SELECTION OF ARBUSCULAR MYCORRHIZA FOR APPLICATION IN BERMUDA GRASS (*Cynodon dactylon*)

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

Suranaree University of Technology

Academic Year 2018

การคัดเลือกอาร์บัสคูลาร์ไมคอร์ไรซาเพื่อการประยุกต์ใช้กับหญ้าเบอร์มิวดาร์ (*Cynodon dactylon*)

นางสาวนิรมล ลักขษร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

สาขาวิชาเทคโนโลยีชีวภาพ

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ปีการศึกษา 2561

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APPLICATION IN BERMUDA GRASS

(*Cynodon dactylon*)

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

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นิรมล ลักขษร : การคัดเลือกอาร์บัสคูลาร์ไมคอร์ไรซาเพื่อการประยุกต์ใช้กับหญ้าเบอร์มิวดาร์ (SELECTION OF ARBUSCULAR MYCORRHIZA FOR APPLICATION IN BERMUDA GRASS (*Cynodon dactylon*)) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.พรรณลดา ติตตะบุตร, 94 หน้า.

ราอาร์บัสคูลาร์ไมคอร์ไรซาสามารถอาศัยในสภาวะพึ่งพาซึ่งกันและกันได้กับพืชจำนวนมากถึงร้อยละเก้าสิบของพืชบนโลกนี้ โดยทำหน้าที่ช่วยในการดูดซับธาตุอาหารจากดินมาสู่พืช ทั้งนี้วัตถุประสงค์ของการศึกษานี้เพื่อคัดแยกราอาร์บัลคูลาร์ไมคอร์ไรซาที่มีประสิทธิภาพสำหรับประยุกต์ใช้เป็นปุ๋ยชีวภาพให้กับหญ้าเบอร์มิวดาร์ (*Cynodon dactylon*) ที่เจริญทั้งในสภาวะดินปกติ และดินกรด การคัดแยกเชื้อทำโดยเก็บตัวอย่างดินจากสนามกอล์ฟ 4 แห่งในประเทศไทย นำมาตรวจสอบความหลากหลายของราอาร์บัสคูลาร์ไมคอร์ไรซาในดินแต่ละแห่ง และความสามารถในการกลับเข้าไปอยู่อาศัยร่วมกับหญ้าเบอร์มิวดาร์ ทั้งนี้จากการตรวจสอบสัณฐานของสปอร์ร่วมกับการอ่านลำดับเบสของดีเอ็นเอที่แยกได้จากสปอร์เดี่ยว และร่วมกับการวิเคราะห์แผนภูมิต้นไม้ แสดงให้เห็นว่าราอาร์บัสคูลาร์ไมคอร์ไรซาที่พบส่วนใหญ่ในดินอยู่ในสกุล *Claroideoglomus* และ *Acaulospora* จากนั้นนำสปอร์ของราอาร์บัสคูลาร์ไมคอร์ไรซาที่พบจำนวนประชากรมากที่สุดจากดินในแต่ละแหล่ง (ไอโซเลท Sur1, PT1, Tos1 และ Tig1) มาทำการเพิ่มจำนวนสปอร์ในพืชอาศัยชนิดต่าง ๆ ผลการทดลองพบว่า สปอร์ของไอโซเลท Tos1สามารถผลิตได้มากเมื่อใช้ต้นข้าวโพด และต้นข้าวฟ่างเป็นพืชอาศัย ในขณะที่สปอร์ของไอโซเลท Sur1 และ PT1 ผลิตได้มากเมื่อใช้หญ้าเบอร์มิวดาร์ และต้นข้าวฟ่างเป็นพืชอาศัย นอกจากนี้ต้นหญ้าเบอร์มิวดาร์ยังเป็นพืชอาศัยที่ใช้สำหรับการผลิตสปอร์ของไอโซเลท Tig1 ได้อีกด้วย แสดงให้เห็นว่า ราอาร์บัสคูลาร์ไมคอร์ไรซามีความชอบ หรือมีการเลือกชนิดของพืชอาศัยเพื่อเพิ่มจำนวน จากนั้นนำสปอร์ของราอาร์บัสคูลาร์ไมคอร์ไรซาที่ได้มาทดสอบเพื่อตรวจสอบความสามารถของราอาร์บัสคูลาร์ไมคอร์ไรซาประจำถิ่นที่คัดแยกได้ในการส่งเสริมการเจริญของหญ้าเบอร์มิวดาร์เมื่อปลูกในสภาวะดินกรด (pH3.29) เพื่อให้สามารถแยกแยะความแตกต่างของประสิทธิภาพการส่งเสริมการเจริญเติบโตของราอาร์บัสคูลาร์ไมคอร์ไรซาไอโซเลทต่าง ๆ เนื่องจากดินกรดมักทำให้พืชขาดธาตุอาหารที่สำคัญ และต้องการใช้ราอาร์บัสคูลาร์ไมคอร์ไรซาเพื่อช่วยในการละลายธาตุอาหารจากดินเข้าสู่พืช ผลการทดสอบพบว่า ราอาร์บัสคูลาร์ไมคอร์ไรซา ไอโซเลท Sur1 และ PT1 สามารถส่งเสริมการเจริญของหญ้าได้ดีกว่าการใช้ไอโซเลท Tos1 และที่ไม่ได้ใช้หัวเชื้ออย่างมีนัยสำคัญ โดยพบว่าประสิทธิภาพการเข้าครอบครองรากของทั้งไอโซเลท Sur1 และ PT1 มีอัตราสูงกว่าการใช้ไอโซเลท Tos1 อย่างชัดเจน ดังนั้นผลการทดสอบนี้ แสดงให้เห็นถึงศักยภาพของการใช้ราอาร์บัสคูลาร์ไมคอร์ไรซาที่คัดแยกได้ในการเป็นปุ๋ยชีวภาพเพื่อส่งเสริมการเจริญของหญ้าเบอร์มิวดาร์ และเพื่อเป็นการเพิ่มประสิทธิภาพของหัวเชื้อราอาร์บัสคูลาร์ไมคอร์ไรซา จึงได้ตรวจสอบผลของการใช้หัวเชื้อราอาร์บัสคูลาร์ไมคอร์ไรซาร่วมกับหัวเชื้อแบคทีเรียที่ส่งเสริมการเจริญของพืช (พีจีพีอาร์) โดยการทดลองนี้ใช้ราอาร์บัสคูลาร์ไมคอร์ไรซาสายพันธุ์การค้า คือ *Rhizophagus irregularis* ทดสอบร่วมกับ

