site. Roots demonstrated a decay, a yellowish-brown discoloration of tissue, and epidermis of rootlets were sloughed off. A symptom of die back was also detected at above ground (data not shown). Hence, it clearly revealed that the isolated *P. nicotianae* was virulent and could cause similar symptoms to those observed in the orchards.

3.5.6 Influence of effective AM fungi on *P. nicotianae* root rot

In this experiment, both AM species (*A. tuberculata* or *G. etunicatum*) and/or *P. nicotianae* were inoculated to citrus roots of Shogun scion/C-35 citrange rootstock combination. After AM species (*A. tuberculata* or *G. etunicatum*) inoculations and *P. nicotianae* inoculation later on, we found that the roots of C-35 citrange rootstocks were not heavily infected and the disease symptoms remain localized and could not be seen externally. Therefore, root visualization was used to compare between the inoculated treatment and control treatment. In addition to external visualization, root dry weight (g), P content (g/100 g dry wt) and AM colonization (%) were also examined as listed in Table 3.5. Visualization of AM citrus roots stainedwith 0.03% trypan blue was observed as shown in Figure 3.2. The citrus rootstocks inoculated by both AM strains (*A. tuberculata* or *G. etunicatum*) and

P. nicotianae were compared to the treatment inoculated only with *P. nicotianae*. The rootstocks infected by both *P. nicotianae* and AM fungi showed root injury at low level. The rootstocks inoculated with AM strain showed healthy roots which were more straight and longer than those of *P. nicotianae* treatment. The data presented in Table 3.5 indicated no significant difference of the root dry weight in all of the treatments. The means recorded were in the range of 2.6–4.1 g. There were the greatest P content and percentage of AM colonization in C-35 citrange rootstock roots inoculated with *G. etunicatum* while treatment infected with *P. nicotianae* provided the lowest P concentration. C-35 citrange roots colonized by both *G. etunicatum* and *P. nicotianae* showed significantly lower P content and AM colonization than those colonized only with *G. etunicatum* (Table 3.5).

Table 3.5 Influence of *Phytophthora nicotianae* on root dry weight, phosphorus (P) content in leave and arbuscular mycorrhizal (AM) colonization in mycorrhizal and non-mycorrhizal citrus scion/rootstock combinations (*n*=5)

Treatments	**Shogun scion/C-35 citrange rootstock combination‡**		
	Root dry weight (g)	**P content (g /100 g dry wt)**	**AM colonization (%)**
Control	3.47[a†](0.08[**])	0.53[bc] (0.04)	ND*
A. tuberculata	3.10[a] (0.16)	0.55[bc] (0.06)	82.13[b] (1.78)
G. etunicatum	4.13[a] (0.14)	0.82[a] (0.04)	94.48[a] (0.78)
A. tuberculata × *P. nicotianae*	3.23[a] (0.14)	0.53[bc] (0.02)	63.46[c] (1.09)
G. etunicatum × *P. nicotianae*	4.10[a] (0.19)	0.68[ab] (0.08)	86.18[b] (1.37)
P. nicotianae	2.67[a] (0.24)	0.39[c] (0.06)	ND

† Means under each parameter followed by the same letter are not significantly different (P < 0.05) according to Duncan's multiple-range test.

‡ Shogun (*C. reticulata* Blanco cv. Shogun) scion grafted on C-35 citrange rootstock.

* Not detected

** Values in the parentheses indicate standard error (SE)

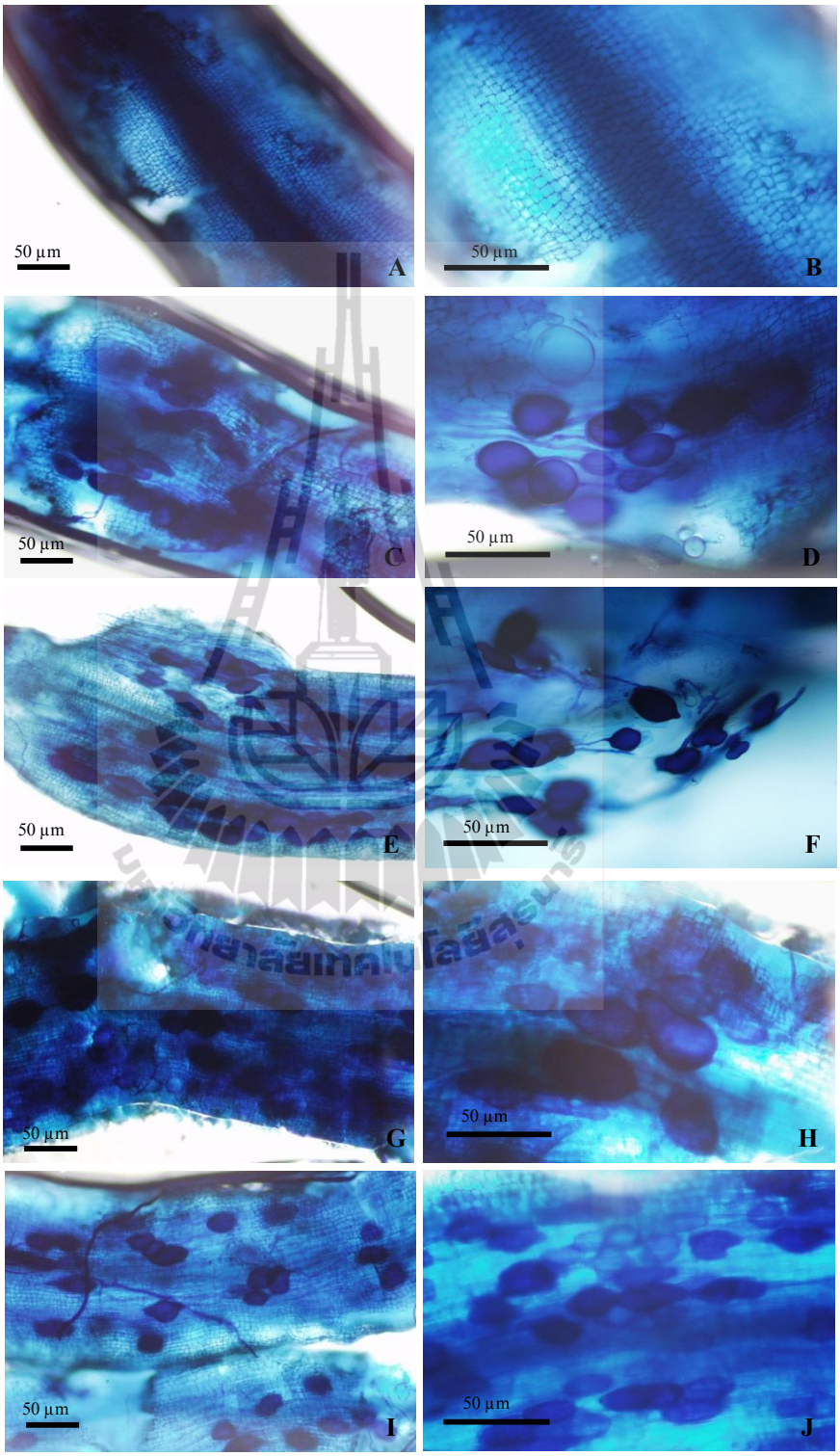

Figure 3.2 Roots of shogun scion/C-35 citrange rootstock combination colonized by AM fungi and *P. nicotianae* stained with 0.03% trypan blue; (A)-(B) control (C)-(D) roots colonized by *G. etunicatum*, (E)-(F) roots colonized by both *G.*

etunicatum and *P. nicotianae*, (G)-(H) roots colonized by *A. tuberculata*, (I)-(J) roots colonized by both *A. tuberculata* and *P. nicotianae*. (v: vesicle, scale bar = 50 μm)

Even though the roots were not heavily infected and the symptoms could not be seen externally, the part of Shogun scions (that grafted onto C-35 rootstock) provided interestingly different results. After 2 weeks of the third re-inoculation, the treatment inoculated only with *P. nicotianae* showed a severe symptom of shoot die back when compared with the control treatment, while plants inoculated with both *A. tuberculata* or *G. etunicatum* and *P. nicotianae* were observed to have lower level of symptoms than those inoculated only with *Phytophthora*. Plants inoculated with both AM and *Phytophthora* initially had low level of die back symptom on the scion shoot, and then newly budding young shoot was regenerated in them within 2 weeks (Figure3.3).

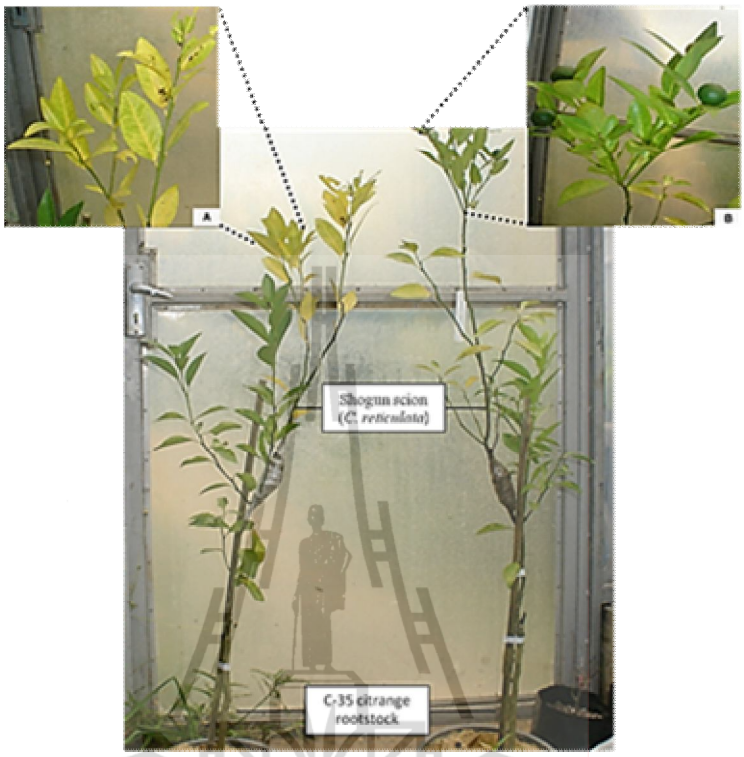

Figure 3.3 Young shoot restoration and root rot symptom of citrus scion (*C. reticulata* Blanco CV. Shogun) grafted on C-35 citrange rootstock; (A) citrus inoculated only with *P. nicotianae* show a symptom of shoot die back, (B) citrus inoculated with both *A. tuberculata* and *P. nicotianae* show a newly budding young shoot.

3.5.7 Influence of effective AM fungi on nutrient uptake

In this experiment, *G. etunicatum* and *A. tuberculata* were selected to test the effectiveness of AM fungi on commercial citrus, Tangerine. After 3 months under greenhouse condition, the parameters were analyzed and compared between treatments. Statistical analyses demonstrated the differences between treatments. Means were compared by a Duncan's test and levels of significance ($P<0.05$) of main treatments and their interactions were calculated. All results were summarized in

Table 3.6.

There was no significant difference in height in all of the treatments (data not shown). The citrus inoculated with *A. tuberculata* and fertilizer (NPK and micronutrients) provided the highest root colonization (58.82%) when compared with that of other treatments ($P<0.05$). In case of N content, the plants showed lowest N content in the control plants and the plants inoculated only with *G. etunicatum* or *A. tuberculata* spores, while the treatment amended with both AM spores (*G. etunicatum* or *A. tuberculata*) and fertilizer (NPK and micronutrients) and the treatment fertilized with NPK and micronutrients provided significantly higher N content. P content accumulation in citrus leaf tissues pointed out no significant differences ($P<0.05$) in all of treatments. The highest K contents were detected in the citrus leaves amended with both *A. tuberculata* spores and fertilizer (NPK and micronutrients), and in the citrus amended only with fertilizer (NPK and micronutrients). In the part of micronutrients (Fe, Mn, Cu, and Zn) absorption (Table 3.6), the citrus inoculated with both AM spores and fertilizer, and the citrus added only with fertilizer exhibited the higher accumulation level of Fe, Cu, and Zn than other treatments whereas Mn accumulation could not be detected in any citrus treatments.

Table 3.6 Effect of arbuscular mycorrhizal (AM) fungi on leaf nutrients accumulation in Tangerine (*C. reticulata*) citrus (*n*=5)

Treatments	Tangerine (*C. reticulata*)							
	AM colonization (%)	N (%)	P (%)	K (%)	Fe (ppm)	Mn (ppm)	Cu (ppm)	Zn (ppm)
Control	15.33^{dt} (0.67**)	0.05^{b} (0.02)	0.04^{a} (0.01)	0.09^{c} (0.04)	2.50^{b} (0.74)	ND*	0.00^{b} (0.00)	1.51^{c} (0.19)
G. etunicatum	55.43^{b} (0.53)	0.05^{b} (0.008)	0.09^{a} (0.006)	0.05^{c} (0.01)	6.05^{b} (0.83)	ND	0.04^{b} (0.03)	1.82^{c} (0.12)
A. tuberculata	56.08^{ab} (0.67)	0.27^{b} (0.05)	0.11^{a} (0.04)	0.09^{c} (0.03)	5.08^{b} (0.97)	ND	0.03^{b} (0.02)	1.75^{c} (0.26)
G. etunicatum × NPK × micronutrient	51.97^{c} (0.66)	0.78^{a} (0.07)	0.13^{a} (0.03)	0.90^{ab} (0.20)	18.30^{a} (2.67)	ND	0.20^{ab} (0.05)	6.52^{c} (0.47)
A. tuberculata × NPK × micronutrient	58.82^{a} (1.23)	0.87^{a} (0.09)	0.15^{a} (0.04)	1.13^{a} (0.11)	18.61^{a} (2.80)	ND	0.33^{a} (0.08)	17.63^{b} (3.05)
NPK × micronutrient	15.31^{d} (0.70)	0.94^{a} (0.13)	0.10^{a} (0.02)	1.11^{a} (0.08)	15.41^{a} (3.50)	ND	0.24^{ab} (0.12)	40.36^{a} (3.12)
NPK	15.55^{d} (0.80)	0.68^{a} (0.10)	0.11^{a} (0.02)	0.63^{b} (0.06)	1.97^{b} (0.18)	ND	0.014^{b} (0.007)	2.96^{c} (0.40)

† Means under each parameter followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple-range test.

* Not detected

** Values in the parentheses indicate standard error (SE)

3.5.8 Molecular identification

Two of AM isolates investigated led to a weak amplification product of about 720 bp visible after the first PCR (primers LR1/FLR2). In the second, nested PCR was necessary to obtain a visible product. All these trials were successful with respect to the amplification of the expected fragment. A nested PCR with the primers FLR3/FLR4 could amplify product of about 380 bp. PCR products were used to construct LSU rDNA libraries from AM spore, and randomly selected clones from each library were sequenced. The most similar sequences were obtained from the sequence database in NCBI using BLAST and the sequences were grouped into *Glomeromycete*. AM fungi sequences, *G. etunicatum* and *A. tuberculata*, have been submitted to the GenBank database under the following accession numbers: FJ687414 (72% similarity) and FJ687415 (71% similarity), respectively.

3.6 Discussion

In order to determine whether different AM species can produce different effects of host (citrus cultivars) growth, our experiments thus were conducted by inoculations of two AM species (*A. tuberculata* and *G. etunicatum*) into the roots of three citrus cultivars (C-35 citrange, Shogun and Tangerine). From this study, *G. etunicatum* showed high percentage of MEI in Shogun and Tangerine citrus, which suggests it to be an effective AM strain. Mycorrhizal efficiency index (MEI) is the index that indicates the potential of AM fungi affecting the citrus growth. For root colonization, both *A. tuberculata* and *G. etunicatum* could colonize the roots of citrus cultivar in this study. AM colonization is usually considered to relate with P content in plant tissue. Normally, P content has the positive effect on root colonization. However, some data of citrus cultivars from this experiment showed no difference between the treatments. This suggests the loss of function of the direct uptake pathway in roots colonized by AM fungi, which can apparently be completed in some AM symbioses. This is in agreement with a previous report (Pearson and Jakobsen, 1993), which indicates a similar effect of *G. caledonium* to reduce the direct uptake capacity of cucumber (*Cucumis sativus*) roots. On the citrus growth, the tangerine root colonized with *G. etunicatum* demonstrated the highest stem dry weight and total dry weight. The highest root dry weight was found in Shogun and Tangerine roots inoculated with *G. etunicatum*. This was suggested that mycorrhizal inoculation appeared effective in improving nutrient content and plant growth. It is well known that AM fungi enhances nutrient uptake, as this fungal symbiosis increases the abilities of the host plants to explore a larger volume of soil than roots alone and to take up phosphate from a greater surface area surface area (Jakobsen et al., 1992; Joner et al., 2000).

Here, we present results from using of traditional spore morphology and molecular-based techniques to determine AM identities. AM spores identified by morphological technique may pose problems and limitation, because morphological characters are scarce in some AM. Furthermore, a great deal of training and expertise of researcher are needed to accurately establish AM identification. Molecular techniques thus were used together with spore-based techniques, because they have the potential to identify some AM that cannot be discerned by spore morphology. For molecular identification, length of PCR products obtained by the nested PCR protocol were about 380 bp in both AM species. The FLR4 primer has a perfect match only with glomalean LSU rDNA sequences and although the FLR3 primer could recognize DNA from some Basidiomycetes as well as from *Glomales*, this primer pair only resulted in the amplification of *Glomales* sequences. The extent of variability of the D1-D2 region of rDNA enabled not only analysis of AM fungal biodiversity by systematic sequencing but also the design of taxon discriminating primers, which could then be used to monitor AM fungi in roots from the field (Clapp et al., 2001; Rodriguez et al., 2001).

As well as citrus growth, combination of AM fungi with *P. nicotianae* could reduce severity of disease symtoms in roots of C-35 citrange rootstocks (see Section 3.5). In the experiment of the *Phytophthora* suppression, it was found that severity of damage by *Phytophthora* was reduced by AM fungi inoculation. For example, consistent with results from many studies, Davis and Menge (1980) demonstrated the reduction of *P. nicotianae* root rot severity in citrus seedlings infected with *G. fasciculatum*. Cordier et al. (1996) also showed that *Phytophthora* development is reduced in AM fungal-colonized and adjacent uncolonized regions of AM root

systems, and that in the former the pathogen does not penetrate arbuscule-containing cells. This means that localized competition occurs, and that even in the absence of systemic resistance, resistance was still induced at some distance from the AM-colonized tissue. In the experiments of AM and *Phytophthora* (Table 3.5), treatments inoculated only with AM provided the higher level of AM colonization than treatments provided with both AM and *Phytophthora*. A similar result was also found in case of P concentrations, the citrus inoculated only AM (*G. etunicatum*) provided the higher P concentration than mycorrhizal citrus infected with *Phytophthora*, uninoculated control, and nonmycorrhizal citrus inoculated with *Phytophthora* alone. There were similar results regarding P concentrations reported by Davis et al. (1978). They revealed that P levels were obviously higher in citrus mycorrhizal seedlings than in nonmycorrhizal seedlings, seedlings inoculated with *Phytophthora* alone, or seedlings inoculated with both *Phytophthora* and *G. fasciculatus*. These results indicate the ability to disrupt AM colonization by *Phytophthora*. This is possible to both pathogen (*P. nicotianae*) and symbiont (AM fungi) to probably compete for the same space and nutrients, therefore the reduction of AM colonization results in the decrease of P concentration.) also suggested that the low P levels measured in mycorrhizal citrus plants infecting *Phytophthora* indicate that mycorrhizal symbiosis may be damaged by *Phytophthora* infections. *Phytophthora* may reduce the inoculum potential of *G. fasciculatus* in the soil, so subsequent colonization by these beneficial fungi also may be reduced. The current results gave us the basic conclusions that different combinations of host (citrus cultivars) and AM species provide different outcomes of growth and pathogen resistance. Similarly, Herre et al. (2005) indicated that the ability of different AM species produced different host protection (defense)

level and promoted different host growth patterns.

From our results, it is implied that citrus perform different response to *P. nicotianae*. Nevertheless, the roots of C-35 citrange rootstock inoculated with *P. nicotianae* remained to tolerate infection and reproduction by pathogen whereas susceptible Shogun scion roots had more severe infection. Fungal resistant compounds in roots have been accepted to reduce the rate of invasion of *Phytophthora* spp. into cortical and vesicular tissues, allowing roots to heal and regenerate (Graham, 1995). Consistent with studying of AM–host–pathogen relationship (Herre et al., 2007), the results reported that AM inoculations showed significantly reduced damage in the leaves due to *Phytophthora palmivora* in *Theobroma cacao*. They have discussed that AM colonization can enhance plant defense against foliar pathogens. They have also suggested that the potential mechanisms influence the outcome of host pathogen interactions. There are indirect effects of mechanisms that can contribute to defense responses. In addition to the possibility that AM-derived products are translocated to the leaf, the anti-pathogen effects observed in the leaves are probable due to indirect effect (Herre et al., 2007). They also pointed out discussions that it is probably because there are enhancing host strength (for defense) due to more ability to approach nutrients.

For citrus cultivars selection, the combination of scion/rootstock use were considered to test AM and *Phytophthora* experiments. C-35 citrange rootstock is more tolerant to most of soil borne diseases and pest particularly to tristeza virus than other cultivars, less susceptible to *Phytophthora* root rot than Troyer citrange, tolerant to the citrus nematode (*Tylenchulus semipenetrans*) and also provides the good fruit quality (Australian Citrus Propagation Association Incorporated (ACP),

2005).Rootstocks are referred to as being tolerant rather than resistant because fibrous roots become infected under inoculations and in infected nursery and orchard soils (Broadbent, 1969; Carpenter and Furr, 1962; Graham, 1990; Agostini et al., 1991). Tolerance was previously defined as the condition in which plants are infected but show little or no root weight loss because either the infected roots do not rot or the root mass density is maintained by root regeneration (Graham, 1990). A previous study revealed that most commercial rootstocks in Florida were intolerant to root rot caused by *P. nicotianae*, while trifoliate orange (*Poncirus trifoliate* (L.) Raf.) and its hybrid, Swingle citrumelo (*C. paradise* Macf. × *P. trifoliate*), were considered tolerant (Graham, 1990). In Florida rootstock, trifoliate orange and Swingle citrumelo supported lower soil populations of *P. nicotianae* (Agostini et al., 1991). It is still unclear whether rootstocks that support lower populations suffer less direct root loss because their roots are resistant to infection or whether tolerant rootstocks have a greater capacity to regenerate roots, or both mechanisms are co-operative. In *Phytophthora* experiment, our data indicated no significant difference of the root dry weight in all of the treatments. This result might be because the rootstocks have the ability to regenerate roots in the presence of *P. nicotianae*. The resistance factors, however, that limit infection to the root tip are not known. Our results were similar to the previous research conducted by Graham (1995) in which tolerance of citrus rootstocks was identified as the capability to regenerate fibrous roots in greenhouse and field soils infected with *P. nicotianae*. They suggested that since the expression of tolerance by trifoliate orange was not strongly related to root growth potential in the absence of the pathogen, tolerance may be more related to biochemical resistance as observed for other citrus tissues (Afek and Sztejnberg, 1988; Sulistyowati et al.,

1990). However, the rootstocks inoculated only with *P. nicotianae* showed an unexpected result of a severe symptom of shoot die back. Nevertheless, no severe root rot symptom was found in the citrus rootstocks. These results may be a consequence of fungal populations in the soil that are maintained by several repeated *Phytophthora* infections. *Phytophthora* cycle can repeat itself as long as conditions are favorable and susceptible tissues are available. We also initially found low level of die back symptom on the scion shoot in citrus inoculated with both AM fungi and *Phytophthora*, and then newly budding young shoot was regenerated. The unexpected results may be because of the ability of AM fungi to restore shoots. Although most commonly scion cultivars are susceptible to bark infection, most rootstocks are at least moderately tolerant. However, though the use of resistant rootstocks is the best solution for control of *Phytophthora* disease, some highly resistant rootstocks are susceptible to other diseases. Moreover, there is not always a good correlation between tolerance to foot rot and to root rot. For instance, Carrizo citrange and sour orange are tolerant to foot rot, but are susceptible to root rot. The mechanisms of resistance of different citrus tissues to *Phytophthora* are little understood. Because of the great differences in the type of tissue affected and the response of different citrus species to infection, there is probably more than one resistance and/or tolerance mechanism involved (*Phytophthora* diseases of citrus, one of a series of the Soil and Water Science Department, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida, linked on http://edis.ifas.ufl.edu).