การใช้หัวเชื้อพีจีพีอาร์ *Pseudomonas* sp. SUT19 ซึ่งเคยมีรายงานมาแล้วว่าสามารถส่งเสริมการเจริญของพืชได้ด้วยกลไกต่าง ๆ เช่น การตรึงไนโตรเจน การสร้างฮอร์โมนพืช และกิจกรรมของเอนไซม์ ACC deaminase ทั้งนี้ทำการทดสอบทั้งในสภาวะดินปกติ (pH6.5) และดินกรด (pH3.29) ผลการทดลองพบว่าค่าความสามารถในการเข้าครอบครองรากทั้งระบบ (M%) หรือการเข้าครอบครองในแต่ละชิ้นส่วนของราก (m%) ตลอดจนการสร้างอาร์บัสคูลในรากทั้งระบบ (A%) เพิ่มขึ้นอย่างมีนัยสำคัญเมื่อใช้หัวเชื้อพีจีพีอาร์ร่วมกันกับหัวเชื้อราอาร์บัสคูลาร์ไมคอร์ไรซา เมื่อปลูกในสภาวะดินปกติ อย่างไรก็ตามไม่เห็นผลความแตกต่างเมื่อทดสอบในสภาวะดินกรด และไม่เห็นผลความแตกต่างทางด้านการเจริญของพืชที่ชัดเจน แสดงให้เห็นว่าการใช้หัวเชื้อ 2 ชนิดร่วมกันนี้จำเป็นต้องพิจารณาถึงอิทธิพลของความเข้ากันได้ระหว่างพืชกับหัวเชื้อพีจีพีอาร์ และความเข้ากันได้ระหว่างหัวเชื้อราอาร์บัสคูลาร์ไมคอร์ไรซากับหัวเชื้อพีจีพีอาร์ เพื่อให้เกิดประโยชน์ต่อการส่งเสริมการเจริญของพืชในแต่ละชนิดร่วมกันต่อไป

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2561

ลายมือนักศึกษา _____
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NIRAMON LAKKASON : SELECTION OF ARBUSCULAR MYCORRHIZA FOR APPLICATION IN BERMUDA GRASS (*Cynodon dactylon*).
THESIS ADVISOR : ASSOC. PROF. PANLADA TITTABUTR, Ph.D., 94 PP.

ARBUSCULAR MYCORRHIZA/BERMUDA GRASS/GOLF COURSE/ PHOSPHORUS/BIOFERTILIZER

Arbuscular Mycorrhizal Fungi (AMF) could symbiosis with 90% of land plants and facilitated nutrients acquisition to their host. The objective of this study was to isolate an effective AM fungus for Bermuda grass (*Cynodon dactylon*) under neutral and acidic soil conditions as biofetilizer. The rhizospheric soils were collected from four golf courses in Thailand to observe the presence of indigenous AM fungi and their ability to re-colonize Bermuda grass. The results showed that AM fungi were found in all soil samples and had the ability to re-colonize the grass. The spore morphology together with single spore DNA sequencing and phylogenetic tree analyses revealed that most fungi belonged to the genera *Claroideoglomus* and *Acaulospora*. The dominant AM fungal species from each soil sample, including isolates Tos1, Sur1, PT1, and Tig1, were selected to propagate using different host plants. Tos1 significantly produced a high spore number when maize and sorghum were used as a host plant, while the spores of Sur1 and PT1 were highly produced in Bermuda grass and sorghum roots. Bermuda grass was also the best host for spore production of the isolate Tig1. These results indicated the preference among AM fungus and host plants for propagation. Then, the potential of these indigenous AM fungi on Bermuda grass growth promotion under strongly acidic soil (pH 3.29) was investigated. The acidic condition could facilitate the distinguishability between effective and non-effective AM fungi. It was found that the AM fungi isolates Sur1 and PT1 promoted greater grass growth than using isolate Tos1 and non-inoculated plants. The colonization efficiency of isolates