In the experiment of micronutrients accumulation, Mn accumulation was not found. This might be caused by the negative interactions between P and Mn. Mn cation has very low solubilities with phosphate, resulting in precipitation which limits

P and Mn availability in the soil and plant. In addition, Kothari et al. (1991) reported that AM affected the microbial populations in the rhizosphere, inhibiting the growth of Fe- and Mn-reducers and consequently diminishing the availability of Mn. The higher levels of Fe, Cu, and Zn in the citrus inoculated with AM and fertilizers indicated that the absorption and assimilation of these relatively immobile micronutrients might be enhanced in root system that supported mycorrhizal colonization. The enhanced accumulation of these micronutrients affects vital physiological processes in citrus, which impact yield and disease resistance (Sena et al., 2002).

3.7 Conclusion

Mycorrhizal fungi differ in their ability to improve growth of each citrus cultivar. There are different responses of growth between citrus cultivars and particular AM species. Davis and Menge (1981) reported that the host plant was a factor affecting the interaction between mycorrhizal fungi and *P. nicotianae*. They suggested that the influence of AM fungi on *Phytophthora* root rot should be determined for each host–AM fungi–pathogen system. However, it is difficult to reach practical conclusions, because of the complexity of the microbe–soil–plant system and influence of environment conditions. Thus, further research in applying AM fungi to improve plant growth and to be the biological control of target diseases in agrosystems, particularly with horticultural crops in the field, is needed.

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CHAPTER IV

EFFECT OF RICE CULTIVATION SYSTEM ON INDIGENOUS ARBUSCULAR MYCORRHIZAL FUNGAL (AM) COMMUNITY STRUCTURE

4.1 Abstract

AM in an agricultural ecosystem is necessary for proper management of beneficial symbiosis. In this study, we explored how AM community structure (based on T-RFLP derived from 18S rDNA) in rice root (*Oryza sativa* L.) were affected by rice cultivation systems (the system of rice intensification (SRI) and the conventional rice cultivation system (CS)), and by applying of compost and plant-growth-promoting rhizobacteria (PGPR) (*Azospirillium largimobile* and *Azotobacter vinelandii*) during different growth stages. Prior to investigate the AM community structure in the rice field, mimic of AM in both CS and SRI systems were carried out in rice pot containing sterilized vermiculite. The results indicated that the inoculation with either *Acaulospora tuberculata* or *Glomus etunicatum* significantly increased total dry weight of rice from both CS and SRI systems. The mycorrhizal responsiveness (MR) exhibited no significant differences between cultivation and between both AM species. The average mycorrhizal responsiveness based on plant dry weight, was ranged between 163.77 % and 167.04 % for *A. tuberculata*, and 116.17% and 163.33% for *G. etunicatum*, under CS and SRI cultivation managements, respectively. Ranking the colonization rates of AM species in both cultivation systems indicated that AM (either *A. tuberculata* or

G. etunicatum) with rice grown in SRI produced the best colonization rate (29.38% or 28.46%, respectively), followed by *A. tuberculata* plants grown in CS (20.54%), while the lowest colonization rate was detected in plants inoculated with *G. etunicatum* under CS management (16.07%). In the rice field experiment, we found that all sequences had high levels of similarity (98% to 100% identity) to AM fungal sequences belonged to members of the phylum *Glomeromycota*, the genera *Glomus* and *Acaulospora*. Surprisingly, our results revealed that all AM sequences obtained from the rice roots under CS field plots belonged to the genus *Glomus*, no *Acaulospora* sequences appeared in the rice grown under this cultivation. In other words, the rice roots sown in SRI showed AM sequences belonged to both AM genera, *Glomus* and *Acaulospora*. AM community structure was compared across the different cultivation types (CS and SRI) and different PGPR application by principle component analysis (PCA). In the 2nd (60 days), the 3rd (90 days) and the 4th (120 days) of sampling times, the AM assemblages of conventionally managed system (CS) were separated from those of SRI management. The distribution of AM community composition based on T-RFLP data showed that the AM fungal communities structure associated with the different cultivation plots (CS and SRI) were separated from each other, while AM fungal community structures associated with the different compost and PGPR treatments (control, compost, and PGPR additions) were not clearly devided. In addition, it was also demonstrated that rice grown in SRI showed higher in Shannon-Weaver indices of diversity (*H'*) than rice plant grown under CS management. The results also revealed that there was an increase of *H'* of rice plants sown under SRI during growth stages (gradually increased from 30 days until 120 days of growth stage).This output indicated that rice plant grown under SRI had more diverse AM fungal community than that grown under CS condition.

ARBUSCULAR MYCORRHIZAL FUNGI/COMMUNITY STRUCTURE/PGPR /*Oryza sativa*/CULTIVATION SYSTEMS

4.2 Introduction

Rice (*Oryza sativa* L.) is one of the important crops grown extensively in many countries. Generally, rice is grown under shallow flooded or wet paddy conditions, but it is also cultured where floodwaters may be several meters deep. Therefore, rice appears to have a high water requirement. Another system for rice cultivation so called system of rice intensification (SRI) originated in Madagascar in early 1980s (Uphoff, 2002). SRI is composed of a set of practices that are used to cultivate rice, and these practices have now been adopted in over 20 countries (Satyanarayana et al., 2007). There are five main differences in paddy management when using SRI compared to conventional practices. These are: (i) transplanting younger seedlings, (ii) transplanting seedlings singly, (iii) using wider spacing, (iv) alternating soil flooding with draining during the vegetative growth phase, and (v) applying compost rather than mineral fertilizer. Increases in rice yields from 2 t ha^{-1} in conventionally managed paddies to 15–20 t ha^{-1} or higher for SRI-managed paddies have been reported (Uphoff, 2002). The mechanisms responsible for the dramatic yield increases obtained from these changes in management practices are still unclear. Several research projects have been undertaken to gain more understanding of the agronomic and scientific bases for the increased yields obtained (Stoop et al., 2002). Previous studies have demonstrated that the increase of root growth (Joelibarison, 2002), the addition of compost, and the presence of nitrogen fixing bacteria, nitrifying bacteria in the rice rhizosphere (Randriamiharisoa and

Uphoff, 2002; Sooksa-nguan et al. 2009) are factors in the increased yields observed in the SRI system. In SRI, paddies are alternately flooded and drained during vegetative growth in contrast to the conventional system where the paddies remain flooded throughout the growing season. Cycling of mineral nutrients, particularly of nitrogen (N), is strongly affected by oxygen (O_2) concentrations; hence, alternating wetting and drying of the soil is likely to strongly influence nutrient availability (Kirk, 2001). Oxygen, nitrate (NO_3^-), and other nitrogen species are introduced into the soil mainly by bulk flow, and as water moves down through the soil profile and those can be transformed by biological processes, many of which are mediated by soil organisms (Bothe et al., 2007). The transformation rates can lead to either removal or release of nitrogen, as determined by the balance among the processes of N mineralization, nitrification, denitrification and nitrogen fixation (Arth et al., 1998; Ghosh and Kashyap, 2003; Rich and Myrold, 2004; Bothe et al., 2007).

In literature, there are rare study providing an overview of the colonizing arbuscular mycorrhizal (AM) fungi in rice roots grown under SRI and there is still no clear picture of how the association may be exploited to benefit crop yield directly in fields. Nevertheless, this SRI systemcan create aerobic conditions in the soil that stimulate colonization of rice roots by AM and other fungi. This symbiosis may enhance the root system's ability to absorb and carry phosphorus and other soil elements of low mobility, by means of a network of mycelia, thus promoting plant growth. The upland rice (Gangopadhyay and Das 1982; Ammani et al. 1985; Brown et al. 1988) normally develop AM (Baby and Manibhushanrao 1992), but cultivation methods can have an impact on root colonization by AM fungi and on associated spore numbers in soils (Pozzebon et al. 1992). The growth of rice plants can be

increased by inoculation with AM fungi (Sanni 1976), and Dhillion (1992) observed increases in dry weight of rice plants irrespective of the associated fungal type.

Rice plants readily form mycorrhizal associations under upland conditions, but under submerged conditions infection is rare due to the anoxic environment (Ilag et al. 1987). Barea (1991) concluded, however, that AM are obligate aerobes in nature but can survive in waterlogged conditions, and this is supported by the fact that *Glomusetunicatum*, a AM, showed fairly high colonization in rice roots and best survival under submerged conditions.Some reports revealed AM growth in wetland rice under flooded conditions, and an increase in yield and promotion of nutrient acquisition in wetland rice by inoculation with AM in both high- and low-fertility soil were observed (Secilia and Bagyaraj 1992; Gupta and Ali 1993; Secilia and Bagyaraj 1994a, b; Solaiman and Hirata 1996). However, the dynamics of AM in wetland rice at different growth stages in high-fertility soil are unknown. It has been established that mycorrhizal colonization of crops increased plant growth in low-fertility soil (Jeffries 1987). However, some reports also revealed growth response to AM in high-fertility soil (Porter et al. 1978; Sylvia and Schenck 1983). In some studies, mycorrhizal rice has been investigated (IIag et al. 1987; Brown et al. 1988; Dhillion and Ampornpan 1990, 1992). Although the AM associations are generally less frequent in wetland rice cultivation than upland cultivation, some reports also indicated that wetland rice plants in submerged fields were highly colonized (Ilag et al. 1987; Sivaprasad et al. 1990; Secilia and Bagyaraj 1992). Sivaprasad et al. (1990) reported that grain and straw yields in rice plants inoculated with *G. faciculatum* were significantly higher than in non-mycorrhizal ones and concluded that AM inoculation by the dry nursery method may enhance AM benefits in lowland (wetland) rice

cultivation. Some studies have shown that plant growth and nutrition (P and Zn) of wetland rice were improved under pot culture conditions following inoculation with AM isolated from paddy and non-paddy soils (Sharma et al. 1988; Secilia and Bagyaraj 1992, 1994).

Arbuscular mycorrhizal (AM) fungi are probably the most important of other microorganisms. AM fungi play a key role in plant phosphorus supply, and in return the host plant provides carbon assimilates (Smith and Read 1997). AM association is a balanced complex system involving a three-way interaction among plant, fungal, and soil. Analysis of both this association and the changes in the population of colonized AM in an agricultural ecosystem is necessary for proper management of beneficial symbiosis. Growth responses of plants associated with AM may depend on the soil moisture, availability of nutrients, pH, AM species, host plant species, type of host root system, and growth stage of host plant (Dhillion and Ampornpan 1992). AM fungi also interact synergistically with other microorganisms such as nitrogen-fixing bacteria (Barea and Azcon 1983; Azcon 1989; Bagyaraj and Menge 1978; Brown and Carr 1984), phosphate-solubilizing bacteria (Azcon et al. 1976; Barea et al. 1975; Villegas and Fortin 2002), biocontrol agents (Barea et al. 1998; Budi et al. 1999; Edwards et al. 1998; Abdel-Fattah and Mohamedin 2000), and plant-growth-promoting microorganisms (Linderman 1988) to enhance plant growth and survival. AM fungi interact with these bacteria directly by providing niche and/or habitat (Bianciotto et al. 2000; Mansfeld-Giese et al. 2002; Walley and Germida 1997) or indirectly by modifying host physiology (Rambelli 1973). Several investigations have indicated that the rhizosphere bacteria also have a strong impact on growth of AM fungi (Linderman 1997). The plant-growth-promoting rhizobacteria (PGPR) have

been described as facilitating colonization of the plants by AM fungi, improving the development of the mycosymbiont, and reducing damage caused by soil-borne plant pathogens.Positive influences on the AM symbiosis after addition of PGPR, which include fluorescent pseudomonads and sporulating bacilli, are frequently reported. For example, dual inoculation of a PGPR (*Pseudomonas putida*) and an AM fungus induced an additive growth enhancement of subterranean clover when added together rather than singly (Meyer and Linderman 1986). Inoculation with the PGPR also increased root colonization by the AM fungi initially (i.e. measured at 6 weeks) although later (at 12 weeks) colonization levels were similar regardless of the presence of the PGPR (Meyer and Linderman 1986). Enhanced mycelial growth from *Glomus mosseae* spores by a PGPR has also been reported (Azcon 1987). Thus, PGPR appear sometimes to promote both mycorrhizal development and functioning. It is believed that the AM symbiosis relieves P stress for the plant which in turn has benefits for the N_2-fixing nitrogenase system of the other symbiont, resulting in enhanced fixation levels and consequently improved N status of the plant thus promoting plant growth and functioning which in turn also benefits mycorrhizal development (Bethlenfalvay 1992). The growth and development of arbuscular mycorrhizal fungi (AM) is affected by many environmental factors. The best documented of these is soil P supply, which correlates negatively with mycorrhizal growth response and with the extent of fungal colonization (Abbott et al. 1984; Bruce et al. 1994; Smith and Read 1997). Cultivation under different cultural practices and soil conditions is likely to affect growth, development and population of AM in the soil.

Understanding the factors that lead to the enormous diversity in communities of soil biota and the impacts on ecosystem functioning, therefore, is one of the major challenges in soil ecology (Fitter et al. 2005). A well studied group of soil organisms are the AM, which have important functions in ecosystems, as they influence nutrient cycling, plant productivity, and diversity (Van der Heijden et al. 1998a; Klironomos et al. 2000; Klironomos and Hart 2002; Smith and Read 2008). Less is known about the factors determining AM community structure colonizing plant roots (Johnson et al. 2004; Borstler et al. 2006). The presence of community structure of arbuscular mycorrhizal fungi (AM) can have important and well established consequences for plant community dynamics. AMaffect the outcome of plant–plant competition (Bray et al. 2003), plant community dominance (Grime et al. 1987), plant diversity, and ecosystem productivity (van der Heijden et al. 1998a, b).Plant host identity, plant priority, soil type, habitat fragmentation, and seasonality are all involved in structuring AM communities (Vandenkoornhuyse et al. 2002; Lekberg et al. 2007; Mangan et al. 2004; Pringle and Bever 2002). Different AM species are also active at different times of the year (Merryweather and Fitter 1998), and different plant species benefit from different AM (Ravnskov and Jakobsen 1995). These observations imply that diverse AM communities may enhance plant productivity and ecosystem functioning as observed in several studies (van der Heijden et al. 1998; Vogelsang et al. 2006).

In this study, we explored how AM community structures in rice root were affected by rice cultivation systems (the SRI and the conventional rice cultivation system (CS)), and by different growth stages. We also investigated whether root AM communities were altered by applying compost and PGPR (*Azospirillium largimobile*

and *Azotobacter vinelandii*). The hypothesis was that the relationships between rice cultivation systems and the additions of compost and PGPR drive patterns of root-associated AM colonization and community structure during rice growth stages. We tested this hypothesis in a field plots experiment. AM community structures in rice roots growing in plots with different cultivation systems were analyzed using terminal restriction fragment length polymorphism (T-RFLP), cloning and sequencing.

4.3 Materials and Methods

4.3.1 Mycorrhizal survival and responsiveness

In order to test whether AM could colonize into rice root grown under anaerobic condition (waterlogged or CS) and aerobic condition (SRI), rice seedlings inoculated with AM, *Glomus etunicatum* or *Acaulospora tuberculata,* were carried out in a pot experiment for 6 weeks. A pot experiment was conducted in growth room under a 16-h-light (25 °c) / 8-h-dark (25 °c). The experiment was done in completely randomized design (CRD) with three replications. The three germinated seeds of wetland rice (*O. sativa* L.) were grown into plastic bottles (8 cm long × 8 cm wide × 50 cm height) containing 2 kg of sterilized sandy soil mixed vermiculite (1:1, v/v). The treatments were; (i) non mycorrhizal control (- AM) and mycorrhizal inoculation (+ AM) with *G. etunicatum* (FJ687414) or *A. tuberculata* (FJ687415) which were performed under aerobic (SRI) and anaerobic (CS) condition. Both AM used were originally isolated from rhizosphere soil in Thailand (Watanarojanaporn et al. 2011). Spores were collected by wet sieving with a 53-µm sieve (Daniels and Skipper 1984), and then surface sterilization of spores was performed by 5 mg/liter streptomycin. After 3-5 days of germination, seedling was inoculated with 100 spores of each AM

isolate into mycorrhizal treatment and watered with Hoagland's solution weekly (Hoagland and Arnon 1938). Under anaerobic (CS) and aerobic (SRI) conditions, the SRI was added with 3-5 cm water whereas the CS was flooded with 20-30 cm water above the soil surface of the pots after seedling emergence.

Harvest was performed at 6 weeks after sowing, and then plant growth parameters (height, plant/hill, root length, root dry weight, shoot dry weight, total dry weight, and P uptake) and AM colonization were determined. Shoots were cut off at ground level and soil was washed from the roots with tap water. Shoots and roots were rinsed in deionized water. Roots were cut into 1 cm segments and mixed thoroughly. A subsample of 0.2 g fresh weight per pot was taken to determine mycorrhizal root colonization as described by Phillips and Haymann (1970). Briefly, 1 cm root samples were cleared in 10% KOH and stained with 0.03% trypan blue in lactoglycerol. Stained roots were observed under a microscope and the percentage of root length colonized by AM was calculated. The shoots and remaining roots were oven dried at 70 °C for 48 h, and weighed. Dried and ground plant samples were digested in acid mixture (HNO_3 + $HClO_4$) for P analysis, and P concentration was analyzed by the vanadomolybdate blue method (Watanabe and Olsen 1965). Mycorrhizal responsiveness based on growth and P uptake were also measured. Mycorrhizal responsiveness (MR) and mycorrhizal P responsiveness (MPR) (Hetrick et al. 1992) were calculated as:

$$MR = \frac{[(Plant\ dw\ (+AMF) - Plant\ dw\ (-AMF))}{Plant\ dw\ (-AMF)]} \times 100$$

$$MPR = \frac{[(P\ uptake\ (+AMF) - P\ uptake\ (-AMF))}{P\ uptake\ (-AMF)]} \times 100$$

All data were tested, in statistical analyses performed with SPSS software (ver. 17.0), by univariate analysis. Significant differences between means were analyzed post hoc with Duncan's multiple range test ($P < 0.05$).

4.3.2 AM community composition

4.3.2.1 Field sites and rice cultivation systems

To study the influence of the rice cultivation systems on indigenous AM composition within rice root, plant roots were collected from experimental rice field plots that differed in growth stages in February-June 2010. The experimental field is located in the Northeastern region, Nakhon Ratchasima. (lat. 102° 10′ E and long. 14° 97′ N), Suranaree University of Technology. The study area is the paddy soil, with mean annual rainfall of 906 mm, mean annual minimum and maximum temperatures of 27 °c and 36 °c, respectively. The soil is a sandy clay loam, pH 7.39, organic matter content of 0.69%, 30.17 mg kg^{-1} P (BrayII; Houbu et al. 1988), 0.3% total N, 3.76 mg kg^{-1} NO_3^-, 1.88 mg kg^{-1} NH_4^+ and 89.53 mg kg^{-1} K (1 M NH_4OAc, pH 7.0; Helmke and Sparks, 1996). Plots measured 8 m×8 m and were separated from each other by bunds. Rice was grown under two types of water management, the conventional flooded system (CS) and system of rice intensification (SRI). Water level was maintained at 30 cm under CS condition throughout rice growth stages, while the SRI was added with 3-5 cm water above the soil surface during tillering to flowering stage. The rice variety *Patum-1* was planted in nursery beds (containing sterilized sand) at the beginning of February 2010 and seedlings were transplanted to the conventional and SRI-managed plots at 30 and 15 days old, respectively. A plant spacing of 30 cm × 30 cm with a single plant per hill was used in SRI, whereas three plants per hill with a plant spacing of 20 cm × 20 cm were done

in CS. In SRI, 4-5 days of flooding was alternated with 4-5 days of draining during the vegetative growth period, while rice plots in the conventional system were kept flooded.

Field plots were arranged by a factorial approach in a randomized complete block design (RCBD) (two factors; 2×5 factorial RCBD) with three replications. The experiment was designed with two factors that consisted of: (i) cultivation system (CS and SRI) and (ii) PGPR inoculations including, the treatments of *A. largimobile*; *Az. vinelandii*; *A. largimobile* + *Az. vinelandii*. Control treatments were also tested to compare with treatments that inoculated with PGPR. Compost (0.88% N, 208 mg kg^{-1} NH_4^+, 1,346 mg kg^{-1} NO_3^-, 3.9% P_2O_5, 0.9% K_2O) was applied to all treatments (except for control) in both cultivation systems at 12.50 t ha^{-1} seven days before rice seedlings were transplanted into the plots. The +/- compost applied treatments were established within these two water management systems.

4.3.2.2 Determination of AM colonization and amount of P uptake

Rice plants were sampled four times at monthly intervals during the dry season, in the middle of each month of March to June 2010. The stages of rice development at each sampling time were: (i) vegetative growth phase after the first draining of the SRI plots (March), (ii) vegetative growth phase one month after the previous sampling (April), (iii) rice panicle initiation (May), and (iv) rice harvest (June). Our timeline represents the period during which cultivation systems and PGPR inoculation effect might be important for establishment and development of AM in roots. Three rice plants were harvested from each plot in an area of 1 m^2 (1 m × 1 m). Roots of each individual were collected by carefully untangling them while briefly immersed in water. Half of the roots were stored in 95% ethanol for analysis of AM colonization rates and half were dried at 50 °c for 16 h and frozen at -80 °c for

DNA extraction. For colonization, roots were stained with trypan blue and all AM structures were scored using the line intersect method at ×400 with 100 intersections (McGonigle et al. 1990). Shoots of each plant were oven dried at 70 °c for 48 h, and ground. Ground plants were digested in acid mixture (HNO_3 + $HClO_4$) for P analysis, and P concentration was analyzed by the vanadomolybdate blue method (Watanabe and Olsen, 1965). The data were analyzed using analysis of variance (ANOVA). Statistical analyses were performed with SPSS software (ver. 17.0). Significant differences between means were analyzed post hoc with Duncan's multiple-range test ($P < 0.05$). We identified AM taxa in roots using a combination of cloning, sequencing, and TRFLP to fingerprint individual root samples that could be keyed to the clone library.

4.3.2.3 AM structure in rice roots

DNA extraction and PCR condition

Dried roots were ground in a microcentrifuge tube using a 4-mm glass bead in a Mixermill MM301 (Retch) at 24 Hz, for 10 min. DNA of 20 mg ground roots was extracted using DNeasy Plant Mini kit (Qiagen) following the manufacturer's instructions. DNA extracts were diluted 10x in PCR-grade water before PCR reactions (see below). A ~800-bp fragment of 18S rDNA was amplified by using the primers AM-specific primer, AML1 (5'-ATC AAC TTT CGA TGG TAG GAT AGA-3') and AML2 (5'-GAA CCC AAA CAC TTT GGT TTC C-3') (Lee et al. 2008). In separate PCR reactions, untagged PCR products were created for cloning and sequencing, and tagged PCR products intended for T-RFLP. PCR reaction mixtures were performed using 0.2 mM of each dNTPs, 10 pmol of each primer AML1 and AML2, 0.725 U of *Taq* polymerase, and the supplied reaction buffer (Promega) in final volume of 20 ml. PCR was run in a PTC100 (MJ research)

in the following condition: 95 °c for 3 min, followed by 30 cycles of 94 °c for 30 s, 63 °c for 45 s, 72 °c for 50 s, and extension step at 72 °c for 5 min. PCR products were loaded on 1% agarose gel and examined under UV light after staining with SYBR safe (Invitrogen). Amplified products (~800 bp) were purified with QIAquick PCR purification kit according to the manufacturer's instructions (Qiagen) for ligation and cloning.