Sur1 and PT1 was clearly better than that of isolate Tos1. These results revealed the potential of using indigenous AM fungal strains as biofertilizer for Bermuda grass. To increase the efficiency of AM fungal inoculum, the strategy of co-inoculation AM fungus with Plant Growth Promoting Rhizobacteria (PGPR) was investigated. The commercial AM fungal strain of *Rhizophagus irregularis* was co-inoculated with *Pseudomonas* sp. SUT19, which has been reported to promote plant growth through nitrogen fixation, indole acetic (IAA) production, and ACC deaminase activity. The experiment was performed in both neutral (pH6.5) and acidic (pH3.29) soils. The results demonstrated that the intensity of mycorrhizal colonization in the root system (M%) and in the root fragment (m%) as well as the arbuscule abundance in the root system (A%) were significantly increased when co-inoculated with SUT19 under neutral soil conditions. However, there was no difference in colonization efficiency between AM and AM+SUT19 plants under acidic soil condition. This indicated that the synergistic effect may be influenced by the specific preference among tripartites, and this factor should be considered if using co-inoculation to promote the specific plant growth.

School of Biotechnology

Academic Year 2019

Student's Signature Niramon Lakkason.

Advisor's Signature

Co-Advisor's Signature

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ACKNOWLEDGEMENT

I would like to express the deepest and sincere gratitude to my research advisor, Assoc. Prof. Dr. Panlada Tittabuther who dedicate and paying attention to me, especially for my research in Master's Degree, it is great privilege and honor to work and study under her guidance. I would also convey the deepest appreciation to Co-Advisors Prof. Dr. Nantakorn Boonkerd and Prof. Dr. Neung Teaumroong, which dynamism, vision, sincerity and motivation have deeply inspired me and guided into the paths of righteousness. I am extremely grateful for the generous of Dr. Pongpan Songwattana for helping in part of molecular works as well as giving me guidance. She has all the love and helpfulness in world right inside her.

I am extremely grateful to my parents for their love, caring, understanding and sacrifices for educating and preparing me for my future. It was pressure to study under the social culture that expected with a career success after graduation which sometime causes discouragement.

I would like to express the deepest appreciation to Dr. Kamonluck Teamtisong and Ms. Prangkhaw Prukhett who are kindness people, caring and understanding me like my own family, to support equipments and providing advice. I also would to say thanks to my friends and all NPN Lab member who have supported me to complete the research work both directly or indirectly ways.

Finally, I would like to thank Suranaree University of Technology OROG scholarship for support my opportunities to study at the master's degree in School of Biotechnology.

Niramon Lakkason

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LIST OF ABBREVIATIONS

°C	=	degree Celsius
µm	=	micrometer
ACC	=	1-aminocyclopropane-1-carboxylic acid
AM	=	Arbuscular mycorrhiza
bp	=	base pair
DNA	=	deoxyribonucleic acid
et al.	=	Et alia (and other)
g	=	gram
IAA	=	Indole-3-acetic acid
l	=	litre
mg	=	milligram
Mg	=	Magnesium
min	=	minute
ml	=	milliliter
mM	=	millimolar
N	=	Nitrogen
OM	=	Organic matter
PCR	=	polymerase chain reaction
PGPR	=	Plant Growth Promoting Rhizobacteria
rRNA	=	ribosomal ribonucleic acid
SD	=	Standard Deviation
SUT	=	Suranaree University of Technology

LIST OF ABBREVIATIONS (Continued)

tRNA	=	transfer ribonucleic acid
USDA	=	United States Department of Agriculture
v/v	=	volume per volume
w/v	=	weight per volume

CHAPTER I

INTRODUCTION

1.1 Significance of this study

Golf is a popular sport for hundreds of years and need large area of good quality green grass for the competition. Grass on the golf courses is often grown under high maintenance conditions to maintain the good quality of the green (Nikbakht et al., 2014). Since the visual quality on golf course is one of the major factors in rating system, using large quantity of chemical substances, such as herbicides, insecticides, fungicide, and also chemical fertilizer are mainly applied on the golf course, which resulted in high investment of maintenance and prone to highs pollution.

To fertilize the grass on golf course, not only nitrogen element that is necessary for plant growth and development, but phosphorus is also an essential nutrient required by plant. Although phosphorus in soil may appear in large dose, it is mostly less available for plant. It was reported that only 0.1% of the total P is available in the natural soil due to the binding of phosphorus with other ions, such as iron, aluminium, and calcium especially in acidic soil, and then transforming into insoluble form of phosphorus (Sharma et al., 2013). Phosphorus availability occurs in narrow ranges of soil pH. The availability is quite low in alkaline soils (pH > 7.3) and in acidic soils (pH < 5.5) (Valsami-Jones and Association, 2004). Therefore, golf course located on the inappropriate soil pH may have direct effect on grass growth due to lack of phosphorus. Moreover, the problem of phosphorus pollutant derived from soil leaching, macropore flow, and mineralization of humus in the golf course can also cause the pollution in the natural water ponds. In this case, the high amount

application of chemical phosphorus fertilizer may not efficiently promote grass growth, while it may create pollution to the environment. The questions are how to increase the phosphorus availability for grass and reduce the utilization of chemical phosphorus fertilizer in the golf course. Since most grasses could form an Arbuscular Mycorrhiza (AM) symbiosis, it is possible to apply AM for golf course. Bermuda grass (*Cynodon dactylon*) is mostly used for growing on the golf green (McCarty and Miller, 2002). It was found that rhizosphere of Bermuda grass was colonized by AM species belonged to genera of *Glomus* and *Acaulospora* (Wu et al., 2010). However, the indigenous AM community might be changed due to several factors, such as plant species, soil condition, or climate in each location (Massenssini et al., 2014). Therefore, the effective strain of AM for Burmuda grass should be selected for plant growth promotion.

To increase the efficiency of AM fungal inoculant, the strategy of co-inoculation AM fungus with Plant Growth Promoting Rhizobacteria (PGPR) has been reported. PGPR are also known to promote plant growth by several mechanisms, such as nitrogen fixation, IAA production, and ACC deaminase activity. In this study, the commercial AM fungus, *Rhizophagus irregularris* and the PGPR, *Pseudomonas* sp. strain SUT19, were used for co-inoculation test and determine their efficiency on Bermuda grass growth promotion. Since strain SUT19 has been reported to have nitrogen fixation, IAA production, and ACC deaminase activities (Piromyou et al., 2011) therefore, it was expected that the co-inoculation may facilitate the application of AM fungal inoculum and promote plant growth better than using AM inoculum alone.

Therefore, this study was focused on investigation the diversity of AM fungi that can colonize Bermuda grass roots and to determine the potential of using the indigenous AM fungi as biofertilizer to support the growth of Bermuda grass. Moreover, the influence of host plant on AM fungal propagation was also determined to evaluate the possibility of further producing as AM fungal inoculant. Finally, the strategy of co-inoculation AM

fungus with PGPR was also performed using the commercial AM fungal strain to investigate their synergistic ability to support Bermuda grass growth under both neutral and acidic pH conditions.

1.2 Hypothesis

1.2.1 The indigenous AM could be found in rhizospheric soil of Bermuda grass.

1.2.2 The combination of AM fungus with PGPR showed high effectiveness to support growth of Bermuda grass in all conditions.

1.3 Objectives

1.3.1 Main objective

To investigate the effect of AM fungi on Bermuda grass growth promotion

1.3.2 Specific objectives

1. To investigate the diversity of AM fungi that can colonize Bermuda grass roots in the soil collected from different golf courses

2. To determine the potential of using the indigenous AM fungi as biofertilizer to support the growth of Bermuda grass

3. To determine the influence of different host plants on indigenous AM fungal propagation

4. To investigate the effectiveness of co-inoculation AM fungus with PGPR on Bermuda grass growth promotion

CHAPTER II

LITERATURE REVIEWS

2.1. Phosphorus

2.1.1 Phosphorus forms and availability in soil

One of the essential plant nutrients is phosphorus (P). Phosphorus in natural soil can be placed in to different three category 1) inorganic compounds, 2) organic compounds of soil humus and 3) organic and inorganic P associated with living matter cells. The amount of phosphorus in the environment, natural and anthropogenic materials are presented in Table 2.1. P is usually form a complex with other elements such as aluminum (Al), iron (Fe), manganese (Mn), and calcium depending on soil pH. For example, P can form a complex with Al, Fe, Mn, under acidic soils (pH less than 5.5), while it strongly react with Ca under alkaline soils (pH more than 7.3). These reactions cause accumulation with many insoluble forms in soil (Valsami-Jones, 2004). Some of common P minerals are presented in Table 2.2

The exogenic phosphorus cycle starts with phosphorus that released from primary sources, such as mineral P and organic, followed by cycling within soil. Although P may appear large amount in natural soil about 0.05% w/w (Illmer and Schinner, 1995), P solution (available P for plant) is lower length from 0.001 to 1 mg/l (Khan and Ahmad, 2014). Soil P is normal fixed form and low length available for plant uptake. Thus, application of phosphatic fertilizers (i.e. super phosphate and triple phosphate) or organic fertilizers which contain soluble P, organic P and inorganic P are applied for several cases to enhance P adequate to plant. However, phosphatic fertilizers are strongly react with soil

component result to unavailable for plant. Insoluble forms and inaccessible P are hydrolyzed into soluble and available forms for plant uptake through the solubilization (inorganic) and mineralization (Organic) process. The lack of an atmospheric phosphorus, released and leached out from rhizosphere zone to river, lakes, sea make it limiting nutrient for plant. Moreover, the excess P can cause water pollution so called eutrophic condition. However, limiting of P nutrient could be alleviated by micro-organism in the rhizospheric soil. The phosphorus cycle was depicted in Figure 2.1