For T-RFLP reaction, AM-specific primer pairs AML1-AML2 were labelled on both ends with different fluorescent labels (HEX-AML1 and FAM-AML2; Invitrogen). PCR mixtures and thermal cycling were performed as described above, except that the PCR-TRFLP was run for 20 cycles. Then, the amplified PCR-TRFLP products were digested with the restriction enzyme having specific recognition sequences. The 3U of the restriction endonuclease *Hin*fI (Promega, Madison, USA) were applied for digestion in a 20 mL reaction volume (three parallel reactions for each). Purified products were digested at 37 °C for 3 h. Digestion reactions were further purified with QIAquick PCR purification kit (Qiagen) and eluted in 12 mL of nuclease-free distilled water (Invitrogen) prior to T-RFLP analysis. Samples were prepared with 6.5 µL of deionized formamide, 0.5 µL of 600 LIZ internal size standard (Applied Biosystems), and 3 µL of *Hin*fI digested products. Prior to T-RFLP run, the mixtures were denatured at 94 °C for 5 min and immediately placed on ice for a few minutes. Fluorescently labelled terminal restriction fragments (T-RFs) were separated on an ABI PRISM 3730 DNA Analyzer Sequencer (Applied Biosystems, Foster City, CA), with three replicates for each digestion mixture to ensure reproducibility. The GeneMapper version 3.7 software (Applied Biosystems) was used to analyze the labelled T-RF sizes and T-RF quantities (peak heights as rfu; relative fluorescent unit) by comparison with the internal size standard. Peak heights

and peak areas were also recorded with a minimum peak amplitude threshold of 50 rfu. In cases by which the strongest peaks in the electropherograms were not in the range 2,000-8,000 relative fluorescence units (rfu), the sample concentration was adjusted accordingly and the sample was rerun.

Cloning and Sequencing, Phylogenetic and Cluster analysis

A clone library was generated using pooled amplicons from three replicates of the same sample. Pooled PCR products were gel-purified to improve the efficiency of the ligation reaction (QIAquick gel purification kit, Qiagen). Purified products were ligated into the pGEM-T-Easy vector system cloning kit according to the manufacturer's recommendation (Promega) and transformed into *Escherichia coli* (JM109). Ninety-six positive transformants were selected randomly by blue-white screening and confirmed by amplifying white colonies. Amplified inserts were then digested by the restriction enzyme *Hin*fI according to the manufacturer's instructions (Promega). Each pattern of RFLP was screened and sequenced to ensure sequence identity with both directions of vector primers SP6 and T7 on ABI PRISM 3730 (Applied Biosystems, Foster City, CA) DNA Analyzer System at Macrogen (Seoul, South Korea).

All sequences were compared in the public database by BLASTN searches (http://www.ncbi.nlm.nih.gov) and twenty AM sequences (phylotype) were deposited in GenBank under the accession numbers JF906731-JF906750. To separate AMsequences from nontarget sequences, the BLAST (Altschul et al. 1990) was used. The resulting sequences (GenBank accession numbers JF906731-JF906750) were subjected to phylogenetic analysis, together with 46 reference sequences and 3 sequences of other fungi were used as the outgroup. Using CLUSTALX version 1.83.1 (Jeanmougin et al. 1998), AM sequences were automatically aligned, their

closest BLAST matches, and additional AM sequences from phylogenetically representative set of *Glomeromycota* downloaded from GenBank. Neighbour-joining distances tree was constructed in MEGA version 4 (Tamura et al. 2007) with 1,000 bootstrap replicates.

All sequences were subjected to *in silico* restriction digestion with the program BioEdit 7.0 (Hall 1999) to predict the fragment sizes of each double-labelled sequences fragment. Using this procedure, predicted fragment lengths were calculated, taking the differences in migration caused by dye properties (HEX vs FAM) into account. These *in silico* RFLP analyses were performed to putatively assign the most common T-RF pairs to specific sequences (AM types). This procedure was only performed for T-RF pairs that were specific for a certain sequence. Related sequences having approx. 2 bp differences in T-RF sizes were considered to be the same, and this criterion was also used to match the observed T-RFs to sequences.

Cluster analysis based on the similarity of AM communities with respect to the numbers of phylotype were performed between the two different cultivation systems. AM sequence profiles were converted to binary data (presence or absence of AM phylotypes). Jaccard similarity matrix was calculated for the set of samples.

T-RFLP analysis and Diversity analysis

Despite the cleanup of products, a relatively broad bining procedure was necessary because of irregular final adenosine incorporation by *Taq* polymerase and stutters resulting from residual polymerase activity acting on the 5' overhang left after digestion. The threshold for peak recognition was set at 50 rfu. The resulting profiles were subjected to the following procedure: (1) exclusion of

peaks < 50 bp; (2) size binning of peaks within a size range of 1.2 bp, and assignment to the maximal peak size to account for A incorporation;(3) peak area calculation within the bins; peaks contributing < 1% to the total area were excluded to achieve the same sensitivity across runs with different total fluorescence. The relative abundance was calculated by dividing the individual T-RF peak area attributable to a given species by the total fluorescence in the profile.

To evaluate richness and evenness, diversity statistics were calculated using the number and area of peaks in each average profile as representations of the number and relative abundance of different phylotypes in a sample. It is understood that any given TRF fragment may represent sequences from multiple phylogenetic groups and may therefore not represent a true phylotype. The term of phylotype was used to indicate groups for richness calculations and also for the sake of consistency during comparisons of the TRF method with restriction fragment length polymorphism (RFLP) analysis of 18S rRNA gene clones. AM community diversity was calculated by three replicate T-RFLP profiles generated from each replicate of root sample (3 replicates of each sample). The TRF-signal in the simulated T-RFLP profile (representing peak area) was assumed to be proportional to the relative abundance of the species. TRF signals were pooled when two species had the same TRF size in base pairs. TRFs smaller than 50 bp and greater than 600 bp were deleted, and TRFs were expressed as relative abundances of the total signal detected in the T-RFLP profile. TRFs below a threshold (less than 1% of the total profile signal) were also deleted, and relative abundances of remaining TRFs were recalculated.Phylotype richness (*S*) was calculated as the total number of distinct TRF sizes (between 50 and 600 bp) in a profile. The Shannon-Weiner diversity index (Margalef 1958) was calculated as follows: $H' = -\sum(p_i)\ (\log_2 p_i)$, where

p is the proportion of an individual peak area relative to the sum of all peak areas. Evenness (Margalef 1958) was calculated from the Shannon-Weiner diversity function: $E = H/H_{max}$, where $H_{max} = \log_2(S)$.

Data and Statistical analysis

Principle component analysis (PCA) was performed to compare the AM communities in rice roots, under the different cultivation systems and different PGPR inoculations during four growth stages, using the AM phylotype (based on clone library; presence / absence of AM sequence) of each field plot as input data. PCA was executed for all samples together, as well as for the first (March), the second (April), the third (May) and the last (June) sampling. PCA analysis was carried out in SPSS version 17.0 software package. To visualize the differences among AM communities (under different cultivation system) determined with by T-RFLP analysis, the TRF- peak area values were subjected as input data to multidimensional Scaling in two dimension by using the ALSCAL in the SPSS version 17.0 software package.

4.4 Results

4.4.1 Plant growth and Mycorrhizal responsiveness (MR)

In order to prove whether AM could colonize into rice root grown under anaerobic condition (waterlogged or CS) and aerobic condition (SRI), mimic of AM in both CS and SRI systems were carried out in a pot experiment. Rice seedlings inoculated with AM, *G. etunicatum* or *A. tuberculata,* and grown under CS and SRI conditions in order to investigate the colonization of the *G. etunicatum*- or *A. tuberculata*- rice root were conducted for 6 weeks. After rice plants harvest, plant growth parameters (height (cm), plant numbers (plants hill^{-1}), root length (cm), plant

(shoot, root and total) dry weight (mg pot^{-1}), P uptake (g pot^{-1})), AM colonization (%), mycorrhizal responsiveness (MR) based on plant dry weight, and mycorrhizal responsiveness based on P uptake (MPR) were analyzed (Table 4.1).

Table 4.1 Plant growth [height (cm), plant numbers (plants hill^{-1}), root length (cm), plant (shoot, root and total) dry weight (mg pot^{-1}), P uptake (g pot^{-1})], AM colonization (%), mycorrhizal responsiveness (MR) based on plant dry weight, and mycorrhizal responsiveness based on P uptake (MPR) of rice that was inoculated with either of the two AMF species (*A. tuberculata* or *G. etunicatum*) under conventional flooded system (CS) and system of rice intensification (SRI).

System	Treatments	Measured parameters									
		Height‡ (cm)	Plant no. (plants hill^{-1})	Root length (cm)	Root dry weight (mg pot^{-1})	Shoot dry weight (mg pot^{-1})	Total dry Weight (mg pot^{-1})	MR (%)	P uptake (mg pot^{-1})	MPR (%)	AM colonization (%)
CS	Control (- AMF)	36.67±0.88^{b†}	4.33±0.33^{c}	23.50±2.78^{a}	166.67±14.53^{ab}	276.67±8.82^{b}	443.33±23.33^{b}	-	99.70.±12.4^{c}	-	-
	AMF (+ *A. tuberculata*)	45.33±1.59^{a}	9.67±1.20^{bc}	21.33±2.18^{a}	510.00±15.10^{a}	670.00±52.91^{a}	1180.00±20.33^{a}	167.04±44.46^{a}	294±6.3^{ab}	193.8±44.1^{ab}	20.54±1.37^{b}
	AMF (+ *G. etunicatum*)	43.17±2.45^{a}	9.67±0.88^{bc}	21.50±0.50^{a}	520.00±18.20^{a}	640.00±10.17^{a}	1160.00±11.55^{a}	163.33±16.19^{a}	270.8±3.8^{ab}	180.5±56.8^{ab}	16.07±1.21^{c}
SRI	Control (-AMF)	34.50±1.75^{b}	5.33±0.88^{c}	25.00±2.64^{a}	103.33±17.64^{b}	310.00±43.59^{b}	413.33±61.19^{b}	-	102.2±7.0^{c}	-	-
	AMF (+ *A. tuberculata*)	19.83±0.44^{d}	24.67±4.10^{a}	20.33±0.33^{a}	426.67±13.50^{ab}	636.67±13.70^{a}	1063.33±37.30^{a}	163.77±55.96^{a}	321.5±3.9^{a}	221.6±55.0^{a}	29.38±1.47^{a}
	AMF (+ *G. etunicatum*)	26.17±3.18^{c}	15.00±5.03^{b}	20.50±0.29^{a}	370.00±14.30^{ab}	436.67±12.03^{ab}	806.67±13.70^{ab}	116.17±72.43^{a}	190.3±13.7^{b}	88.9±23.8^{c}	28.46±1.29^{a}

† Means under each parameter followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple-range test. Values are means± standard errors calculated by three replicates.

‡ Means are calculated by each column.

The mycorrhizal rice roots stained with trypan blue were visualized as*Arum*-type association (Figure 4.1). The highest height was found in mycorrhizal rice inoculated with *A. tuberculata* or *G. etunicatum* (45.33 cm and 43.17 cm, respectively) under CS condition (Figure 4.2). For plant numbers per hill, mycorrhizal plants grown under SRI significantly showed higher numbers than mycorrhizal plants grown in CS. The greatest plant numbers per hill were observed in mycorrhizal plants that were inoculated with *A. tuberculata*, or *G. etunicatum* under SRI management. The rice plants colonized with AM (*A. tuberculata*, or *G. etunicatum*) under SRI had significantly higher plant numbers per hill than non-mycorrhizal plants (control) (Table 4.1). There was no significant differences of root length in all treatments. Considering the treatments, plants inoculated with AM revealed the best root dry weight, shoot dry weight, and total dry weight in both cultivation systems. Inoculation with either *A. tuberculata* or *G. etunicatum* significantly increased total dry weight of both CS and SRI (Table 4.1). The mycorrhizal responsiveness (MR) exhibited neither significant differences between both cultivations nor between both AM species. The average mycorrhizal responsiveness based on plant dry weight, were ranged between 163.77 % and 167.04 % for *A. tuberculata*, and 116.17% and 163.33% for *G. etunicatum*, under CS and SRI cultivation managements, respectively. P uptake of all plants were ranged between 99 mg pot^{-1} to 321.50 mg pot^{-1}. P uptake was significantly increased by inoculation with either of the two AM species. There was AM treatments variation ($P < 0.05$) in P uptake among the cultivation systems. Inoculation of *A. tuberculata* or *G. etunicatum* significantly increased P uptake of rice plants grown in SRI with 321.50 mg pot^{-1} or 190.30 mg pot^{-1}, respectively, and CS conditions with 294 mg pot^{-1} or 270.8 mg pot^{-1}, respectively. The mycorrhizal P

responsiveness (MPR) exhibited no significant differences among AM species under CS condition. The averages of *A. tuberculata* and *G. etunicatum* mycorrhizal P responsiveness were varied between 88.9 % and 221.6 % for SRI and between 180.5 % and 193.8 % for CS, respectively. For AM colonization, no root colonization by AM was observed in the uninoculated plants (control). Variation in AM colonization was found ($P < 0.05$) among cultivation systems. Root colonization of AM-inoculated plants ranged from 16.07% to 29.38%. Ranking the colonization rates of AM species in both cultivation systems indicated that AM (either *A. tuberculata* or *G. etunicatum*) plants grown in SRI produced the best colonization rate (29.38% or 28.46%, respectively), followed by *A. tuberculata* plants grown in CS (20.54%), while the lowest colonization rate was detected in plants inoculated with *G. etunicatum* under CS management (16.07%) (Table 4.1)

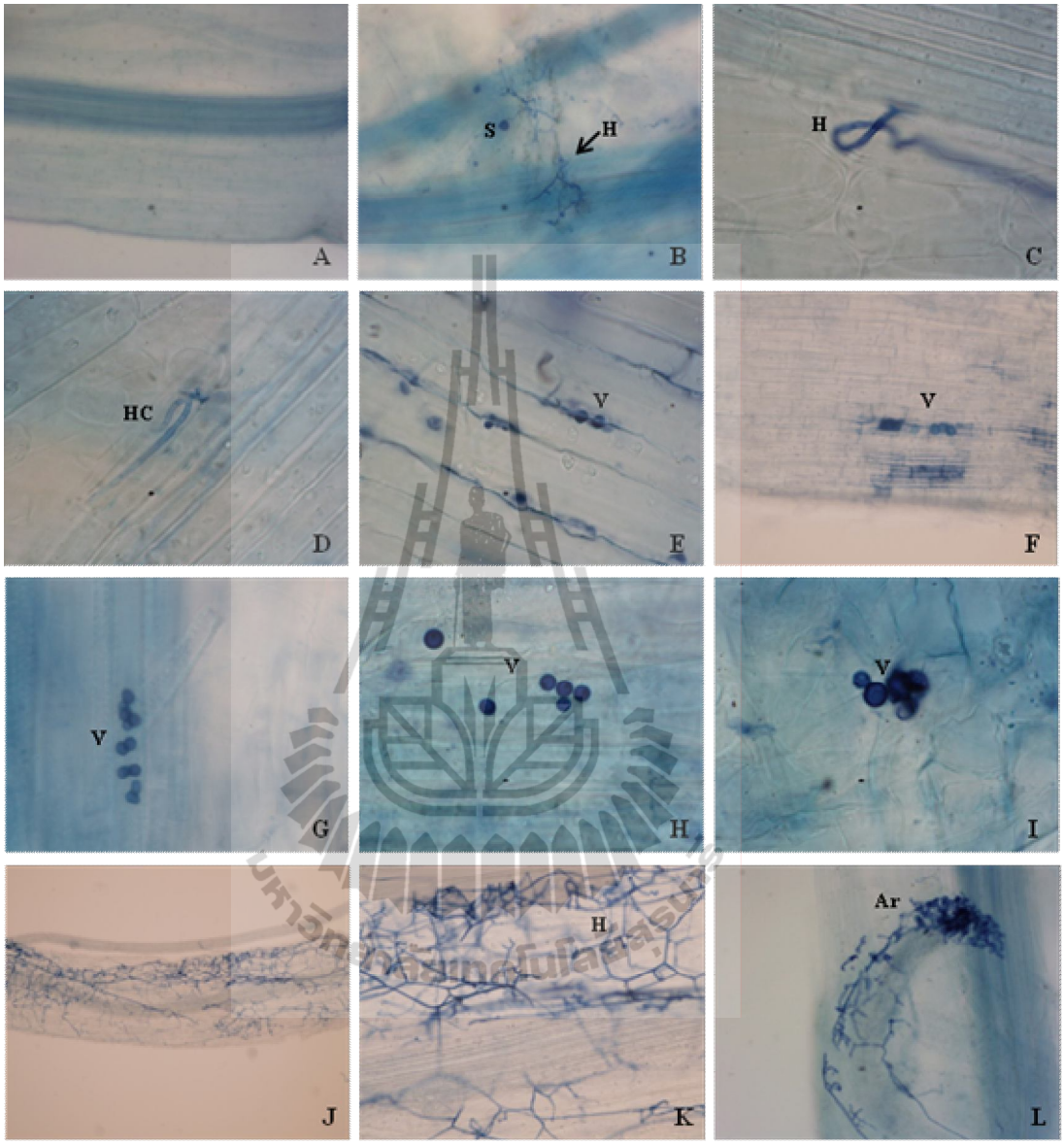


Figure 4.1 Roots of rice grown under aerobic (no flooding) and anaerobic (flooding) colonized by AM fungi stained with 0.03% trypan blue; (A) control, (B)-(G) roots colonized by *A. tuberculata*, (H)-(L) roots colonized by *G. etunicatum*. (V: vesicle, H: hyphae: HC: hyphal coil, Ar: arbuscule, scale bar = 50 μm)

Figure 4.2 Rice plants grown under aerobic (no flooding, SRI) and anaerobic (flooding, CS) colonized by AM fungi; (a) control, (b) roots colonized by *A. tuberculata*, (c) roots colonized by *G. etunicatum*.

4.4.2 AM community structure in field condition

4.4.2.1 AM colonization and P uptake

In order to determine colonization rates of indigenous AM and P uptake of rice plants grown under different cultivation systems (SRI and CS) during 4 stages of rice growth, roots, and shoots were analyzed and compared between treatments. Means were calculated by a Duncan's test, and levels of significance ($P < 0.05$) were summarized in Table 4.2 for AM colonization and for P uptake (Table 4.3). The results revealed that there were variations in AM colonization and P uptake among different growth stages and PGPR inoculation. Statistical analysis

demonstrated that there were interactions in AM colonization and P uptake between growth stages (sampling times) and PGPR inoculation (treatments) ($P < 0.01$).

The growth stages, 90 and 120 days, provided the greatest AM colonization rates in all treatments of PGPR inoculation. For PGPR inoculation (treatments), we found that rice plants grown under SRI cultivation and inoculated with compost × *A. largimobile,* and rice plants inoculated with compost × *Az. vinelandii* × *A. largimobile*, gave higher colonization rate than other treatments in all growth stages (Table 4.2). At 90 and 120 days of growth stage, rice plants grown in SRI and amended with compost × *Az. vinelandii* × *A. largimobile* significantly showed the highest percentage of colonization at 50.03% and 49.80%, respectively, followed by rice plants inoculated with compost × *A. largimobile* (48.67% and 48.52%), while the lowest colonization rates were detected in the first stage of control treatment and treatment amended compost alone under CS management (Table 4.2).

In case of P uptake, statistical results indicated that there were interactions between sampling times and PGPR treatments ($P < 0.01$) (Table 4.3). For growth stage, 90 days, produced the best P uptake in all PGPR treatments. Considering the PGPR treatments, rice plant sown in SRI and amended with compost × *Az. vinelandii* × *A. largimobile* exhibited the highest P uptake in all stages of rice growth. The results presented that the P uptake at different stages differed between treatments ($P < 0.01$). Ranking the P uptake of rice plant demonstrated that rice grown under SRI (at 90 and 120 days) and inoculated with compost × *Az. vinelandii* × *A. largimobile* provided the best value (7.73 and 8.13 g plant^{-1}, respectively), followed by compost treatment (6.87 g plant^{-1}) and compost mixed with *Az. vinelandii* treatment (7.03 g plant^{-1}) of SRI condition at 90 days of growth stage, while control treatment gave the lowest value (3.69 and 4.29 g plant^{-1}) at 90 and 120 days, respectively (Table 4.3).

Table 4.2 Effect of PGPRs inoculation under conventional cultivation systems (CS) and system of rice intensification (SRI) on mycorrhizal root colonization of rice field plot at 30, 60, 90 and 120 days of growth stages.

Sampling times	Treatments	AM colonization (%)
30 days[c]	CS-control [d]	11.06 ± 0.20 w[†]
	CS-compost [d]	10.23 ± 0.20 w
	CS-compost × *Az. vinelandii* [d]	13.40 ± 0.10 v
	CS-compost × *A. largimobile*[d]	12.67 ± 0.40 v
	CS-compost × *Az. vinelandii*× *A. largimobile*[d]	14.33 ± 0.33 v
	SRI-control [c]	20.67 ± 0.17 t
	SRI-compost [b]	29.15 ± 0.30 m
	SRI-compost × *Az. vinelandii* [b]	32.11 ± 0.64 k
	SRI-compost × *A. largimobile*[a]	30.16 ± 0.16 l
	SRI-compost × *Az. vinelandii* × *A. largimobile*[a]	35.27 ± 0.27 i
60 days[b]	CS-control [d]	17.24 ± 0.50 u
	CS-compost [d]	18.33 ± 0.83 u
	CS-compost × *Az. vinelandii* [d]	20.10 ± 0.40 t
	CS-compost × *A. largimobile*[d]	24.54 ± 0.30 p
	CS-compost × *Az. vinelandii*× *A. largimobile*[d]	25.23 ± 0.13 o
	SRI-control [c]	27.83 ± 0.15 n
	SRI-compost [b]	34.11 ± 0.11 j
	SRI-compost × *Az. vinelandii* [b]	36.24 ± 0.44 h
	SRI-compost × *A. largimobile*[a]	45.53 ± 0.20 d
	SRI-compost × *Az. vinelandii* × *A. largimobile*[a]	47.02 ± 0.12 c
90 days[a]	CS-control [d]	19.21 ± 0.32 u
	CS-compost [d]	20.33 ± 0.43 t
	CS-compost × *Az. vinelandii* [d]	20.20 ± 0.10 t
	CS-compost × *A. largimobile*[d]	22.40 ± 0.30 r
	CS-compost × *Az. vinelandii*× *A. largimobile*[d]	24.87 ± 0.37op
	SRI-control [c]	35.52 ± 0.30 i
	SRI-compost [b]	40.02 ± 0.20 f
	SRI-compost × *Az. vinelandii* [b]	41.20 ± 0.30 e
	SRI-compost × *A. largimobile*[a]	48.67 ± 0.17 b
	SRI-compost × *Az. vinelandii* × *A. largimobile*[a]	**50.03[‡] ± 0.23 a**
120 days[a]	CS-control [d]	20.10 ± 0.40 t
	CS-compost [d]	20.83 ± 0.70 t
	CS-compost × *Az. vinelandii* [d]	21.33 ± 0.22 s
	CS-compost × *A. largimobile*[d]	21.50 ± 0.30 s
	CS-compost × *Az. vinelandii*× *A. largimobile*[d]	24.03 ± 0.13 q
	SRI-control [c]	37.02 ± 0.52 g
	SRI-compost [b]	39.80 ± 0.28 f
	SRI-compost × *Az. vinelandii* [b]	41.14 ± 0.24 e
	SRI-compost × *A. largimobile*[a]	48.52 ± 0.23 b
	SRI-compost × *Az. vinelandii* × *A. largimobile*[a]	**49.80 ± 0.30 a**
**	**	**

† Means under each parameter followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple-range test. Values are means± standard errors calculated by three replicates.