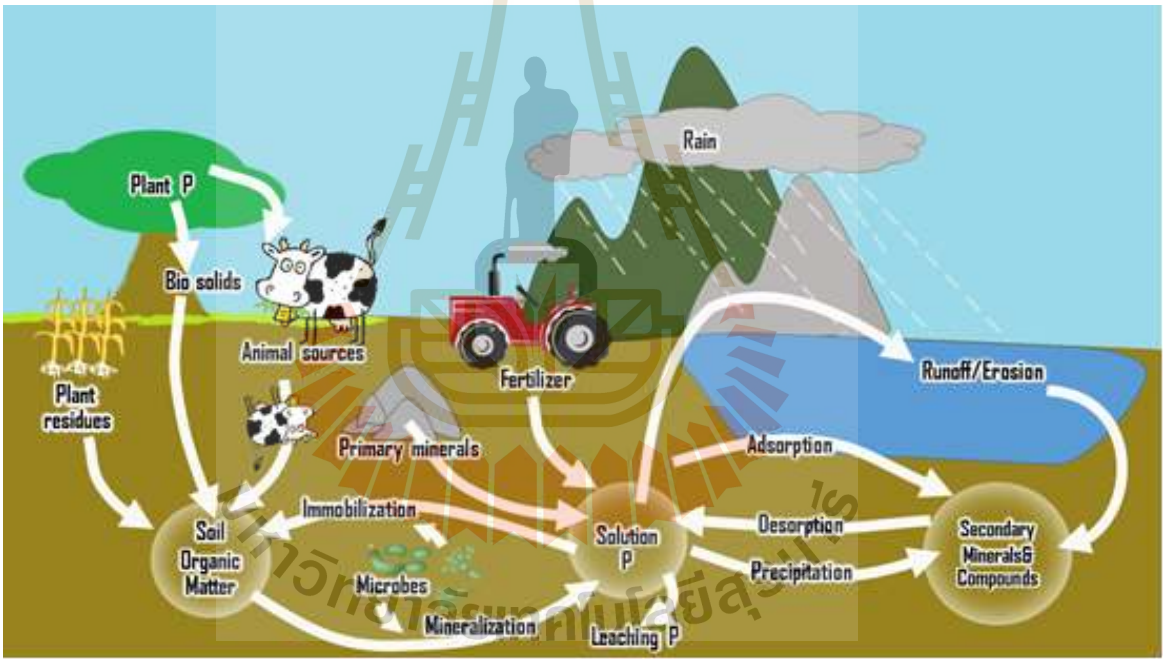

Figure 2.1 The soil phosphorus cycle, labile and fixed phosphorus pools and relative behavior of organic and inorganic pools (adapted from Valsami-Jones, 2004)

2.1.2 Plant acquisition and utilization of phosphorus

Phosphorus is a component of the complex nucleic acid structure which are generate protein synthesis for plant development and growth about 0.2% of plant dry weight (Schachtman et al., 1998). Therefore, P insufficient plants which P concentration in

leaves lower than 0.2% resulting in stunted growth, mature at slower rate than adequate P plants, and dark leaves symptom caused by carbohydrate buildup. Since P is deficiency available for plant, application of P-based fertilizers is normally use to overcome P insufficient and to support agriculture productivity (Richardson et al., 2009). Plant uptake P as Pi form which has concentration less than 10 µM in soil solutions even in fertile soils. In contrast, the modal concentration of potassium, calcium, and magnesium has concentration about 90, 700, and 1000 µM, respectively (Bieleski, 1973). This is the reason why plant must have special membrane transporters for extracting Pi from solutions between root-soil interface. Most study in higher plants have been demonstrated that Pi uptake under pH range between pH 5.0-6.0 are the highest as monovalent form ($H_2PO_4^-$) (Schachtman et al., 1998).

Table 2.1 The amount of phosphorus (wt % P) in the environment, in natural, and anthropogenic materials (Valsami-Jones, 2004)

Environment	wt% P	Natural materials wt% P		Anthropogenic materials wt% P	
Air	0.00	Plant	0.05-1.0	Concrete	0.1-0.05
Sea water	0.0001-0.001	Body	1.0	Window glass	< 0.01
Rain water	0-0.001	Blood	0.04	Wood ash	4.0-9.0
Igneous rocks	0.1	Bones	12.0	Wrought iron	0.1-0.2
Phosphate rocks	10.5-15.0	Teeth	8.0	Steel	0.02-0.05
Soil	0.02-0.50	Brain	0.3	Seaweage sludge	2.6
Meteorites	0.2	Milk (cow)	0.1	(dried)	
		Brewer’s yeast	1.8		

Table 2.2 Common phosphorus minerals found in acid, neutral and calcareous soils (Valsami-Jones, 2004)

Minerals	**Chemical formula**
Acid soils	
Strengite	$FePO_4 \cdot 2H_2O$
Variscite	$AlPO_4 \cdot 2H_2O$
Neutral and calcareous soils	
B-tricalcium phosphate	$Ca_3(PO_4)_2$
Dicalcium phosphate	$CaHPO_4$
Dicalcium phosphate dehydrate	$CaHPO_4 \cdot 2H_2O$
Fluorapatite	$Ca_5(PO_4)_3\,F$
Hydroxyapatite	$Ca_5(PO_4)_3\,OH$
Octacalcium phosphate	$Ca_4(PO_4)_3 \cdot 2\text{-}5H_2O$

2.1.3 Genetics and molecular biology of phosphorus nutrition

Specific transport systems are necessary for Pi uptake by plant. The Pi transporters had been studied using molecular techniques. It has been proven through the plant model such as *Arabidopsis*, *Medicago* and rice. Specific transporter Pht1 family which including with nine members in *Arabidopsis* genome, indicated that most of genes show high expression in P stressed plants.

2.2 Arbuscular Mycorrhizal Fungi

2.2.1 General background

The word “Mycorrhiza” is derived from classical Greek word for “mushroom and root” (μύκης mýkēs, "fungus", and ῥίζα rhiza, "root"). Arbuscular mycorrhiza (AM) fungi were originally known as vesicular-arbuscular mycorrhiza (VAM) since this group of fungi contains vesicle. Although AM is wildly used for calling this type of fungus, VAM is also still used by some publication (Abbasi et al., 2015). From the fossil evidence of plants like Aglaophyton (Remy et al., 1994) and the DNA sequence analysis (Simon et al., 1993) suggested that this mutualism may be appeared around 400-460 million years ago. AM can associate with several land plants (vascular plant families) in almost all terrestrial ecosystems, including tropical to temperate forests, sand dunes, deserts, and grasslands (Brundrett, 1991; Wang and Qiu, 2006). AM fungi are considered to be an obligate biotrophs which they unable to complete their life cycle in the absent of living plant root (Smith and Read, 2008). This AM fungi are known as beneficial because they do not cause any harm to their host, while they often support the plant growth by increase nutrients uptake, production of plant growth promoting substances, enhance plant tolerance to drought and salinity, as well as having synergistic interactions with other beneficial microorganisms (Sreenivasa and Bagyaraj, 1979). AM is considered as natural bio fertilizer since it provides the host with nutrient, especially phosphorus in exchange for carbon from photosynthesis product from plant provide to AM. Moreover, AM fungi can also protect their host to pathogen. Since AM fungi have low level of host specificity, they are suitable for a wide range of plant and in different environmental conditions.

2.2.1.1 Taxonomy

The history and complexity of the taxonomy and systematics of these obligate biotrophs is addressed differently in four periods. The initial discovery period was in 1974-1984, AM fungi were characterized by description mainly of sporocarp-forming

species which were classified into three groups. The next period was in 1975-1989 (alpha taxonomy period), this period established a solid morphological basis for species identification and classification. It was resulting in a profuse description of new species and need to standardize the nomenclature of spore subcellular structures. Then, the cladistics period started from 1990 to 2000. The first cladistic classification of AMF was based on only the phenotypic characters. The genetic characters played a role in the end of this period for defining taxa and elucidating evolutionary relationships within the group. The most recent phylogenetic synthesis period (2001 to present) started with the proposal of a new classification based on genetic character using sequences of multicopy of rRNA genes.

Focusing on the phylogenetic synthesis period (from 2001 to present), this ultimate period is characterized by: (1) the propose of a new classification based on genetic characters (SSU rRNA gene), (2) description of new taxa based on the fossil record, and (3) the creation of new taxa and a new classification based on a combination of phenotypic and genetic characters. Based on the mycorrhizal morphology in combination with rDNA sequences, fatty acid profiles, and immunological reactions provided the recognition of two families and two genera: (1) the genus *Archaeospora* in the family Archaeosporaceae including species forming monomorphic spores (acaulosporoid) or dimorphic spores (acaulosporoid and glomoid), and (2) the genus *Paraglomus* in the family Paraglomeraceae encompassing species forming glomoid spores which is indistinguishable from those formed in *Glomus*. Mycorrhizal structures formed by either genus stain very weakly in trypan blue. Schwarzott et al. 2001 provided a phylogenetic analysis based on the nearly full-length SSU rRNA gene sequences from 30 isolates of *Glomus* species. Their results indicated that *Glomus* is not monophyletic but can be separated into three clades, two of which are phylogenetically distant enough to warrant a family level distinction (*Glomus* group A and B although no formal classification was proposed at the family level) and one of which is closely related to the families Acaulosporaceae and Gigasporaceae.

The most important event in this period was discovered of *Geosiphon pyriforme* that forms a symbiosis with Nostoc (Schüßler and Kluge, 2001). The proposed phylum Glomeromycota was based on a phylogenetic analysis of SSU rRNA gene sequences. With this classification, AMF were removed from the polyphyletic phylum Zygomycota and placed in their own phylum that is hypothesized to share a common ancestry with the phyla Ascomycota and Basidiomycota. Four new orders (Paraglomerales, Archaeosporales, Diversisporales, and Glomerales) and new families were proposed (Table 2.3). New classes and orders have been also proposed by Oehl et al. (2011) in the phylum Glomeromycota. These authors proposed the classes Archaeosporomycetes and Paraglomeromycetes to contain in the orders Archaeosporales and Paraglomerales, respectively. They also proposed the order Gigasporales to be placed within the class Glomeromycetes. In the same year, new genera and families were proposed by Oehl and co-workers. *Scutellospora pernambucana* and *S. projecturata* were transferred to the newly genus *Orbispora*, which was hypothesized to be ancestral to species of glomeromycotan fungi forming spores with a bulbous base (Oehl et al., 2011). In 2012 a new classification proposed base on combined molecular and morphological Goto and co-worker, based on combined molecular and morphological studies, a new family Intraornatosporaceae was erected including with two new genera *Intraornatospora* and *Paradentiscutata*, this Intraornatosporaceae family was placed within order Gigasporales (Goto et al., 2012). Until now, several AMF have been characterized by descriptions and proposals of new families and genera for both ancient and extant AMF, with some of the taxa proposed still in debate among taxonomists.