‡ Bold face type indicates the highest value in AM colonization and superscript letters (a, b, c, d) indicates significant differences among sampling times and treatments

** Statistical level indicates significant differences among sampling times and treatments at $P < 0.01$

Table 4.3 Puptake in rice plants grown under conventional cultivation systems (CS) and system of rice intensification (SRI).

Sampling times	Treatments	P uptake (g/plant)
30 days[c]	CS-control [cd]	2.38 ± 0.95 k[†]
	CS-compost [cd]	2.98 ± 0.88 jk
	CS-compost × *Az. vinelandii* [cd]	2.71 ± 0.43 k
	CS-compost × *A. largimobile*[c]	3.18 ± 0.84 jk
	CS-compost × *Az. vinelandii*× *A. largimobile*[cd]	2.87 ± 0.75 jk
	SRI-control [d]	0.97 ± 0.51 k
	SRI-compost [b]	3.28 ± 2.54 jk
	SRI-compost × *Az. vinelandii* [ab]	3.98 ± 0.76 efghij
	SRI-compost × *A. largimobile*[b]	4.40 ± 0.76 efghi
	SRI-compost × *Az. vinelandii* × *A. largimobile*[a]	3.99 ± 1.13efghij
60 days[b]	CS-control [cd]	3.74 ± 1.00efghij
	CS-compost [cd]	4.29 ± 0.35 efghij
	CS-compost × *Az. vinelandii* [cd]	5.37 ± 0.68 bcdef
	CS-compost × *A. largimobile*[c]	4.91 ± 0.61 cdefg
	CS-compost × *Az. vinelandii*× *A. largimobile*[cd]	4.72 ± 1.18 defgh
	SRI-control [d]	2.33 ± 1.54 k
	SRI-compost [b]	3.36 ± 1.55 jk
	SRI-compost × *Az. vinelandii* [ab]	4.05 ± 0.34 efghij
	SRI-compost × *A. largimobile*[b]	4.32 ± 0.22efghi
	SRI-compost × *Az. vinelandii* × *A. largimobile*[a]	4.60 ± 0.06defghi
90 days[a]	CS-control [cd]	3.976 ± 0.57efghij
	CS-compost [cd]	4.293 ± 1.48efghij
	CS-compost × *Az. vinelandii* [cd]	4.751 ± 1.06 defgh
	CS-compost × *A. largimobile*[c]	4.309 ± 0.67 efghi
	CS-compost × *Az. vinelandii*× *A. largimobile*[cd]	4.044 ± 0.59 efghij
	SRI-control [d]	3.690 ± 1.40efghij
	SRI-compost [b]	6.874 ± 1.89 ab
	SRI-compost × *Az. vinelandii* [ab]	7.035 ± 1.60 ab
	SRI-compost × *A. largimobile*[b]	6.410 ± 1.05 abcd
	SRI-compost × *Az. vinelandii* × *A. largimobile*[a]	**7.730[‡] ± 0.59 a**
120 days[b]	CS-control [cd]	2.040 ± 0.65 k
	CS-compost [cd]	2.136 ± 0.09 k
	CS-compost × *Az. vinelandii* [cd]	2.096 ± 0.41 k
	CS-compost × *A. largimobile*[c]	2.825 ± 0.58 jk
	CS-compost × *Az. vinelandii*× *A. largimobile*[cd]	1.743 ± 0.28 k
	SRI-control [d]	4.292 ± 0.35efghij
	SRI-compost [b]	5.567 ± 1.69 bcdef
	SRI-compost × *Az. vinelandii* [ab]	6.583 ± 1.68 abc
	SRI-compost × *A. largimobile*[b]	5.681 ± 0.13 bcde
	SRI-compost × *Az. vinelandii* × *A. largimobile*[a]	**8.130 ± 0.96 a**
**	**	**

† Means under each parameter followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple-range test. Values are means± standard errors calculated by three replicates.

‡ Bold face type indicates the highest value in AM colonization and superscript letters (a, b, c, d) indicates significant differences among sampling times and treatments

** Statistical level indicates significant differences among sapling times and treatments at $P < 0.01$

4.4.2.2 Phylogenetic analyses of AM fungal groups

The topology of the phylogenetic tree derived from partial 18S rDNA sequences of rice root DNA was illustrated in Figure 4.3. All sequences had high levels of similarity (98% to 100% identity) to AM fungal sequences belonged to members of the phylum *Glomeromycota*, the genera *Glomus,* and *Acaulospora*. The constructed alignment included the 46 different glomalean sequences and 3 other fungal sequences defined as the outgroup. Some clones produced the same sequence and were represented just once in the alignment. The sequences obtained in this study were clustered into twenty discrete groupings or fungal sequence types (JF906731-JF906750). Fifteen of these sequences belonged to genus *Glomus*, which was the most frequently obtained from rice root DNA. Another group of the 5 AM fungal sequences belonged to the genus *Acaulospora*. The majority of AM fungal types showed a high level of similarity, root-derived sequences in GenBank belonging to uncultured *Glomus*. The phylogenetic tree demonstrated that all *Glomus* and *Acaulospora* sequences from the present study, as expected, grouped into their own genera (Figure 4.3). Surprisingly, our results revealed that all AM sequences obtained from the rice roots under CS field plot belonged only to the genus *Glomus*, no *Acaulospora* sequences appeared in the rice grown under this cultivation. In other words, the rice roots sown in SRI showed AM sequences belonged to both AM genera, *Glomus* and *Acaulospora* (Figure 4.3).

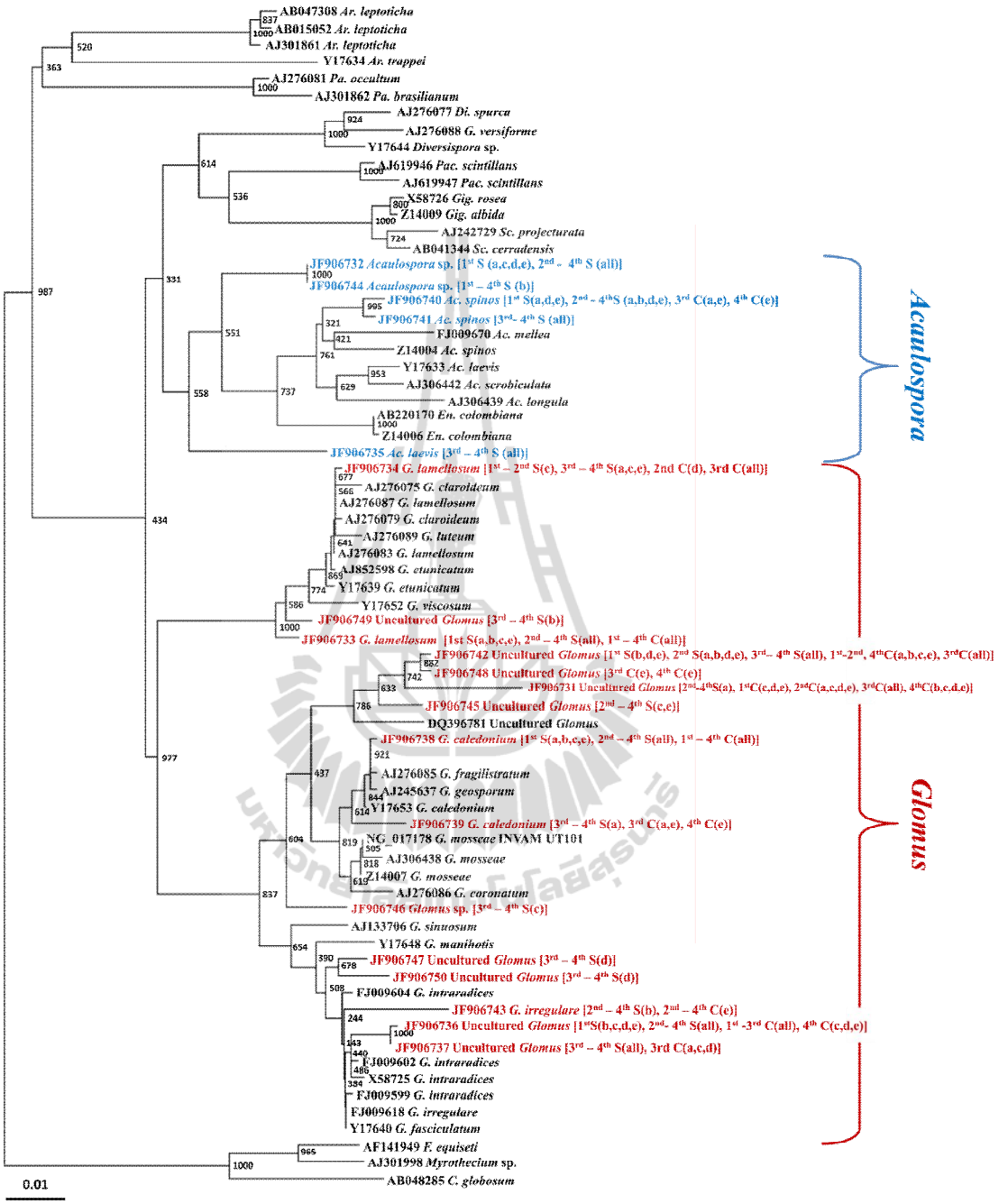
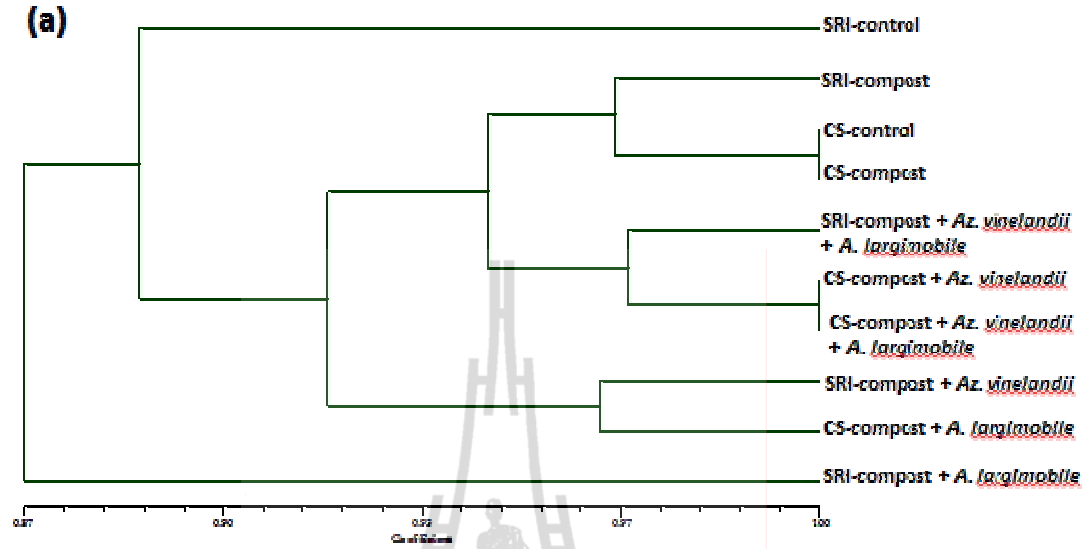


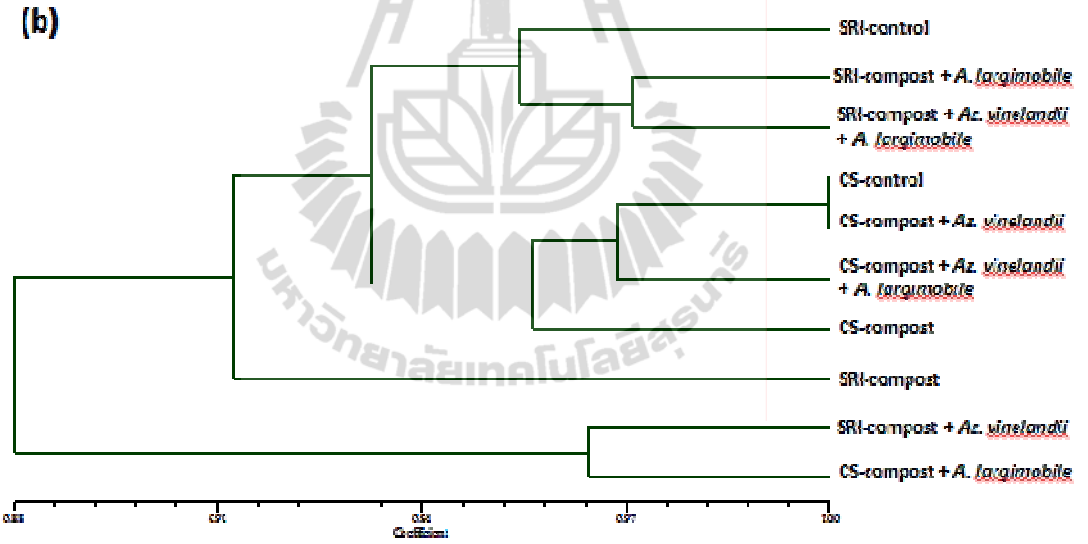
Figure 4.3 Phylogenetic relationships derived from partial 18S rDNA sequences (1,000 bootstrap) of AM colonizing the rice roots grown under the five different PGPRs inoculations treatments (control, compost, compost × *Az. vinelandii*, compost × *A. largimobile* and compost × *Az. vinelandii* × *A. largimobile*) in two different rice cultivation systems (conventional cultivation system, CS and

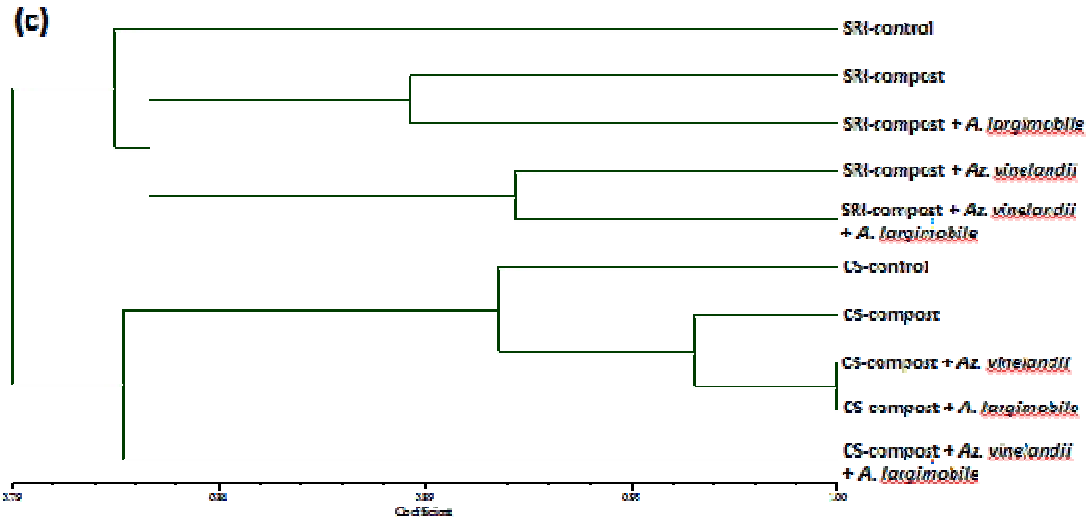
system of rice intensification, SRI). GenBank accessions with the letters in parentheses indicated that AM sequences obtained from rice roots, which were grown under conventional (C) and system of rice intensification (S) and inoculated with different PGPRs treatments during the 4 growth stages (vegetative, 1st; tillering, 2nd; flowering, 3rd; and harvest, 4th). The letters (a, b, c, d and e) denote the AM sequences obtained from the rice roots under treatments of control, compost, compost × *Az. vinelandii*, compost × *A. largimobile* and compost × *Az. vinelandii* × *A. largimobile*, respectively, (**i.e**., [1st S(b)] indicates AM colonizing the roots of rice collected at 1st stage of growth, under SRI condition, and inoculated with compost).

4.4.2.3 Cluster analyses of AM communities and AM distribution

Cluster analysis based on the similarity of AM communities with respect to the numbers of AMsequence showed that the ten AM communities were divided into two distinct clade (Figure 4.4). At the first and second times of sampling (Figure 4.4a and 4.4b), the AM fungal communities associated with the different cultivation plots (CS and SRI) were not divided into different clusters, while AM fungal communities under CS and SRI plots were separated into different clusters during the third and the last stages of rice growth. Each cluster contained five AM communities from each different cultivations (Figure 4.4c and 4.4d).


(a)
SRI-control
SRI-compost
CS-control
CS-compost
SRI-compost + Az. vinelandii + A. largimobile
CS-compost + Az. vinelandii
CS-compost + Az. vinelandii + A. largimobile
SRI-compost + Az. vinelandii
CS-compost + A. largimobile
SRI-compost + A. largimobile


(b)
SRI-control
SRI-compost + A. largimobile
SRI-compost + Az. vinelandii + A. largimobile
CS-control
CS-compost + Az. vinelandii
CS-compost + Az. vinelandii + A. largimobile
CS-compost
SRI-compost
SRI-compost + Az. vinelandii
CS-compost + A. largimobile


(c)
SRI-control
SRI-compost
SRI-compost + A. largimobile
SRI-compost + Az. vinelandii
SRI-compost + Az. vinelandii + A. largimobile
CS-control
CS-compost
CS-compost + Az. vinelandii
CS compost + A. largimobile
CS-compost + Az. vinelandii + A. largimobile

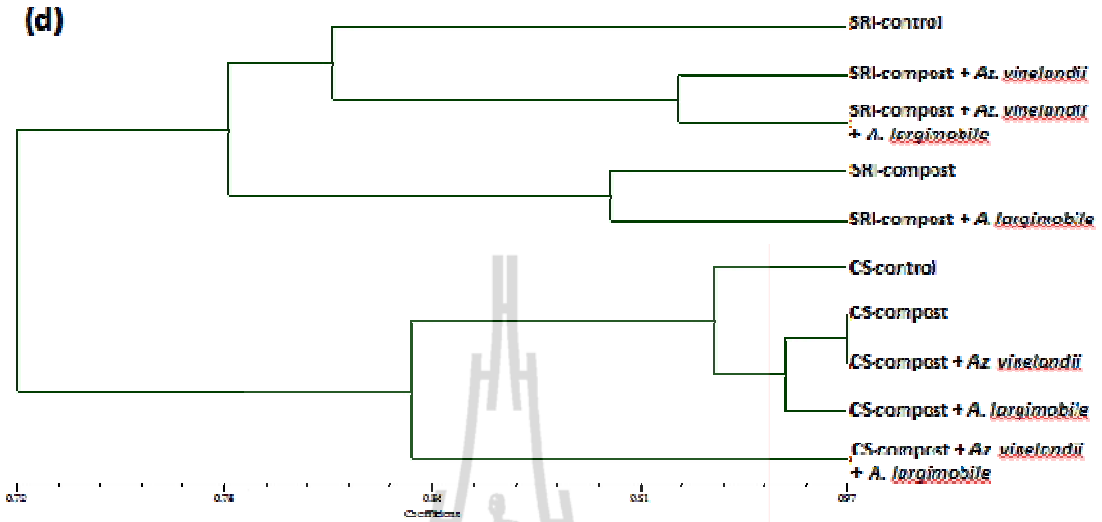
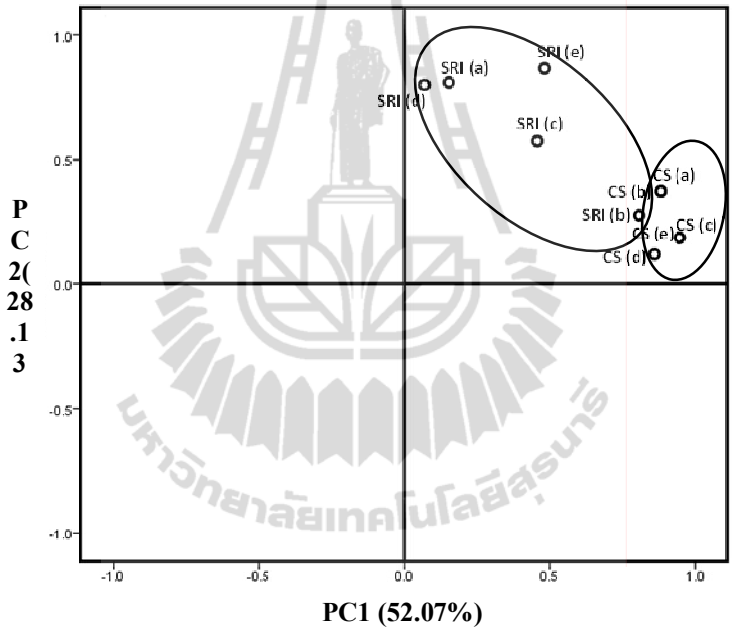


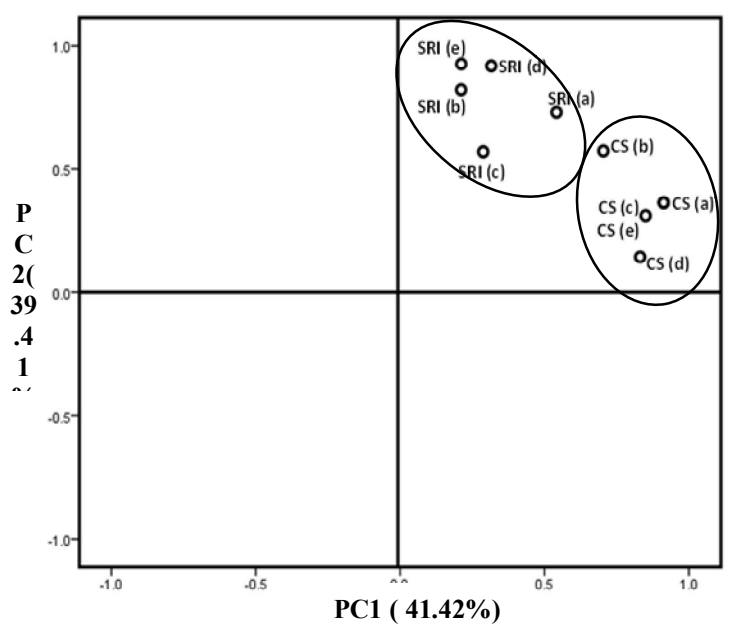
Figure 4.4 Dendogram of cluster analysis based on the similarity of the ten AM communities (presence and absence of AM sequences) in rice roots grown under different cultivation systems (conventional cultivation system, CS and system of rice intensification, SRI) and different PGPR inoculations (control, compost, compost × *Az. vinelandii*, compost × *A. largimobile* and compost × *Az. vinelandii* × *A. largimobile*). Rice roots sampled at (a) the first stage; (b) the second stage; (c) the third stage; and (d) the last stage of growth.

The effect of cultivation managements on AM community structure (based on AM sequences obtained from clone library) of rice plots were illustrated in Figure 4.5. AM community structure was compared across the different cultivation types (CS and SRI) and different PGPRs application by principle component analysis (PCA). In the 2nd (60 days), the 3rd (90 days) and the 4th (120 days) sampling times, the AM assemblages of conventionally managed system (CS) were separated from those of SRI management (Figure 4.4b, 4.4c and 4.4d, respectively). During the growth stages (or sampling times), AM community

compositions appeared in the principle component (PC) plot with small shifts between CS and SRI management schemes (Figure 4.5). Additionally, there was considerably more variation in AM composition between PGPR applications from SRI plot that between those from CS plot, which can be seen by a more scattered distribution of the SRI samples than of the CS samples in the PC Analysis (Figure 4.5).