Table 2.3 Proposals of classification of glomeromycotan fungi within the kingdom Fungi (Stürmer, 2012; Goto, et al., 2012)

Phylum	**Class**	**Order**	**Family**	**Genera**
Gerdemann and Trappe (1974)				
Zygomycota	Zygomycetes	Endogonales	Endogonaceae	*Glomus*
				Sclerocystis
				Acaulospora
				Gigaspora
Morton and Benny (1990)				
Zygomycota	Zygomycetes	Glomerales	Glomeraceae	*Glomus*
				Sclerocystis
			Acaulosporaceae	*Acaulospora*
				Entrophospora
			Gigasporaceae	*Gigaspora*
				Scutellospora
Schüßler et al. (2001)				
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	*Glomus*

Table 2.3 Proposals of classification of glomeromycotan fungi within the kingdom Fungi (Stürmer, 2012; Goto, et al., 2012) (continued)

Phylum	**Class**	**Order**	**Family**	**Genera**
		Diversisporales	Gigasporaceae	*Gigaspora*
				Scutellospora
			Acaulosporaceae	*Acaulospora*
				Entrophospora
			Diversisporaceae	*Diversispora*
		Paraglomerales	Paraglomeraceae	*Paraglomus*
		Archaeosporales	Archaeosporaceae	*Archaeospora*
			Geosiphonaceae	*Geosiphon*
Walker and Schüßler (2010)				
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	*Glomus*
				Funneliformis
				Sclerocystis
				Rhizophagus
			Claroideoglomeraceae	*Claroideoglomus*

Table 2.3 Proposals of classification of glomeromycotan fungi within the kingdom Fungi (Stürmer, 2012; Goto, et al., 2012) (continued)

Phylum	Class	Order	Family	Genera
		Diversisporales	Gigasporaceae	*Gigaspora*
				Racocetra
				Scutellospora
			Acaulosporaceae	*Acaulospora*
			Entrophosporaceae	*Entrophospora*
			Pacisporaceae	*Pacispora*
			Diversisporaceae	*Diversispora*
				Otospora
				Redeckera
		Paraglomerales	Paraglomeraceae	*Paraglomus*
		Archaeosporales	Archaeosporaceae	*Archaeospora*
			Ambisporaceae	*Ambispora*
			Geosiphonaceae	*Geosiphon*

Table 2.3 Proposals of classification of glomeromycotan fungi within the kingdom Fungi (Stürmer, 2012; Goto, et al., 2012) (continued)

Phylum	**Class**	**Order**	**Family**	**Genera**
Oehl et al. (2011)				
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	*Glomus*
				Funneliformis
				Simiglomus
				Septoglomus
			Claroideoglomeraceae	*Claroideoglomus*
				Viscospora
		Diversisporales	Diversisporaceae	*Diversispora*
				Redeckera
				Otospora
			Entrophosporaceae	*Entrophospora*
			Acaulosporaceae	*Acaulospora*
				Kuklospora
			Pacisporaceae	*Pacispora*

Table 2.3 Proposals of classification of glomeromycotan fungi within the kingdom Fungi (Stürmer, 2012; Goto, et al., 2012) (continued)

Phylum	Class	Order	Family	Genera
		Gigasporales	Gigasporaceae	*Gigaspora*
			Scutellosporaceae	*Scutellospora*
				Orbispora
			Racocetraceae	*Racocetra*
				Cetraspora
			Dentiscutataceae	*Dentiscutata*
				Fuscutata
				Quatunica
	Archaeosporomycetes	Archaeosporales	Archaeosporaceae	*Archaeospora*
				Intraspora
			Ambisporaceae	*Ambispora*
			Geosiphonaceae	*Geosiphon*
	Paraglomeromycetes	Paraglomerales	Paraglomeraceae	*Paraglomus*

Table 2.3 Proposals of classification of glomeromycotan fungi within the kingdom Fungi (Stürmer, 2012; Goto, et al., 2012) (continued)

Phylum	**Class**	**Order**	**Family**	**Genera**
Goto et al. (2012)				
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	*Glomus*
				Septoglomus
				Funniliformis
				Simiglomus
			Entrophosporaceae	*Entrophospora*
				Claroideoglomus
				Albahypha
				Viscospora
	Glomeromycetes	Diversisporales	Diversiporaceae	*Diversispora*
				Redeckera
				Otospora
				Tricispora
			Sacculosporaceae	*Sacculospora*

Table 2.3 Proposals of classification of glomeromycotan fungi within the kingdom Fungi (Stürmer, 2012; Goto, et al., 2012) (continued)

Phylum	Class	Order	Family	Genera
			Pacisporaceae	*Pacispora*
			Acaulosporaceae	*Acaulospora*
				Kuklospora
	Glomeromycetes	Gigasporales	Scutellosporaceae	*Scutellospora*
				Orbispora
			Dentiscutataceae	*Dentiscutata*
				Quantunica
				Fusculata
			Racocetraceae	*Racocetra*
				Cetraspora
			Gigasporaceae	*Gigaspora*
	Archaeosporomycetes	Archaeosporales	Ambisporaceae	*Ambispora*
			Archaeosporaceae	*Intraspora*

Table 2.3 Proposals of classification of glomeromycotan fungi within the kingdom Fungi (Stürmer, 2012; Goto, et al., 2012) (continued)

Phylum	**Class**	**Order**	**Family**	**Genera**
				Archaeospora
			Geosiphonaceae	*Geosiphon*
	Paraglomeromycetes	Paraglomerales	Paraglomeraceae	*Paraglomus*