(a)



(b)

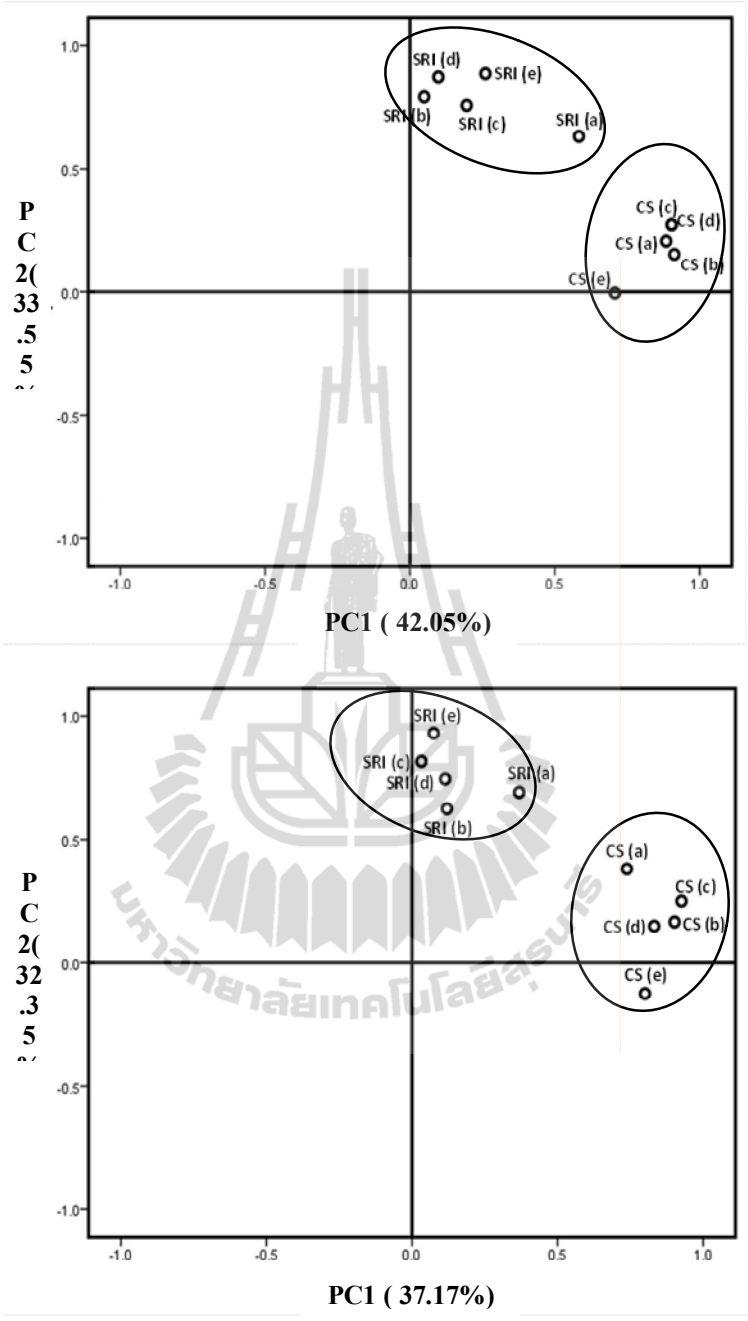


Figure 4.5 Principle components analysis (PCA) of arbuscular mycorrhizal fungal (AM) community composition (based on AM sequences obtained from clone library) in rice plants from conventionally managed field (CS) and from system of rice intensity (SRI) field. Rice roots were sampled at a) the first stage; b) the second stage; c) the third stage; and d) the fourth stage of rice growth. Percentages of total explained variation by the first two PCA axes are

given in parentheses. The letters (a, b, c, d and e) indicate AM community composition of rice roots under treatments of control, compost, compost × *Az. vinelandii*, compost × *A. largimobile* and compost × *Az. vinelandii* × *A. largimobile*, respectively.

The distribution of AM community composition (based on T-RFLP data) for all sampling times was given in the multidimensional scaling ordination (Figure 4.6). During the time of sampling (1st, 2nd, 3rd, and 4th), AM communities (derived from TRF-peak area data input) in the rice field plots did not shift in community composition, but increased the level of AM diversity (see appendix 1). For this all sampling time analyses, the same trend appeared that there was more variation in AM composition between PGPR inoculations from SRI management than between those from CS plot, which can be seen by a more scattered distribution in the SRI plot than in the CS plot (Figure 4.6). The AM fungal community structures associated with the different compost and PGPR treatments (control, compost, and PGPR additions) were not clearly devided while the AM fungal community structures associated with the different cultivation plots (CS and SRI) were separated from each other. Each group contained AM communities from each different PGPR treatments, four AM communities for SRI and three communities for CS (Figure 4.6).

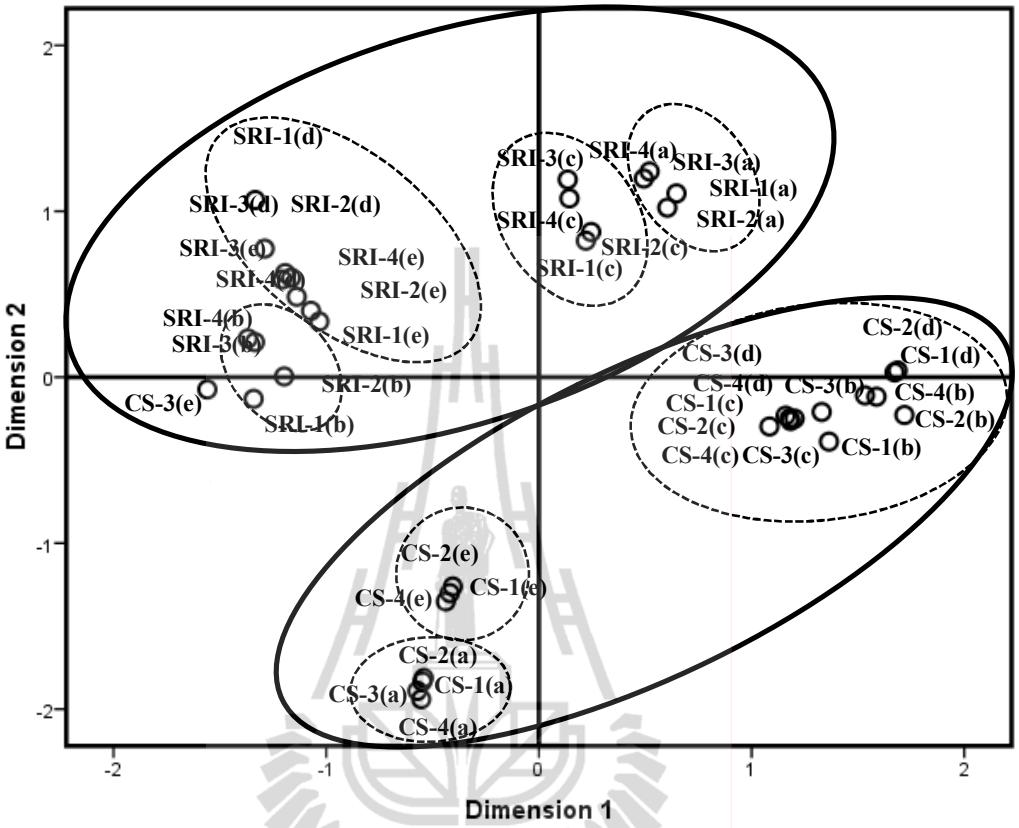


Figure 4.6 Multidimensional scaling (distances as interval; SPSS 17.0) plot of the AM fungal community composition found in the roots of rice plants grown under the two different cultivation, and the five different PGPRs and compost inoculations. The eigenvalues of the first and second axes in the two-dimensional ordination diagrams are as follows: dimension 1, 0.5; dimension 2, 0.4. Oval with full, and oval with dashed represent the distributions of AM community in rice roots grown under SRI and CS plots, and applied with PGPR, respectively. The letters (a, b, c, d and e) indicate AM community composition of rice roots under treatments of control, compost, compost × *Az. vinelandii*, compost × *A. largimobile* and compost × *Az. vinelandii* × *A. largimobile*, respectively, and the number (1, 2, 3 and 4) represent the sampling times of the first, the second, the third and the last, respectively.

4.4.2.4 AM community diversity

Diversity statistics (Table 4.4) were calculated on T-RFLP profile (TRF indices). Statistical result demonstrated that there were interactions in TRF-peak numbers and Shannon-Weaver indices of diversity (*H'*) between growth stages and between treatment ($P < 0.01$), while species evenness (E) showed no interactions between two factors (growth stages and treatments). There was no significant difference in evenness (E) between cultivation systems and between PGPR inoculations (Table 4.4).

Table 4.4 Diversity statistics calculated from T-RFLPs of AM 18S rDNA amplified from rice root DNA.

Sampling times	Treatments	Diversity indices		
		TRF-S‡	TRF-*H′*	E
30 days	CS-control	5 ± 0.00e†	1.5488 ± 0.00f	0.9815 ± 0.13a
	CS-compost	11 ± 0.00ab	1.8969 ± 0.04d	0.7931 ± 0.04b
	CS-compost × *Az. vinelandii*	10 ± 0.00bc	1.9358 ± 0.01c	0.8430 ± 0.04b
	CS-compost × *A. largimobile*	12 ± 0.00a	2.0001 ± 0.02c	0.8063 ± 0.03b
	CS-compost × *Az. vinelandii* × *A. largimobile*	6 ± 0.00de	1.5586 ± 0.00f	0.8798 ± 0.09ab
	SRI-control	11 ± 0.00ab	2.0641 ± 0.02b	0.8625 ± 0.03ab
	SRI-compost	9 ± 0.00c	1.9473 ± 0.00c	0.8894 ± 0.04ab
	SRI-compost × *Az. vinelandii*	11 ± 0.00ab	2.1347 ± 0.01a	0.8921 ± 0.03ab
	SRI-compost × *A. largimobile*	9 ± 0.00c	1.8595 ± 0.00d	0.8494 ± 0.04b
	SRI-compost × *Az. vinelandii* × *A. largimobile*	7 ± 0.00d	1.7714 ± 0.01e	0.9170 ± 0.07ab
60 days	CS-control	5 ± 0.00e	1.5530 ± 0.00e	0.9839 ± 0.13a
	CS-compost	12 ± 0.00ab	1.9632 ± 0.00c	0.7914 ± 0.03c
	CS-compost × *Az. vinelandii*	10 ± 0.00c	1.9346 ± 0.00c	0.8425 ± 0.04bc
	CS-compost × *A. largimobile*	13 ± 0.00a	2.0862 ± 0.03b	0.8144 ± 0.03bc
	CS-compost × *Az. vinelandii* × *A. largimobile*	6 ± 0.00de	1.5797 ± 0.00e	0.8916 ± 0.09abc
	SRI-control	12 ± 0.00ab	2.2252 ± 0.01a	0.8970 ± 0.03abc
	SRI-compost	10 ± 0.00de	2.0825 ± 0.00b	0.9069 ± 0.04ab
	SRI-compost × *Az. vinelandii*	11 ± 0.00bc	2.1322 ± 0.00a	0.8911 ± 0.03abc
	SRI-compost × *A. largimobile*	10 ± 0.00c	1.9907 ± 0.00c	0.8669 ± 0.04bc
	SRI-compost × *Az. vinelandii* × *A. largimobile*	7 ± 0.00d	1.7709 ± 0.00d	0.9168 ± 0.07ab

Table 4.4 (cont.) Diversity statistics calculated from T-RFLPs of AM 18S rDNA amplified from rice root DNA

Sampling times	Treatments	Diversity indices		
		TRF-S‡	TRF-*H´*	E
90 days	CS-control	6 ± 0.00e†	1.6736 ± 0.01d	0.9445 ± 0.09a
	CS-compost	13 ± 0.00bc	2.1041 ± 0.01b	0.8215 ± 0.03b
	CS-compost × *Az. vinelandii*	10 ± 0.00d	1.9313 ± 0.00d	0.8410 ± 0.04b
	CS-compost × *A. largimobile*	15 ± 0.00a	2.2240 ± 0.02a	0.8220 ± 0.02b
	CS-compost × *Az. vinelandii* × *A. largimobile*	7 ± 0.00e	1.7193 ± 0.00d	0.8899 ± 0.07ab
	SRI-control	14 ± 0.00ab	2.3223 ± 0.00a	0.8810 ± 0.03ab
	SRI-compost	13 ± 0.00bc	2.2700 ± 0.01a	0.8862 ± 0.03ab
	SRI-compost × *Az. vinelandii*	13 ± 0.00bc	2.2673 ± 0.00a	0.8852 ± 0.03ab
	SRI-compost × *A. largimobile*	12 ± 0.00c	2.0636 ± 0.00b	0.8319 ± 0.03b
	SRI-compost × *Az. vinelandii* × *A. largimobile*	10 ± 0.00d	1.9407 ± 0.00c	0.8452 ± 0.04b
120 days	CS-control	4 ± 0.00f	1.3344 ± 0.00e	1.0020 ± 0.20a
	CS-compost	11 ±0.00bcd	1.8895 ± 0.00c	0.7949 ± 0.06b
	CS-compost × *Az. vinelandii*	10 ± 0.00cd	1.9365 ± 0.00c	0.8433 ± 0.04b
	CS-compost × *A. largimobile*	14 ± 0.00a	2.1928 ± 0.01a	0.8319 ± 0.02b
	CS-compost × *Az. vinelandii* × *A. largimobile*	6 ± 0.00e	1.5587 ± 0.01d	0.8797 ± 0.08ab
	SRI-control	13 ± 0.00ab	2.2706 ± 0.00a	0.8902 ± 0.05ab
	SRI-compost	11 ± 0.00bcd	2.1795 ± 0.01a	0.9108 ± 0.03ab
	SRI-compost × *Az. vinelandii*	12 ± 0.00abc	2.2319 ± 0.00a	0.8997 ± 0.03ab
	SRI-compost × *A. largimobile*	12 ± 0.00abc	2.0766 ± 0.00b	0.8414 ± 0.06b
	SRI-compost × *Az. vinelandii* × *A. largimobile*	9 ± 0.00d	1.9053 ± 0.01c	0.8703 ± 0.05ab

† Means under each parameter followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple-range test.
Values are means± standard errors calculated by three replicates.

‡ Means are calculated by each column.

The numbers of TRF-peak detected significantly differed between growth stages and between treatments. At ninety-days of sampling times provided the greatest TRFs peak numbers in all treatments. There were variation in TRFs peak numbers among different cultivations and PGPR applications. Under CS condition of all growth stages, rice plants amended with compost × *A. largimobile* produced the highest peak numbers when compared with others (Table 4.4). The *H′* had significant different between growth stages and treatments. The *H′* were varied between 1.33-2.27. The highest *H′* was detected in rice plant sampled at 90 days among all growth stages. At 30 days of sampling times, rice plants sown in SRI and inoculated with compost × *Az. vinelandii* gave the most *H′* (*H′* = 2.13), while the lowest AM fungal diversity (*H′* = 1.54-1.56) was found in rice plant grown in CS condition. In the second stage of growth (60 days), we observed that control treatment (no PGPR inoculation), and rice plant grown under SRI and amended with compost × *Az. vinelandii* exhibited the highest *H′* (2.22 and 2.13, respectively). Additionally, our study pointed out that rice grown in SRI showed higher *H′* than rice plant grown under CS management (Table 4.4). Considering the third (90 days) and the last (120 days) stages of growth, the highest *H′* was found in the treatments of rice plant grown in CS and inoculated with compost × *A. largimobile* (2.22 and 2.19, respectively), control treatment under SRI (2.32 and 2.27, respectively), compost treatment under SRI (2.27 and 2.17, respectively) and compost mixed with *Az. vinelandii* treatment under SRI (2.26 and 2.23, respectively). The results presented in Table 4.4 also revealed that there was an increase of *H′* of rice plants sown under SRI during growth stages (gradually increased from 30 days until 120 days of growth stage). The outputs indicated that rice plant grown under SRI had more diverse AM fungal community than that grown under CS condition (Table 4.4).

4.5 Discussion

4.5.1 Plant growth and Mycorrhizal responsiveness

Mycorrhizal benefits have been reported for many cereals, including pearl millet, wheat, and maize (Rao et al. 1983; Lu and Miller 1989). We demonstrated a beneficial effect of AM inoculation on height, plant numbers per hill, plant dry root (root, shoot and total dry weight), P uptake, AM colonization, and mycorrhizal responsiveness (based on plant dry weight and P uptake) under SRI and CS conditions (Table 4.1). A beneficial effect of AM inoculation on plant growth is frequently attributed to increased nutrient uptake, especially of P uptake (Jeffries 1987). Consistent with our experiment, the highest plant dry weight and P uptake were found in rice inoculated with either *A. tuberculata* or *G. etunicatum* under CS and SRI management. This suggested that mycorrhizal inoculation appeared more effective in improving P content and plant dry weight than uninoculated plants (control). It is well known that AM fungi enhances nutrient uptake, as this fungal symbiosis increases the abilities of the host plants to explore a larger volume of soil than roots alone and to take up phosphate from a greater surface area (Jakobsen et al. 1992; Joner et al., 2000).

There was no variation in mycorrhizal responsiveness (MR) among AM species and among cultivation systems. In contrast, there was variation in AM colonization between SRI and CS condition, which was related to variation in mycorrhizal P uptake responsiveness (MPR) (Table 4.1). Though wetland rice has previously been considered non-mycorrhizal, a positive response to AM inoculation has been observed (Sharma et al. 1988). Our results showed that AM (both *A. tuberculata* and *G. etunicatum*) associated in rice roots could survive under

waterlogged condition (anaerobic condition) and could colonize rice roots to increase the uptake of P and plant growth. Solaiman and Hirata (1998) also reported 28% colonization by *Glomus* spp. at six weeks of growth stage (after growing in dry nursery) still persisted under wetland. Though AM is an obligate aerobic in nature, it probably can survive in association with rice root under anaerobic conditions because it obtains O_2 from the atmosphere through rice aerenchymatous tissue. AM colonization enhanced the P concentration and uptake, and thereby improved the rice growth. The enhancement of P uptake in response to the AM infection may have been the result of the increased absorption of nutrients due to the greater surface area of AM hyphae extending from the roots (Sharma et al. 1988; Secilia and Bagyaraj 1992;Solaiman and Hirata 1996). There are several reports on AM inoculation in wetland rice under high- or low- fertility soil conditions (Secilia and Bagyaraj 1994a, b; Solaiman and Hirata 1995). Secilia and Bagyaraj (1994a) evaluated 18 different inoculants of AM on nursery seedlings for 15 days of growth under dry conditions and then up to 28 days of growth under wet conditions. *Acaulospora* sp., *G. fasciculatum*, and *G.mosseae* were found to be best in improving plant height, tillering, total biomass, panicle number, grain weight, and plant P and Zn contents under nonsterilized soil conditions (available Bray II P was 11.6 mg kg^{-1}) (Secilia and Bagyaraj 1994a).

4.5.2 AM community structure in field condition

The topology of the phylogenetic tree as well as the results of BLAST indicated that the AM sequences obtained from the roots of rice plants were assigned to the genera *Glomus* and *Acaulospora.* In the present study, the majority of sequences detected in the rice roots, belonged to the genus *Glomus*. Other reports on

molecular diversity of AM also showed that *Glomus* was the dominant genus of AMcommunities in different ecosystems (Daniell et al. 2001; Wirsel 2004; Hijri et al. 2006). The sequences clustering in the genera *Acaulospora* were found only in the plots managed under SRI, not detected in CS condition. The occurrence of *Glomus* groups in the rice roots grown under CS plots suggests that these fungi might be more tolerant to anaerobic condition. As in previously reported studies carried out for other eco-systems such as tropical forests (Husband et al. 2002), agricultural sites (Alguacil et al. 2008; Hijri et al. 2006), wetland soils (Wirsel 2004), gypsum soil (Alguacil et al. 2009), or polluted soils (Vallino et al. 2006), we also found an AM fungal community dominated by *Glomus* species. They survive and propagate more easily thanks to the high sporulation rate and the ability to colonize via pieces of mycelium or mycorrhizal root fragments of this genus (Daniell et al. 2001). These attributes might explain why *Glomus* species are better adapted to disturbed environments.

In this study, the change in the AM fungal diversity in response to the different cultivation systems, and the application of PGPR and compost that we observed might be due to several changes: in the soil factors, such as pH, nutrient content, total soil C and N, moisture, and temperature, which are known to influence AM fungal distribution (Husband et al. 2002); in the soil fertility due to increases in the nutrient content and organic matter of the soil; in weed populations; or in microbial activity. Previous studies that reported the use of organic amendments in eroded soils show that they can improve soil productivity and increasing the soil nutrient status (Cox et al. 2001). It was previously proposed that nutrient availability affects the composition of AM fungal communities in the soil (Ezawa et al. 2000; Johnson 1993).

Here, we questioned whether differences in diversity between communities can be meaningfully portrayed by common diversity indices applied to T-RFLP profiles derived from AM communities. Our results demonstrated diversity estimates presented by TRF-richness (numbers of TRFs in profile), *H′* and evenness (frequency distribution of TRFs) of AM communities derived from T-RFLP data. The *H′* is a well-known and widely used diversity index integrating both species richness and evenness, although interpretation of the index itself is not without some conceptual difficulties (Hill et al. 2003; Jost 2006). Use of TRF profiles to provide relative measures of phylotype richness for comparison of bacterial communities was originally projected as a capability of the method (Liu et al. 1997). The TRF method has previously been shown to be capable of assessing phylotype richness in simple, artificial communities containing only four or six members (Avaniss-Aghajani et al. 1994; Liu et al. 1997; Moeseneder et al. 1999). It is possible that use of TRF profiles to measure relative phylotype richness is only possible for simple communities. However, when typical peak area thresholds (~1%) were used, richness and diversity indices (i.e., Shannon-Weaver indices of diversity (*H′*)) applied to T-RFLP profiles had limited capabilities to discriminate between levels of diversity in the underlying community. Some researchers reported that the numbers of peak in T-RFLP profiles (TRF-S) did a very poor job at predicting the number of taxa in the communities (S) and found that the numbers of peak did not correspond with S for other community profiling methods (Loisel et al. 2006). The lower resolution of the TRF method (i.e., the substantial probability of multiple phylotypes being represented by single fragment size in TRF profile) would tend to obscure differences in phylotype richness and evenness that might be detected by other methods.

Interestingly, our results showed that the high percentage of AM colonization was observed for the high diversity of the AM fungal community in the roots growing in the SRI plots. SRI plots sampled at 90 days and 120 days appeared the highest level of colonization and higher AM fungal diversity (based on H' derived from SSU) than the other plots (Table 4.2 and Table 4.4). High AM fungal diversity was reported in the dry Afromontane forest (H' = 2.58 based on ITS) and in the tropical forest (H' = 2.33 based on SSU) (Husband et al. 2002). The higher AM diversity in the SRI plot existed potentially due to the following reason that the higher AM diversity may reflect high colonization of AM in aerobic rice field plots.

There are very little study providing a knowledge of the colonizing arbuscular mycorrhizal (AM) fungi in rice grown under SRI. This symbiosis may enhance the root system's ability to absorb and carry phosphorus and other soil elements of low mobility, by means of a network of mycelia, thus promoting plant growth. Generally, the SRI systemcreate aerobic conditions in the soil which stimulate colonization of rice roots by AM and other fungi, and the aerobic upland (Gangopadhyay and Das 1982; Ammani et al. 1985; Brown et al. 1988) normally develop AM(Baby and Manibhushanrao 1992), though cultivation methods can have an impact on root colonization by AM fungi and on associated spore numbers in soils (Pozzebon et al. 1992). Therefore, this may be the reason why SRI plots appeared the higher level of colonization and higher AM fungal diversity (based on H' derived from SSU) than the CS plots.

4.6 Conclusion

In an attempt to understand how root AM community structure (based on T-RFLP derived from 18S rDNA) were affected by rice cultivation systems (the SRI

and the CS cultivations), and by applying of compost and PGPR (*A. largimobile* and *Az. vinelandii*) during different growth stages. Mimic of AM in both CS and SRI systems were also carried out in a pot experiment in which rice plants were grown for study of mycorrhizal responsiveness. In the pot experiment, inoculation with either *A. tuberculata* or *G. etunicatum* significantly increased total dry weight of rice from both CS and SRI systems, and also showed the mycorrhizal responsiveness (MR). The average mycorrhizal responsiveness based on plant dry weight, was ranged between 163.77 % and 167.04 % for *A. tuberculata*, and 116.17% and 163.33% for *G. etunicatum*, under two cultivation managements.Ranking the colonization rates of AM species in both cultivation systems indicated that AM (either *A. tuberculata* or *G. etunicatum*) plants grown in SRI produced the best colonization rate, followed by *A. tuberculata* plants grown in CS, while the lowest rate was detected in plants inoculated with *G. etunicatum* under CS management.In the rice field experiment, all sequences belonged to members of the phylum *Glomeromycota*, the genera *Glomus* and *Acaulospora*. Our results revealed that all AM sequences obtained from the rice roots under CS field plots belonged to the genus *Glomus*, no *Acaulospora* sequences appeared in the rice grown under this cultivation. In contrast, the rice roots sown in SRI showed AM sequences belonged to both AM genera, *Glomus* and *Acaulospora*.Comparison of AM community structure done across the different cultivation types (CS and SRI) and different PGPR application by principle component analysis (PCA) indicated that the AM assemblages of conventionally managed system (CS) were separated from those of SRI management at the 2nd (60 days), the 3rd (90 days) and the 4th (120 days) sampling times. The distribution of AM community composition based on T-RFLP data showed that the AM fungal community structures associated with the different cultivation plots (CS and SRI) were separated each other, while AM fungal community structures associated with the different

compost and PGPR additions were not clearly devided. The results revealed that there was an increase of *H´* of rice plants sown under SRI during growth stages (gradually increased from 30 days until 120 days of growth stage), suggesting that rice plant grown under SRI had more diverse AM fungal community than that grown under CS condition.

4.7 References

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CHAPTER V

BIASES FOR DETECTING ARBUSCULAR MYCORRHIZA FUNGAL COMMUNITY BY TERMINAL RESTRICTION FRAGMENT LENGTH POLYMORPHISM (T-RFLP)

5.1 Abstract

Terminal restriction fragment length polymorphism (T-RFLP) analysis of amplified ribosomal RNA genes was used for profiling microbial communities and sometimes for species richness and relative abundance estimation in environmental samples. However, T-RFLP fingerprint might be subjected to biases during the procedure, influencing the detection of real community structures in environment. In order to investigate T-RFLP community profiles in terms of relative abundance representing real community structures, artificial pairwise mixtures of 18S ribosomal RNA gene clones derived from two arbuscular mycorrhiza (AM) fungi sequences, were designed to construct simple "pairwise mixes". PCR T-RFLP was used for estimation of T-RFs products to answer the questions regarding how PCR cycle number could affect T-RFs product in pairwise mixes, and how effect of plant genomic DNA purification method and varied AM rDNA template ratio could affect the bias of template-to-product ratio in relative T-RFs peak area. The result of varying cycle numbers indicated that T-RFs products were still be in the exponential phase at

the cycle numbers lower than 18 cycles before approaching the plateau. These small cycle numbers were therefore allowed for the comparison of template-to-product quantities. We then tested the effect of different purification procedures and template-to product ratio on relative T-RF abundance of AM by mixing various quantity ratios of both SSU rRNA gene clones. The results demonstrated that the relative abundance calculations based on T-RFLP patterns varied with different purification procedures and were also reflected by input quantity ratios. The best T-RFs product quantities based on T-RF peak areas were obtained by using phenol-chloroform purification. Non-purified plant genomic DNA might create T-RFLP pattern that was not consistent with theoretical yield, therefore, this generated a bias towards lower or overestimation of relative abundance in current study.

T-RFLP/PAIRWISE MIXES/AM PLASMID DNA/PLANT GENOMIC DNA/
T-RFs PEAK AREA/T-RFs PRODUCT/PCR CYCLE NUMBER/PURIFICATION METHOD

5.2 Introduction

Arbuscular mycorrhizal (AM) fungi generally occur in ecosystems to form as diverse communities by colonizing most terrestrial plant roots (Clapp et al. 1999; Rillig 2004). AM are obligate biotrophs which have hyphae extensions into plant root systems, resulting in an increase of nutrient uptake and plant growth (Auge 2000; Newsham et al. 1995). However, the analysis of microbial communities has been one of the difficulties in the identification and assessment. This is because of unculturable microbes in various environments, the extreme complexity of the physical, chemical

and biological properties of soil, abundance and variation in the microbial diversity, as well as the appropriate molecular approaches to estimate microbial diversity in soil. Due to these difficulties, molecular approaches have been increasingly used for species identification, such as restriction fragment length polymorphism analysis (RFLP), denaturing gradient gel electrophoresis (DGGE) (Kowalchuk et al. 2002; Opik et al. 2003; Bougoure and Cairney 2005; Ma et al. 2005), clone libraries (Renker et al. 2006), as well as terminal restriction fragment length polymorphism (T-RFLP). T-RFLP refers to the use of fluorescently labelled primers combined with restriction digests to visualize sequence variation in single or multi-species DNA samples. This technique was developed as a tool for assessing bacterial diversity and comparing the community structure of bacteria in environmental samples (Liu et al. 1997; Marsh 1999; Kitts 2001). The data obtained are the sizes of the fragments of polymerase chain reaction (PCR) amplicons that contain the labelled primer, observed as electropherogram "peaks". Variation in the presence and location of cutting sites results in different species giving terminal fragments of different lengths. Over the last several years, there has been an increase in the use of T-RFLP technique for the identification of AM. It has been suggested that T-RFLP is more sensitive than DGGE for fungi (Singh et al. 2006), although obtaining sequences directly from samples may be more easily conducted with DGGE (Ma et al. 2005). In T-RFLP methodology, the technique can be applied as a quantitative method for estimation of the relative abundance based on peak heights or peak areas (Liu et al. 1997). Nonetheless, almost each step of the molecular approach or even in T-RFLP technique can introduce biases or errors to the analysis (von Wintzingerode et al. 1997). In the reasons of biases, the ratio of DNA to biomass seems to vary a great deal among different fungal species, because of variation in the number of rDNA

repeats per genome and in the number of genomes per unit of biomass (Maleszka and Clark-Walker 1993). The PCR amplification of a species is known to be directly influenced by the presence of other species in the PCR mixture, resulting in the apparent abundance of a species in PCR reaction. Annealing temperature (Ishii and Fukui 2001), template concentration and cycle number have been thought to have a significant effect on biases caused by preferential amplification (Chandler et al. 1997; Dohrmann and Tebbe 2004). Furthermore, the sequence composition of DNA can also influence PCR amplification (Leckie 2005). It is estimated that microbial community analysis can only detect 1–2% of the microbe population, which represents only the dominant species present in an environmental sample (MacNaughton et al. 1999). Therefore, molecular ecological analysis of a microbial community requires efficient and unbiased DNA extraction and purification methods. However, there are no available molecular methods that can assess microbial diversity without any bias.

To be useful as a marker, T-RFLP data need to be highly reproducible and manifest actual microbial community composition. Obtaining microbial DNA that accurately represents the real community is therefore very important. However, the study of T-RFLP profiles representing actual microbial communities has been scarce. Therefore, artificially designed model communities represent one way to assess the extent of biases induced during T-RFLP analysis. In this study, T-RFLP profiles standing for the real community structures were investigated in terms of relative abundance based on T-RF peak areas. AM pairwise communities were designed by mixing cloned 18S rRNA gene sequences that were derived from a gene library constructed by AM species. We investigated the bias in PCR amplification affecting relative abundance from mixtures of 18S rDNA templates constructed as described

below (materials and methods). In order to examine the effect of the relative amount of a specific template in AM plasmid DNA pairwise on amplification and T-RF abundance, we mimicked a realistic condition by adding plant genomic DNAs of Ri-T-DNA-transformed carrot roots (*Daucus carota* L.) into mixtures of AM 18S rRNA gene clones constructed from library. Hence, this simple community was created in order to estimate how (i) PCR cycle numbers; (ii) plant genomic DNA purification procedures, and (iii) multi-plasmid DNA template ratio would affect relative abundance based on peak areas of T-RF profile between different sequences. For these purposes, T-RFLP analyses were used to demonstrate biases on peak profiles of a simple AM community.

5.3 Objectives

In this work, a simple community was created in order to estimate how **(i)** PCR cycle numbers; **(ii)** plant genomic DNA purification procedures, and **(iii)** multi-plasmid DNA template ratio would affect relative abundance based on peak areas of T-RF profile between different sequences. For these purposes, T-RFLP analyses were used to demonstrate biases on peak profiles of a simple AM community.

5.4 Materials and Methods

5.4.1 Plant and AM materials

Ri-T-DNA-transformed carrot roots (*Daucus carota* L.) used in this study, which were provided in petri dishes on the modified Strullu-Romand (MSR) medium, were purchased from GINCO (Louvain-la-Neuve, Belgium). Ten distinct morphotypes of AM spores were obtained from trap cultures isolated from North Yorkshire (UK) and used to test throughout the experiment.

5.4.2 Characterization of the AM clones used

AM SSU rRNA gene clones were characterized in order to create artificial pairwise mixtures. Briefly, DNA were extracted from distinct morphotypes AM spores by crushing with a needle, and used directly as template for PCR. Partial SSU rRNA gene fragments were amplified using primers AML1 (5'-ATC AAC TTT CGA TGG TAG GAT AGA-3') and AML2 (5'-GAA CCC AAA CAC TTT GGT TTC C-3') developed by Lee et al. (2008). PCR reaction mixtures were performed using 0.2 mM of each dNTPs, 10 pmol of each primer AML1 and AML2, 0.725U of *Taq* polymerase and the supplied reaction buffer (Promega) in final volume of 20 mL. PCR was run in a PTC100 (MJ Research) in the following condition: 95 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 63 °C for 45 s, 72 °C for 50 s and extension step at 72 °C for 5 min. PCR products were loaded on 1% agarose gel and examined under UV light after staining with SYBR Safe (Invitrogen). Amplified products (800 bp) were purified with QIAquick PCR purification kit according to the manufacturer's instructions (Qiagen). Purified products were cloned into pGEM-T Easy Vector System cloning kit according to the manufacturer's recommendation (Promega, Madison, WI) and transformed into *Escherichia coli* (JM109). Ninety-six positive transformants were selected randomly by blue-white screening and confirmed by amplifying white colonies. Amplified inserts were then digested by the restriction enzyme *Hin*fI according to the manufacturer's instructions (Promega). Each pattern of RFLP was screened and sequenced to ensure sequence identity with both directions of vector primers SP6 and T7 on ABI PRISM 3730 (Applied Biosystems, Foster City, CA) DNA Analyzer System at Macrogen (Seoul, South Korea).

Seven out of 96 clones were selected from the gene library and blasted in GenBank for species identification. The sequenced inserts (7 clones) were created by the restriction map using BioEdit 7.0 (Hall 1999) for determining the sizes of terminal restriction fragments (T-RFs). There were two criteria to select AM sequences for all subsequent experiments. First, the sizes of terminal restriction fragments (T-RFs) had to be precisely ranged between 20 and 600 basepairs (bp), and T-RFs sizes of each individual sequence had to be clearly distinguished on T-RFLP electropherogram. Second, sequences had to originate from different species as determined by sequence identification using the blastn database. The selected two clones: sequence clone 01 (accession number: HQ588777) and sequence clone 02 (accession number: HQ588778) were illustrated T-RFs sizes of 516 and 376 bp as shown in Figures 5.1 (A and B), and were extracted using QIAprep spin miniprep kit (Qiagen) following the manufacturer's protocols. Insert of isolated AM plasmid DNA was amplified in order to obtain the correct size by fluorescently labelled primers AML1 (HEX-labelled) and AML2 with PCR condition as previously described. Isolated AM plasmid DNA was quantified with Quant-iT PicoGreens dsDNA reagent (Molecular Probes, Eugene, OR) on a fluorescence microplate reader (Polar Star OPTIMA, BMG LABTECH) in accordance to the manufacturer's instructions. Then, AM plasmid DNA quantities of both clones were equally adjusted to 3 ng mL^{-1}, and quality was controlled by electrophoresis in agarose gels (1.2% w/v) and ethidium bromide staining. The result of plasmid DNA quality controlled by electrophoresis revealed equal band intensities. For all steps, the plasmid DNA were handled.

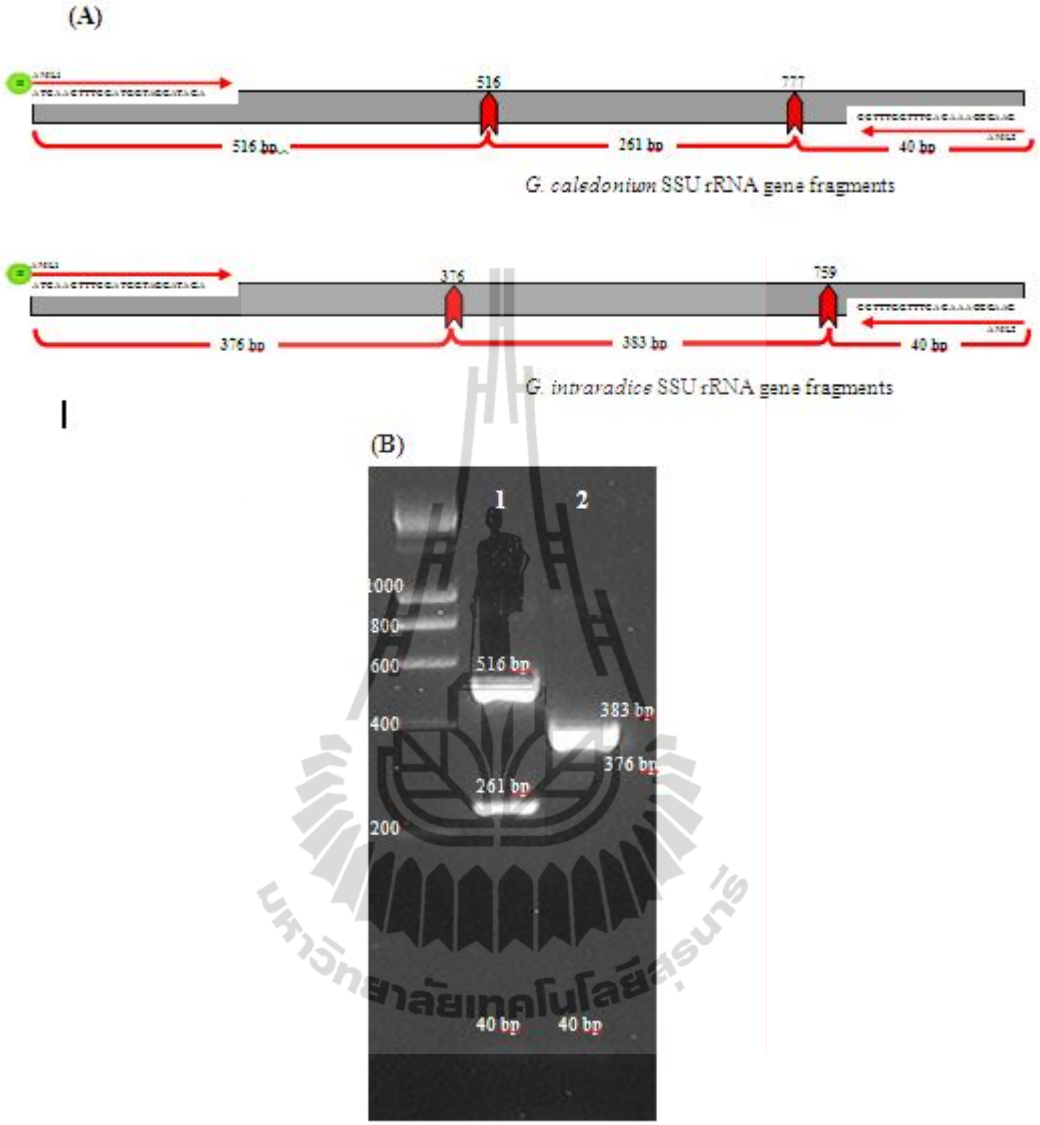

Figure 5.1 Two AM SSU rRNA of clone 01 (*G. caledonium)* and clone 02 *(G. intraradices)* illustrating T-RFs sizes; (A) SSU rRNA fragments were amplified using primers AML1 (HEX-labelled) and AML2 (B) T-RFs products digested by *Hin*fI restriction enzyme were separated on 1% agarose gel (lane 1: PCR products of clone 01 *(G. caledonium)* SSU rRNA gene, lane 2: PCR products of clone 02 *(G. intraradices)* SSU rRNA gene).

5.4.3 Plant genomic DNA extraction and purification

The effect of varying the AM rDNA template ratios on bias in template-to-product ratios in reaction mixtures, the effect of different AM species, and the effect of purifying genomic DNAs of Ri-T-DNA-transformed carrot roots (*D. carota* L.) amended to AM mixtures were explored as potential major biases of T-RF relative abundance originated from PCR amplification.

Briefly, Ri-T-DNA-transformed carrot roots (*D. carota* L.) that did not contain any microbial infection were washed, dried at 50 °C for 16 h and ground in a Mixermill MM301 (Retch) at 24 Hz, for 10 min. DNA of 20 mg ground roots was extracted using DNeasy® Plant Mini Kit (Qiagen). Quality and quantity of extracted DNA were measured by Multi-Sample Micro-Volume UV-Vis Spectrophotometer (NanoDrop 8000, Thermo Scientific). Extracted DNA was purified using protocol of ethanol precipitation adapted from Sambrook et al. (1989) and using phenol chloroform purification (Kang and Yang 2004). Purified and non-purified plant genomic DNA were quantified and their quality was controlled by Multi-Sample Micro-Volume UV-Vis Spectrophotometer (NanoDrop 8000, Thermo Scientific). The purified and non-purified nucleic acids were also adjusted to be equal to volumes of DNA yield for all subsequent PCR-TRFLP experiments. In all experiments, bias due to template saturation was prevented by adjusting reaction parameters so that the plateau phase was not reached.

5.4.4 Generation of AM pairwise mixes

In order to estimate detection of a specific peak interest in a mixed realistic community, the varied ratios of AM pairwise mixes (sequence clone 01 and sequence clone 02) of the SSU rRNA gene clone were generated by mixing into genomic DNAs of Ri-T-DNA-transformed carrot roots (*D. carota* L.). AM plasmid

DNA template mixtures were generated for PCR amplification and subsequent T-RFLP experiments, to serve the questions that (i) how cycle numbers could affect T-RFs peak area in pairwise mixes, and (ii) plant genomic DNA purification method and varying the AM rDNA template ratios could affect the bias of template-to product ratios in relative T-RFs peak area ratio between different AM sequences.

5.4.4.1 Effect of varying PCR cycle numbers on amplification

Simple community was constructed by mixing pairwise template AM plasmid DNA (clone 01 and clone 02) whose concentrations were defined in the ratio 1:1 (0.6 ng of clone 01: 0.6 ng of clone 02). For the pairwise mixes, 20 ng uL^{-1} plant genomic DNA of Ri-T-DNA-transformed carrot roots (*D. carota* L.) was added to mimic real community condition. The 1X TE buffer (Invitrogen) was also composed with those pairwise mixes in the equal concentration as described above to minimize bias that might originate from the presence of plant genomic DNA in PCR amplification from mixtures of 18S rDNA templates. All treatments were each done in triplicate. In experiments with varying cycle numbers, pairwise mixtures as described were used as PCR templates. The mixture of plasmid inserts was amplified with primers AML1 (HEX-labelled) and AML2 in a total volume of 20 ml containing 0.2 mM of each dNTPs, 10 pmol of each primers AML1 and AML2, 0.725U of *Taq* polymerase and the supplied reaction buffer (Promega). PCR was run in a PTC100 (MJ Research) using the following condition: 94 °C for 3 min, followed by 94 °C for 30 s, 58 °C for 40 s, 72 °C for 55 s and extension step at 72 °C for 5 min. Reactions were stopped after 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33 and 36 cycles, followed by incubation at 72 °C for 5 min. PCR products were separated on 1% agarose gel stained with SYBR Safe (Invitrogen) and visualized under UV light. Each PCR was performed in triplicate using templates obtained by independent mixing procedures.

5.4.4.2 Effect of plant genomic DNA purification and effect of AM plasmid DNA template-to-product ratio on PCR amplification

Artificial pairwise communities (clone 01 and clone 02) were designed by mixing pairwise template AM plasmid DNA whose concentrations were in the following proportions: 0.2:1 (0.12 ng: 0.6 ng), 0.4:1 (0.24 ng: 0.6 ng), 0.8:1 (0.48 ng: 0.6 ng), 1:0.2 (0.6 ng: 0.12 ng), 1:0.4 (0.6 ng: 0.24 ng), 1:0.8 (0.6 ng: 0.48 ng), and 1:1 (0.6 ng: 0.6 ng) of clone 01 : clone 02. For minimization of bias generated by plant genomic DNA purity, plant genomic DNA that had been purified by two different methods as described above were mixed with pairwise template AM plasmid DNA in PCR amplification. In order to mimic the realistic community, pairwise mixes were combined to 20 ng uL^{-1} plant genomic DNA (Ri-T-DNA-transformed carrot roots (*D. carota* L.)). For generation of ideal condition model, the 1X TE buffer (Invitrogen) was also composed of those pairwise mixes in the equal ratio as described above to eliminate the bias which may be caused by impurity of genomic DNA in PCR amplification. All treatments were each done in triplicate. The mixture of plasmid insert was amplified with primers AML1 (HEX-labelled) and AML2 in a total volume of 20 ml containing 0.2 mM of each dNTPs, 10 pmol of each primers AML1 and AML2, 0.725U of *Taq* polymerase and the supplied reaction buffer (Promega). PCR was performed in a PTC100 (MJ Research) and run 18 cycles with the condition as previously described. Amplified products were stained on 1% agarose gel by SYBR Safe (Invitrogen) and visualized under UV light. Each PCR was performed in triplicate using templates obtained by independent mixing procedures.

5.4.5 PCR products purification and restriction enzyme digestion

Amplified pairwise PCR products were purified using QIAquick PCR purification kit (Qiagen), and were characterized using SSU rRNA gene-based

terminal restriction fragment length polymorphism (T-RFLP) analysis. The method involves end-labeling polymerase chain reaction (PCR) amplicons through the use of fluorescent molecules attached to the 5'-end of each PCR primer. The amplified pairwise PCR products were digested with selected restriction enzyme having specific recognition sequences. The 3U of the restriction endonuclease *Hin*fI (Promega, Madison, USA) were applied for digestion in a 20 mL reaction volume (three parallel reactions for each). Purified products were digested at 37 °C for 3 h. Digestion reactions were further purified with QIAquick PCR purification kit (Qiagen) and eluted in 12 mL of nuclease-free distilled water (Invitrogen) prior to T-RFLP analysis.

5.4.6 T-RFLP analysis

For T-RFLP analysis, each of the SSU rRNA gene sequences mixtures model described in part no. 5.4.4 was individually analyzed, i.e. in mixtures of plasmid DNA clone 01 and clone 02 varying template ratios or purification methods or cycle numbers. Samples were prepared with 6.5 uL of deionized formamide, 0.5 uL of 600 LIZ internal size standard (Applied Biosystems) and 3 uL *Hin*fI digested products. Prior to T-RFLP run, the mixtures were denatured at 94 °C for 5 min and immediately placed on ice for a few minutes. Fluorescently labelled terminal restriction fragments (T-RFs) were separated on an ABI PRISM 3730 DNA Analyzer Sequencer (Applied Biosystems, Foster City, CA), with three replicates for each digestion mixture to ensure reproducibility. The GeneMapper version 3.7 software (Applied Biosystems) was used to analyze the labelled T-RF sizes and T-RF quantities (peak heights as rfu; relative fluorescent unit) by comparison with the internal size standard. Peak heights and peak areas were also recorded with a minimum peak amplitude threshold of 50 rfu. The relative abundance was calculated

by dividing the individual T-RF peak height or area attributable to a given species by the total fluorescence in the mixes.

5.4.7 Statistical data analysis

T-RF peak area values in a profile were divided by the sum of all peak area values in the corresponding profile. All discriminative statistics were performed using SPSS software (ver. 13.0 SPSS Inc. 1989-2004). The peak area values were calculated for evaluating the effect of different purifications, different 18S rDNA template ratios or different PCR cycle numbers variables on T-RFLP profiles. The effects of categorical factors, i.e. sequence type, purification procedures, cycle numbers and varying template ratios, were carried out for significance using one-way analysis of variance (ANOVA). The mean and standard deviation of peak area among different purification methods on individual peaks of each triplicate mixture were calculated and analyzed post hoc with Duncan's multiple-range test ($P < 0.05$).

5.5 Results

5.5.1 Effect of cycle numbers on T-RFs product in terms of peak area

An equal ratio (1:1) of plasmid DNA clone 01 and clone 02 was mixed with TE buffer and also with plant genomic DNA, then AM plasmid DNA mixes were amplified by using AML1-AML2 primers. As expected that the bias should increase with the PCR cycles numbers, we used T-RF products in terms of peak area to compare amplified products by theoretical yield as noted in Table 5.1. In order to interpret better outcome of quantitative PCR products, we converted T-RFs peak area value into the logarithm of the base 2 (log2), also illustrated in Figure 5.2. In AM plasmid DNA pairwise mixed with TE buffer and with plant genomic DNA, the observed T-RF products based on T-RF peak area varied considerably from

theoretical products. There was no difference between the theoretical yield and observed products produced by amplification of AM plasmid DNA mixes in TE buffer when the cycle numbers run on 3, 6, 9, 12, 15 and 18 cycles (Table 5.1).

Table 5.1 Comparison of theoretical yield and observed 18S rDNA gene T-RF products by varying cycle numbers of plasmid DNA pairwise mixes of clone 02 and clone 01 in 1: 1 ratio

Cycle number (n)	Yield (2^n)	Product (log2)[a]		
		Theoretical yield	AMF Plasmid DNA mixed in TE buffer	AMF Plasmid DNA mixed in plant genomic DNA
3	2^3	$3^a \pm 0.00$	$2.87^a \pm 0.01^*$	$2.22^b \pm 0.01$
6	2^6	$6^a \pm 0.00$	$5.96^a \pm 0.01$	$5.64^b \pm 0.02$
9	2^9	$9^b \pm 0.00$	$9.96^b \pm 0.05$	$11.57^a \pm 0.11$
12	2^{12}	$12^b \pm 0.00$	$12.04^b \pm 0.04$	$12.76^a \pm 0.04$
15	2^{15}	$15^a \pm 0.00$	$14.97^a \pm 0.01$	$14.59^b \pm 0.02$
18	2^{18}	$18^a \pm 0.00$	$17.03^a \pm 0.02$	$16.24^b \pm 0.05$
21	2^{21}	$21^a \pm 0.00$	$18.55^b \pm 0.02$	$17.31^c \pm 0.07$
24	2^{24}	$24^a \pm 0.00$	$19.89^b \pm 0.04$	$18.24^c \pm 0.04$
27	2^{27}	$27^a \pm 0.00$	$20.68^b \pm 0.01$	$18.88^c \pm 0.02$
30	2^{30}	$30^a \pm 0.00$	$21.61^b \pm 0.02$	$19.10^c \pm 0.07$
33	2^{33}	$33^a \pm 0.00$	$22.01^b \pm 0.04$	$19.37^c \pm 0.02$
36	2^{36}	$36^a \pm 0.00$	$22.39^b \pm 0.01$	$19.49^c \pm 0.02$

* Means ± standard deviations were calculated from data in triplicates runs for each of triplicate PCR reactions, and mean values within a row that are statistically significant ($P<0.05$) are indicated by a different letter.

a The data shown T-RF products in term of peak area were transformed into logarithm (log2).

The trend of PCR products produced by amplification of AM plasmid DNA mixes in TE buffer was closer to the theoretical line than that of PCR products from AM plasmid DNA mixes with plant genomic DNA. While the PCR products produced by amplification of AM plasmid DNA mixes with plant genomic DNA appeared significantly different from theoretical product in all cycle numbers. At the higher number of cycles (>18 cycles), we found that the observed products of AM

plasmid DNA mixes (with TE buffer and with plant genomic DNA) were always lower than the theoretical yield, corresponding to result illustrated in Figure 5.2. This implied that different cycle numbers resulted in similar product quantities, since amplification might reach the plateau. Therefore, the plateau effect significantly limited the equivalent of product quantities at higher number of cycles (>18 cycles). Then, optimizing PCR cycle numbers was the best way to avoid amplification approaching the PCR plateau and bias of amplification. This result indicated that the products were still in the exponential phase at the lower number of cycles (≤18 cycles). Thus, lower PCR cycle numbers applied in further experiment was 18 cycles. Small PCR cycle numbers applied in the current study to allow for the comparison of product quantities in the exponential phase of amplification before reaching the plateau.

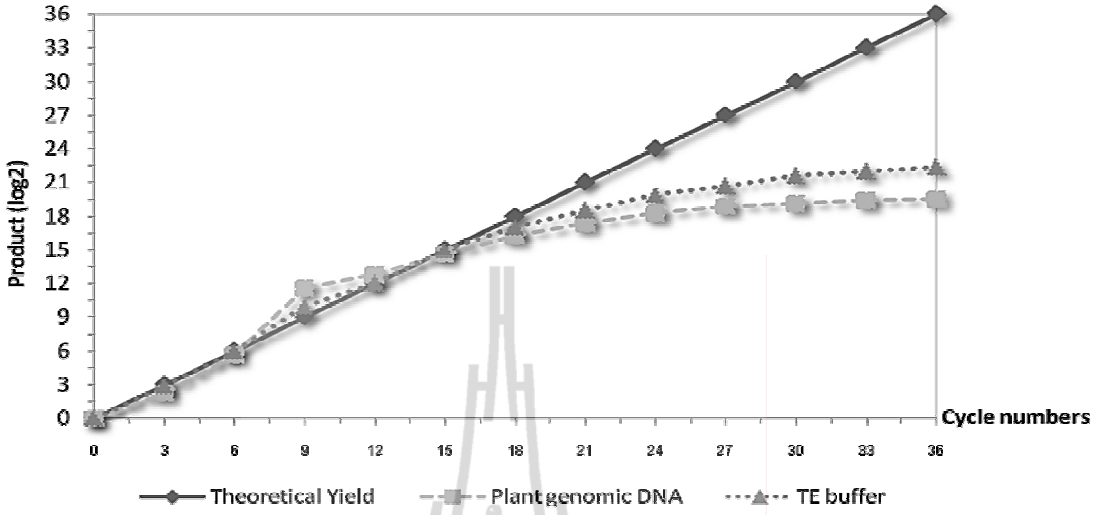

Figure 5.2 The effect of cycle number on T-RFs product based on peak area transformed into logarithm (log2), the product (log2) was compared with theoretical yield. T-RFs product generated from plasmid DNA pairwise mixes of clone 02 (*G. intraradices*) and clone 01 (*G. caledonium)* in 1: 1 ratio. PCR product amplified using the primer pair AML1-AML2, digested with *Hinf*I, and run PCR reaction at cycle number 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33 and 36.

5.5.2 Effect of plant genomic DNA purification methods on the template-to-product ratios in terms of relative T-RFs peak area ratio between different AM sequences

AM plasmid DNA mixtures were prepared in the 84 individual tube among triplicates (28 × 3 replicates = 84 reactions). All samples revealed equal band intensities in agarose gel electrophoresis after amplification (see appendix 2). To demonstrate the effect of plant genomic DNA purification, the DNA isolated from Ri-T-DNA transformed carrot root (*D. carota* L.) was mixed by AM plasmid DNA mixtures in different template ratios, which were amplified by PCR using

labelled primer (HEX-AML1 and AML2). As expected, plasmid DNA clone 02 (*G. intraradices*) was preferentially amplified over plasmid DNA clone 01 (*G. caledonium*) in all purification methods as shown by T-RF peak area (rfu; relative fluorescent unit) in Figure 5.3A and 5.3B. We assessed the influence of purifications on peak area value of individual peaks that were separated as T-RF fragment of plasmid DNA clone 02 (376 bp) and T-RF fragment of plasmid DNA clone 01 (516 bp) in the TRFLP profile. Different purification techniques, ethanol precipitation and phenol chloroform purification of plant genomic DNA appeared significantly different in peak area value (p <0.05, n=3, ANOVA) when the value of peak area was compared with presence of the non purified plant genomic DNA and absence of the plant genomic DNA (TE buffer used as a substitute). The peak area values were varied significantly in all DNA purifications obtained from AM sequence clone 01 and clone 02. The peak area values were higher in samples purified by the phenol chloroform than both the ethanol precipitation and without purification, if the equal plasmid template ratio was used. The absence of the mixed plant genomic DNA (TE buffer used instead) represents no contamination, provided the highest peak area values in both AM sequence clone 01 and clone 02 samples.

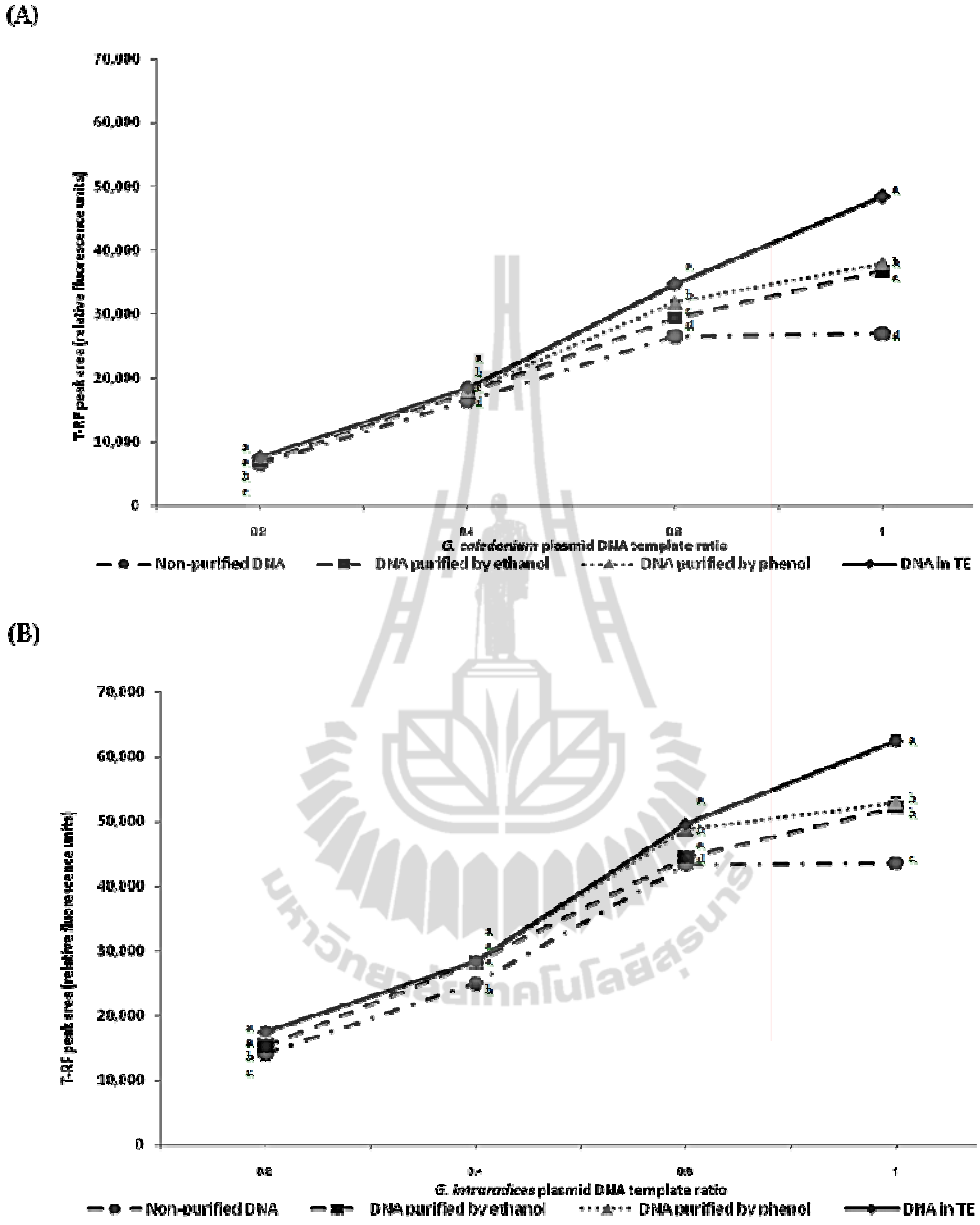

Figure 5.3 Comparison of T-RF peak area derived from mixing plasmid DNA of clone 01 (*G. caledonium)* : clone 02 (*G. intraradices)* in different plant DNA purification methods (A) T-RF peak area of clone 01 (*G. caledonium*) plasmid DNA in varying the ratio: 0.2, 0.4, 0.8, 1; (B) T-RF peak area of clone 02 (*G. intraradices)* plasmid DNA in varying the ratio: 0.2, 0.4, 0.8, 1; a, b, c, d: the letters within a line graph that are statistically significant ($P < 0.05$) are indicated by a different letter.

5.5.3 Bias comparison between expected and observed T-RF product ratios

Evaluation of the bias that caused variations in T-RFLP profile was demonstrated in Table 5.2 and 5.3. The underlying rationale of the experiments was that if bias is due to stochastic fluctuations (PCR drift), it would not be reproducible in replicate reactions, whereas if it is due to a property of the templates (PCR selection), the same pattern of bias would be observed in the individual amplifications. When signal ratios (referring to relative peak area ratios) for the different species pairs were compared, the ratios of AM plasmid DNA templates never corresponded to the ratios of the T-RF products (Table 5.2). If all AM plasmid DNA templates had been amplified with the same efficiency or the same procedure, all of the T-RF product ratios would have been similar to the ratios of the templates. The error due to variability among replications in the electrophoresis runs was small because a large number of replicates were done for all treatments. To estimate the magnitude of bias caused in pairwise mixing, we assumed that the T-RF proportions calculated from AM plasmid DNA mixes reflected PCR amplification bias (in the presence of complex plant genomic DNA without purification). Furthermore, we expected each template to yield equivalent T-RF proportions if no bias occurred. However, the results indicated that template and T-RF product proportions were not equivalent. The largest bias of AM plasmid DNA amplification was significantly observed in the treatment of non-purified plant genomic DNA mixtures (as shown in observed ratio I, Table 5.3), which was amplified with much higher efficiency than the treatment of plant genomic DNA purified by phenol chloroform (observed ratio III, Table 5.3) and ethanol precipitation (observed ratio II, Table 5.3). The extreme biases of template-to-product ratios occurred in terms of relative peak area ratio of AM plasmid DNA clone 02/clone 01 which were revealed in Table 5.2 as 12.79 ±

0.0002, 4.42 ± 0.0002, 2.07 ± 0.0056, and 1.62 ± 0.0024 following AM plasmid DNA ratio 1/0.2, 1/0.4, 1/0.8 and 1/1, respectively. While the same pattern in terms of relative peak area ratio of AM plasmid DNA clone 01/clone 02 was less amplified than theoretical yield, the differences between template and product ratios were considerably smaller in all cases. When the two AM plasmid DNA templates were compared, plasmid DNA clone 01 was less amplified than plasmid DNA clone 02. The data also revealed variations among the ratios of the individual T-RF products. However, some extreme cases, illustrated by the 1/0.2 ratio of plasmid DNA clone 02/clone 01 paired in observed proportion of relative peak area ratio I, differed 7.79-fold (bias magnitude) that was calculated under the difference between the expected (5-fold) and observed (12.79-fold) proportion (Table 5.3). Overamplification of plasmid DNA clone 02 template was decreased to ratios of 1/0.4, 1/0.8 and 1/1 in observed proportion of relative peak area ratio I as well as in observed proportion of relative peak area ratio II, III, and IV that decreased to ratios of 1/0.4, 1/0.8 and 1/1, sequentially (Table 5.3).

Table 5.2 Comparison of expected and observed AM 18S rDNA gene templates and T-RF products in terms of relative peak area ratio[1] using PCR-TRFLP for amplifications of plasmid DNA clone 02 / plasmid DNA clone 01 mixtures and plasmid DNA clone 01 / plasmid DNA clone 02 mixtures.

Proportion of plasmid DNA template mixtures	**[2]Plasmid DNA Proportion clone 02/clone 01**				**[3]Plasmid DNA Proportion clone 01/clone 02**			
	1 / 0.2	**1 / 0.4**	**1 / 0.8**	**1/1**	**1 / 0.2**	**1 / 0.4**	**1 / 0.8**	**1/1**
Expected plasmid DNA template-to-product ratios	5	2.5	1.25	1	5	2.5	1.25	1
(Proportion of relative peak area ratios)	(0.8333/0.1667)[4]	(0.7143/0.2867)[4]	(0.5556/0.4444)[4]	(0.50/0.50)[4]	(0.8333/0.1667)[5]	(0.7143/0.2867)[5]	(0.5556/0.4444)[5]	(0.50/0.50)[5]
Observed proportion of Relative peak area ratio I [6]	$12.79^{a} \pm 0.0002^{*}$	$4.42^{a} \pm 0.0002$	$2.07^{a} \pm 0.0056$	$1.62^{a} \pm 0.0024$	$4.51^{b} \pm 0.0058$	$2.17^{b} \pm 0.0051$	$0.82^{c} \pm 0.0026$	$0.62^{c} \pm 0.0024$
(Proportion of relative peak area ratios)	(0.9275/0.0725)	(0.8154/0.1846)	(0.6739/0.3261)	(0.6176/0.3824)	(0.8187/0.1813)	(0.6847/0.3152)	(0.45/0.55)	(0.3824/0.6176)
Observed proportion of Relative peak area ratio II [7]	$12.02^{b} \pm 0.0001$	$4.06^{b} \pm 0.0005$	$1.83^{b} \pm 0.0009$	$1.42^{b} \pm 0.0047$	$4.52^{b} \pm 0.0001$	$2.17^{b} \pm 0.0108$	$0.82^{c} \pm 0.0122$	$0.71^{b} \pm 0.0047$
(Proportion of relative peak area ratios)	(0.9232/0.0768)	(0.8023/0.1977)	(0.6470/0.3530)	(0.5865/0.4135)	(0.8189/0.1811)	(0.6848/0.3152)	(0.45/0.55)	(0.4135/0.5865)
Observed proportion of Relative peak area ratio III [8]	$11.14^{c} \pm 0.0016$	$4.00^{c} \pm 0.0014$	$1.80^{b} \pm 0.0109$	$1.39^{b} \pm 0.0115$	$5.23^{a} \pm 0.0097$	$2.27^{b} \pm 0.0084$	$0.95^{b} \pm 0.0120$	$0.72^{b} \pm 0.0115$
(Proportion of relative peak area ratios)	(0.9176/0.0824)	(0.80/0.20)	(0.6432/0.3568)	(0.5822/0.4178)	(0.8396/0.1604)	(0.6938/0.3062)	(0.4867/0.5133)	(0.4178/0.5822)
Observed proportion of Relative peak area ratio IV [9]	$11.14^{c} \pm 0.0002$	$3.88^{d} \pm 0.0002$	$1.79^{b} \pm 0.0054$	$1.29^{c} \pm 0.0015$	$5.08^{a} \pm 0.0011$	$2.58^{a} \pm 0.0033$	$1.03^{a} \pm 0.0057$	$0.77^{a} \pm 0.0015$
(Proportion of relative peak area ratios)	(0.9176/0.0824)	(0.7952/0.2048)	(0.6422/0.3578)	(0.5635/0.4365)	(0.8356/0.1644)	(0.7206/0.2794)	(0.5070/0.4930)	(0.4365/0.5635)

*****Means ± standard deviations were calculated from data in triplicates runs for each of triplicate PCR reactions, and mean values within a column that are statistically significant ($P<0.05$) are indicated by a different letter.

1Relative peak area ratio was calculated from dividing individual T-RF peak area values by the sum of all peak area values in the corresponding profile.

2The data were generated from dividing relative peak area ratio of plasmid DNA clone 02 by relative peak area ratio of plasmid DNA clone 01.

3The data were generated from dividing relative peak area ratio of plasmid DNA clone 01 by relative peak area ratio of plasmid DNA clone 02.

4Expected relative peak area ratio in pairwise mixing of plasmid DNA clone 02/plasmid DNA clone 01.

5Expected relative peak area ratio in pairwise mixing of plasmid DNA clone 01/plasmid DNA clone 02.

6Observed proportion of relative peak area ratio I were determined by PCR-TRFLP amplifications of AM plasmid DNA mixtures that were mixed with Ri-T-DNA-transformed carrot root genomic DNA (non-purified DNA).

7Observed proportion of relative peak area ratio II were determined by PCR-TRFLP amplifications of AM plasmid DNA mixtures that were mixed with Ri-T-DNA-transformed carrot root genomic DNA purified by ethanol precipitation.

8Observed proportion of relative peak area ratio III were determined by PCR-TRFLP amplifications of AM plasmid DNA mixtures that were mixed with Ri-T-DNA-transformed carrot root genomic DNA purified by phenol chloroform.

9Observed proportion of relative peak area ratio IV were determined by PCR-TRFLP amplifications of AM plasmid DNA mixtures that were mixed with TE buffer.

Table 5.3 Comparison of expected and observed proportion of T-RFs relative peak area ratios in pairwise mixing experiment and calculated bias.

Proportion of plasmid DNA template mixtures	[1]Plasmid DNA Proportion clone 02/clone 01				[2]Plasmid DNA Proportion clone 01/clone 02			
	1 / 0.2	**1 / 0.4**	**1 / 0.8**	**1/1**	**1 / 0.2**	**1 / 0.4**	**1 / 0.8**	**1/1**
[3]Expected	5	2.5	1.25	1	5	2.5	1.25	1
[4]Observed proportion of relative peak area ratio I	$12.79^{a} \pm 0.0002^{*}$	$4.42^{a} \pm 0.0002$	$2.07^{a} \pm 0.0056$	$1.62^{a} \pm 0.0024$	$4.51^{b} \pm 0.0058$	$2.17^{b} \pm 0.0051$	$0.82^{c} \pm 0.0026$	$0.62^{c} \pm 0.0024$
[5]Bias I	$7.79^{(+)}$	$1.92^{(+)}$	$0.82^{(+)}$	$0.62^{(+)}$	$0.49^{(-)}$	$0.33^{(-)}$	$0.43^{(-)}$	$0.38^{(-)}$
[4]Observed proportion of relative peak area ratio II	$12.02^{b} \pm 0.0001$	$4.06^{b} \pm 0.0005$	$1.83^{b} \pm 0.0009$	$1.42^{b} \pm 0.0047$	$4.52^{b} \pm 0.0001$	$2.17^{b} \pm 0.0108$	$0.82^{c} \pm 0.0122$	$0.71^{b} \pm 0.0047$
[5]Bias II	$7.02^{(+)}$	$1.56^{(+)}$	$0.58^{(+)}$	$0.42^{(+)}$	$0.48^{(-)}$	$0.33^{(-)}$	$0.43^{(-)}$	$0.29^{(-)}$
[4]Observed proportion of relative peak area ratio III	$11.14^{c} \pm 0.0016$	$4.00^{c} \pm 0.0014$	$1.80^{b} \pm 0.0109$	$1.39^{b} \pm 0.0115$	$5.23^{a} \pm 0.0097$	$2.27^{b} \pm 0.0084$	$0.95^{b} \pm 0.0120$	$0.72^{b} \pm 0.0115$
[5]Bias III	$6.14^{(+)}$	$1.50^{(+)}$	$0.55^{(+)}$	$0.39^{(+)}$	$0.23^{(+)}$	$0.23^{(-)}$	$0.30^{(-)}$	$0.28^{(-)}$
[4]Observed proportion of relative peak area ratio IV	$11.13^{c} \pm 0.0002$	$3.88^{d} \pm 0.0002$	$1.79^{b} \pm 0.0054$	$1.29^{c} \pm 0.0015$	$5.08^{a} \pm 0.0011$	$2.58^{a} \pm 0.0033$	$1.03^{a} \pm 0.0057$	$0.77^{a} \pm 0.0015$
[5]Bias IV	$6.13^{(+)}$	$1.38^{(+)}$	$0.54^{(+)}$	$0.29^{(+)}$	$0.08^{(-)}$	$0.08^{(-)}$	$0.22^{(-)}$	$0.23^{(-)}$

[*]Means ± standard deviations were calculated from data in triplicates runs for each of triplicate PCR reactions, and mean values within a column that are statistically significant ($P<0.05$) are indicated by a different letter.

[1]The data were generated from dividing relative peak area ratio of plasmid DNA clone 02 by relative peak area ratio of plasmid DNA clone 01.

[2]The data were generated from dividing relative peak area ratio of plasmid DNA clone 01 by relative peak area ratio of plasmid DNA clone 02.

[3]Expected proportion of relative peak area ratio in AM pairwise mixing

[4]Observed proportion of relative peak area ratio in AM pairwise mixing with

I:Ri-T-DNA-transformed carrot root genomic DNA (non-purified DNA)

II: Ri-T-DNA-transformed carrot root genomic DNA purified by ethanol precipitation

III: Ri-T-DNA-transformed carrot root genomic DNA purified by phenol chloroform

IV: TE buffer

[5]The magnitude of bias attributable to PCR amplification is the difference between expected and observed mean proportion of relative peak area ratios

I: AMF plasmid DNA mixtures that were mixed with Ri-T-DNA-transformed carrot root genomic DNA (non-purified DNA)

II: AMF plasmid DNA mixtures that were mixed withRi-T-DNA-transformed carrot root genomic DNA purified by ethanol precipitation

III: AMF plasmid DNA mixtures that were mixed withRi-T-DNA-transformed carrot root genomic DNA purified by phenol chloroform

IV: AMF plasmid DNA mixtures that were mixed with TE buffer

[(+)]The magnitude of bias is overestimated from expected proportions.

[(-)] The magnitude of bias is underestimated from expected proportions.

5.6 Discussions

Most of the studies describing the use of T-RFLP to monitor microbial populations have focused on the qualitative characterization of environmental samples, but very few have analyzed the quantitative possibilities of this technique (Bruce, 1997; Liu *et al*., 1997; Osborn *et al*., 2000; Schütte *et al.,* 2008). The limitations of T-RFLP for quantitative characterization of communities have been well argued, since it is subject to drawbacks related to PCR amplifications. These problems become more evident when working with complex ecosystems in which diversity of the microorganisms is high, with differences in the ability to extract DNA from different organisms, genome size, and G+C content, all of which may lead to differential amplification (Suzuki *et al*., 1998; Marsh *et al*., 2000). In experiments presented here, pairwise mixtures in different proportions were used as templates for T-RFLP amplification. To measure bias produced by PCR, we compared the proportions of genes in the T-RF products with their proportions in the template mixture. Our results indicated that T-RF products ratios can be significantly biased in amplifications of mixed templates (Table 5.2 and 5.3). The bias was caused by the mixed templates amplification competition during PCR reaction. The amplification competition might occur by different melting temperatures of templates due to different GC contents (Reysenbach et al. 1992), and occur if the target DNA is sufficiently degraded or impure. Sample purify and DNA template concentration during PCR are crucial parameters when analyzing environmental nucleic acids, due to the interferences of humic acids or phenolic compounds on *Taq* polymerase and other commonly used enzymes (Tebbe and Vahjen, 1993). Frequently, environmental nucleic acids are diluted to a point where the concentration of interference material in

the extract is no longer inhibitory to the PCR (Herrick et al., 1993). However, template dilution also reduces the number of target genes during PCR. In our experiment tested here, the constructed realistic plasmid DNA pairwise mixtures contained sufficient nanogram quantities of templates (~0.12-0.60 ng plasmid DNA) or pictogram quantities plasmid DNA templates in PCR reaction (6-30 picogram plasmid DNA) for amplification. The observed bias was caused by these factors may alter the amplification efficiency of individual sequences in multi-template samples and appeared to have influenced individual T-RFs peak area ratio in our study. Errors associated with multi-template mixtures could occur in amplification or subsequent steps (digestion or TRF-run). In our study, it is difficult to assess which step is more problematic, as we did not test each separately. Polz and Cavanaugh (1998) suggest that PCR protocols typically designed for single species sample amplification can lead to bias when multi-species samples are targeted, because of low DNA template amounts and an increasing probability of PCR bias with more PCR cycles.

Primers AML1-AML2 (Lee et al. 2008) were considered to use in our study because they give much more specificity to the *Glomeromycota* and provide more accurate representation of the AM community. The possibility that the bias was caused by primer mismatches was excluded since sequencing of 18S rDNA showed that the primers completely matched the AM plasmid DNA clones. Preferential amplification may also be induced by different flanking sequences of the target (Hansen et al., 1998) or different accessibility of the target within the genome (Farrelly et al. 1995; Trotha et al. 2002). Using cloned homologous 18S rRNA gene fragments, the influence of these factors was intentionally excluded. Although the orientation of the insert within the vector might influence amplification efficiency, no

correlation was found between insert orientation and amplification efficiency. Differences in the GC contents and consequently in the melting temperatures of the target sequence may lead to variations in amplification efficiency and product quantity (Reysenbach et al. 1992; Dutton et al. 1993). The extreme over amplification of the plasmid DNA clone 02 template might be caused by higher primer affinity for the priming region due to higher GC content. Differences in the GC contents of the 18S rDNA templates or the whole genomes may lead to differential denaturation of templates. However, in the case of plasmid DNA clone 02 and clone 01 used, their GC contents are only slightly different; insert of plasmid DNA clone 02 contain 43% GC, whereas insert of plasmid DNA clone 01 contain 42% GC.

Disagreement in template to product ratios may originate from different PCR plateau levels reached by each sequence at larger cycle numbers, e.g. those induced by substrate limitation, polymerase inactivation or inhibition, and different template denaturation and reannealing kinetics (Mathieu-Daud´e et al. 1996; Suzuki and Giovannoni 1996; Kainz 2000). Other studies, however, reported a small influence of increasing cycle number on the reproducibility of bacterial (Osborn et al. 2000) and archaeal T-RFLP profiles (Lueders and Friedrich 2003). Adjustment of PCR conditions by adding sufficient template and PCR components, applying low cycle numbers, and using primers with high binding temperatures will help in reducing PCR-related biases although not in avoiding them.

5.7 Conclusions

The results presented here had implications for studies using T-RFLP to amplify AM 18S rDNAs from DNA samples of natural ecosystems. Based on our results pointed out in this paper that preferential amplification induced remarkable

shifts in the T-RFLP community profiles and limit the quantitative assessment of real community compositions using PCR-based approaches. Even in the simple two-species community tested, relatively large bias was observed. This emphasized the fact that quantitative interpretation of PCR-based results should still be viewed with caution. A major limitation of the established DNA fingerprinting techniques for microbial community analysis is the complexity of rDNA fragments derived from the extreme complexity of ecosystems (Nakatsu et al. 2000). Furthermore, different species will have different gene copy numbers and this can also bias the interpretation of fingerprinting results (Liu et al. 1997). As a result of these biases, representation of the real microbial community and diversity in ecosystems will probably not be possible. However, the T-RFLP approach appeared to reliably detect the relative changes in microbial community structures with a high quantitative precision, which is a prerequisite for monitoring effects on microbial communities.

5.8 References

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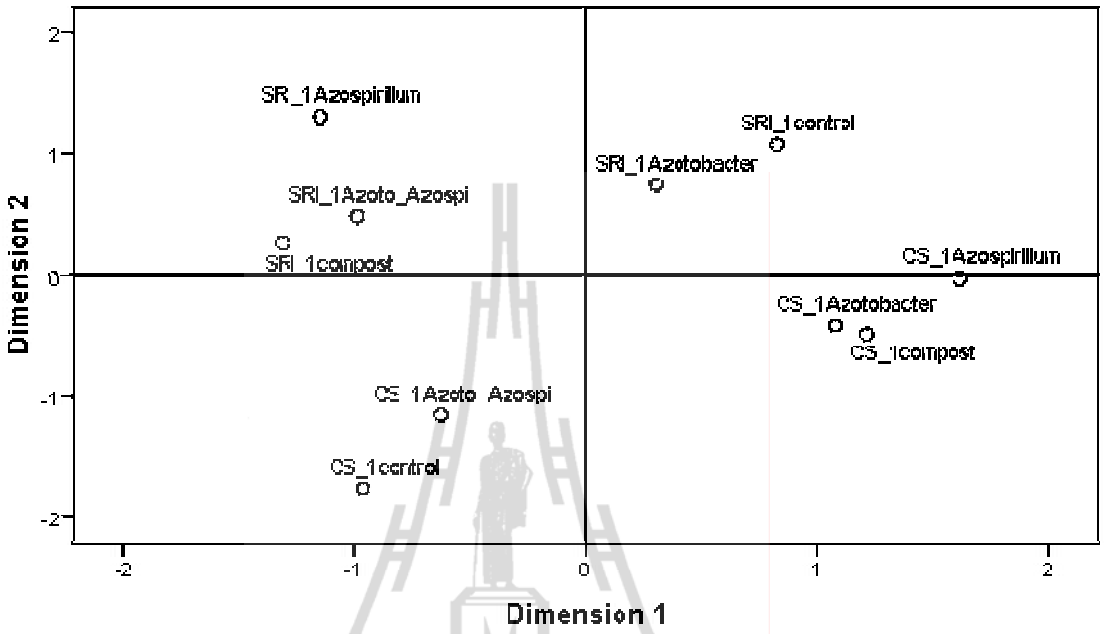
APPENDICES

(a)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2
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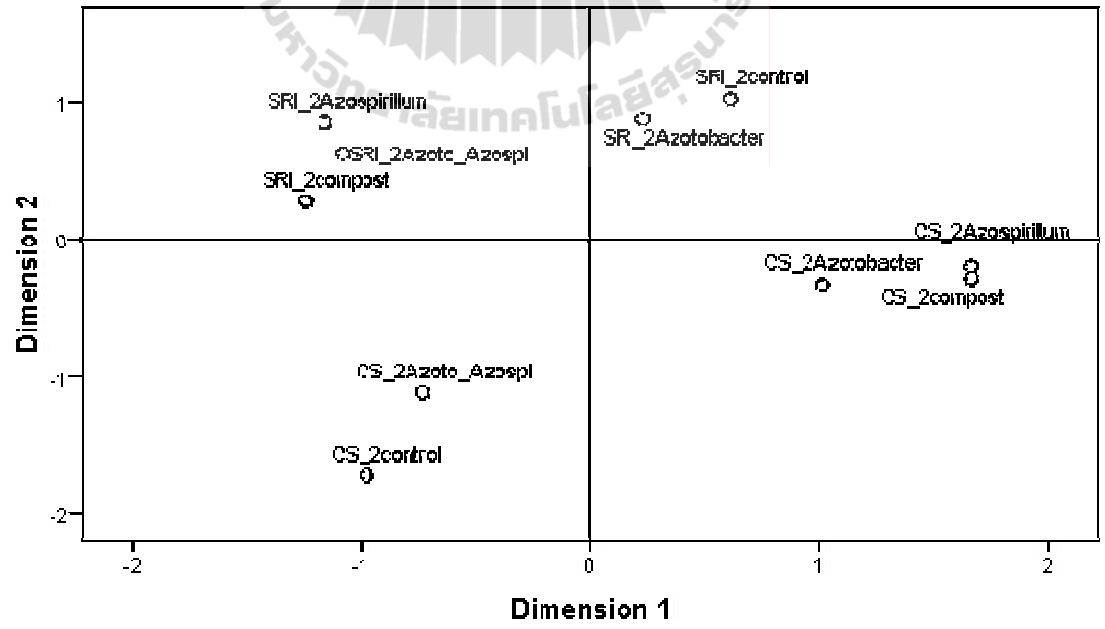
0
-1
-2

(b)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CS_2Azospirillum
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CS_2Azoto_Azospi
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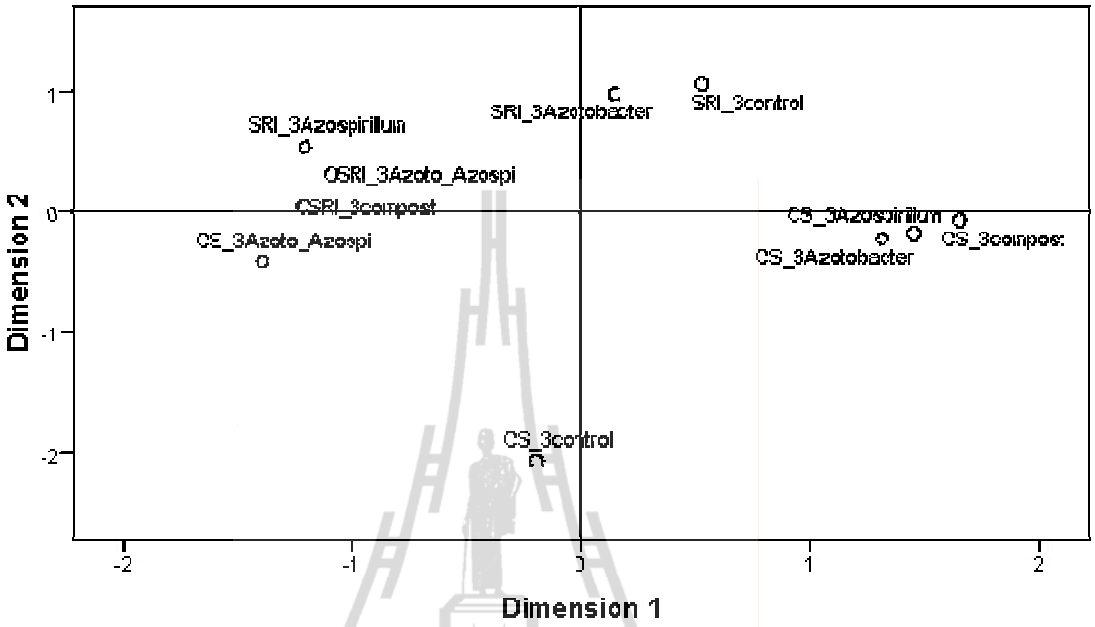
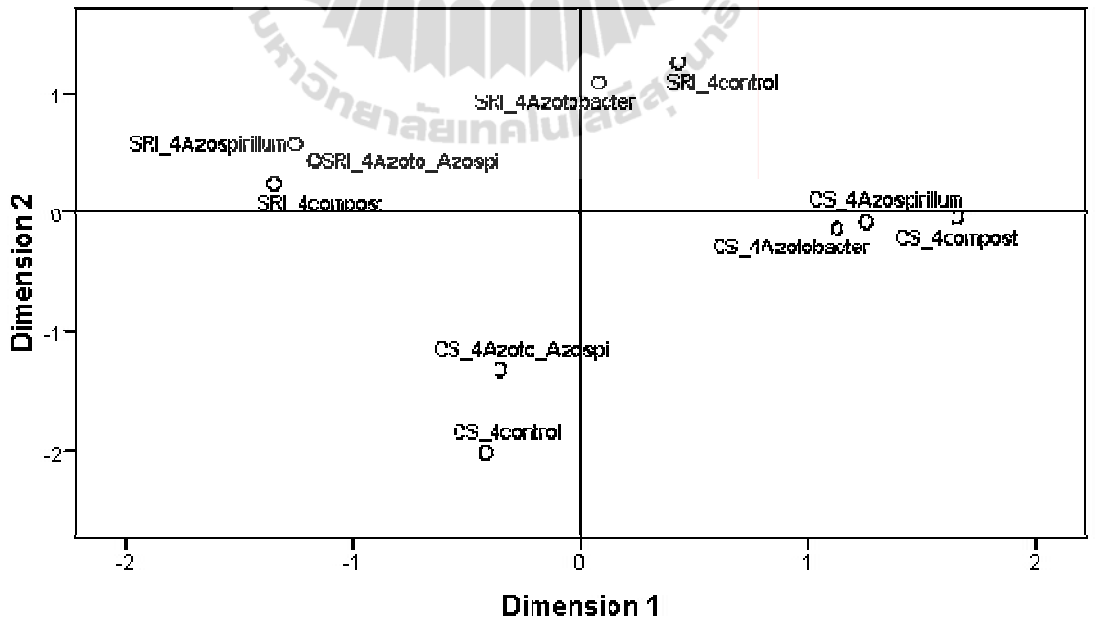
0
-1
-2

(c)

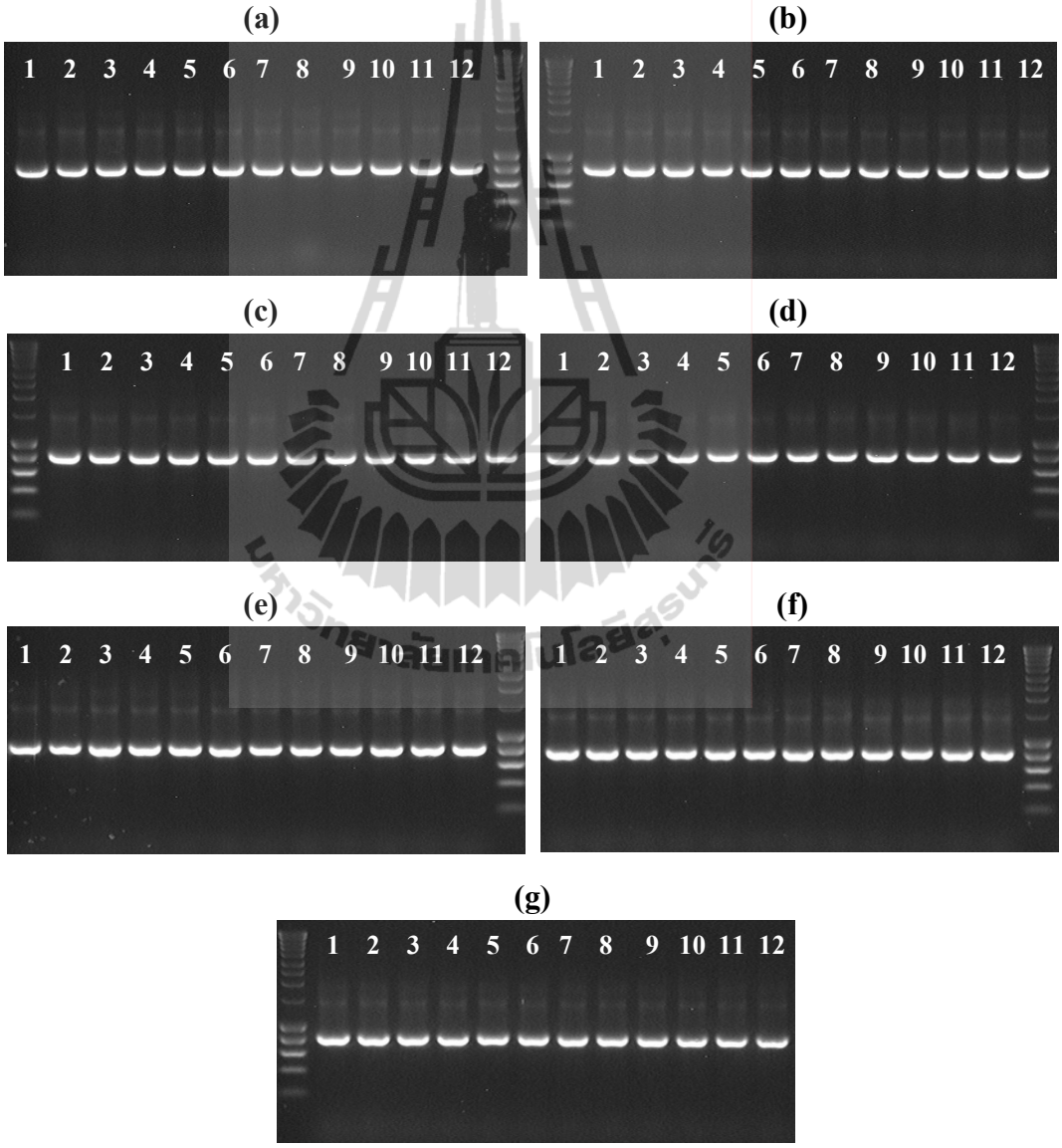


(d)



APPENDIX 1 Multidimensional scaling (distances as interval; SPSS 17.0) plot of the AM fungal community composition found in the roots of rice plants grown under the two different cultivations (CS and SRI), and the five

different PGPR and compost inoculations (control, compost, compost × *Az. vinelandii*, compost × *A. largimobile* and compost × *Az. vinelandii* × *A. largimobile*). Rice roots sampled at (a) the first stage; (b) the second stage; (c) the third stage; and (d) the last stage of growth.



APPENDIX 2 SSU rRNA products were amplified from mixing plasmid DNA *G.caledonium* and *G.intraradices* with different methods of purified plant DNA (non purified plant DNA, plant DNA purified by ethanol

precipitation, plant DNA purified by phenol chloroform and absence of plant DNA, TE) and different ratio using primers AML1 and AML2. PCR products (~800 bp) were separated on 1% agarose gel with three replicates (lane 1-3: absence of plant DNA (added TE buffer), lane 4-6: phenol-chloroform method, lane 7-9: ethanol precipitation, lane 10-12: no purification of plant DNA). The letters indicate **(a)** plasmid DNA mixture of *G. caledonium* and *G. intraradices* in ratio 1:1; **(b)** plasmid DNA mixture of *G. caledonium* and *G. intraradices* in ratio 0.8:1; **(c)** plasmid DNA mixture of *G. caledonium* and *G. intraradices* in ratio 0.4:1; **(d)** plasmid DNA mixture of *G. caledonium* and *G. intraradices* in ratio 0.2:1; **(e)** plasmid DNA mixture of *G. caledonium* and *G. intraradices* in ratio 1:0.8; **(f)** plasmid DNA mixture of *G. caledonium* and *G. intraradices* in ratio 1:0.4; **(g)** plasmid DNA mixture of *G. caledonium* and *G. intraradices* in ratio 1:0.2.

BIOGRAPHY

Miss Nantida Watanarojanaporn was born on April 18th, 1980 in Bangkok, Thailand. She graduated with the Bachelor Degree of Science (Soil Science), King Mongkut's Institute of Technology Ladkraband, Thailand in 2002. During her Doctoral Degree enrollment in School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology (2004-2010), she had received a scholarship from the Royal Golden Jubilee (RGJ) grant of the Thailand Research Fund in 2005 and she had presented research work in the RGJ-Ph.D. Congress XI, the title of "Biases for detecting microbial community by terminal restriction fragment length polymorphism (T-RFLP)" (Poster presentation), April 1-3, 2010, Jomtien Palm Beach Hotel and Resort, Chonburi, Thailand. She had presented research work in the 1st Asian Conference on Plant-Microbe Symbiosis and Nitrogen Fixation (Poster presentation), September 20-24, 2010, Aoshima Palmbeach Hotel, Miyazaki, Japan, and also attended the 3rd SUT Graduate Conference 2010, in the title of "Effect of PGPR inoculation on indigenous arbuscular mycorrhiza (AM) fungi community under two rice growing systems" (Poster presentation), November 21-23, 2010, Suranaree University of Technology, Nakhon Ratchasima, Thailand. Her works have been published in Scientia Horticulturae in the topic of "Selection of arbuscular mycorrhizal fungi for citrus growth promotion and *Phytophthora* suppression" 2011.