2.2.1.2 AM fungi symbiosis

AM fungi could form a symbiosis with over 80% of all vascular plants (broad spectrum of plants). These fungal symbioses started from the fungi come to contact with host from which the fungi obtain either metabolites or nutrients from plant host, while AM fungi make more nutrients availability for plant roots (Lewis, 1985; Smith and Read, 1997). The symbioses among plant and fungi are often classified by either arbuscular mycorrhizal fungi (AM fungi) or ectomycorrhizal fungi (EM fungi). AM fungi have been found not only in vascular plants, but also in liverworts, Moses, fern, gymnosperms, and angiosperms. However, AM fungi did not form symbiosis with all flowering plants in the Brassicaceae (canola, mustards, cabbages, etc.) and the Chenopodiaceae (sugar beets, spinach and the large genus Chenopodium). A few aquatic plant families may have low level of AM fungal colonization. The evolution of the plant species involved in AM fungal symbiosis were summarized in Figure 2.2.

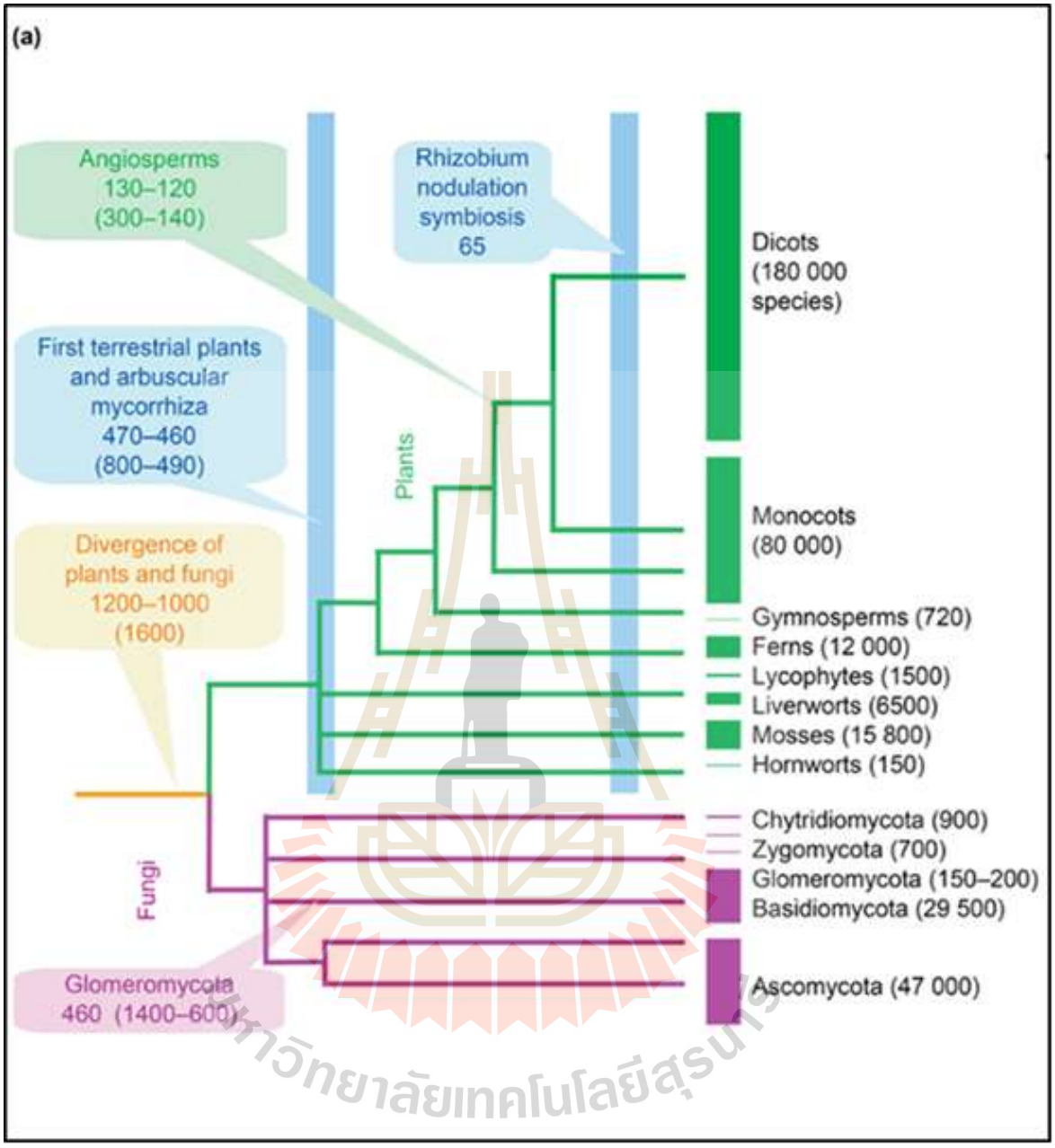

Figure 2.2 Evolution and systematics of fungi and land plants. The numbers show possible ages in millions of years, calculated from fossils and molecular (in brackets) data. The tree is not scaled and does not reflect actual evolutionary distances. Bars to the right reflect the approximate numbers of modern species described for each lineage. (Modified from Karandashov and Bucher, 2005).

The simplified phylogenetic tree of major fungal and land plant lineages showed evolutionary events involved in the appearance of arbuscular mycorrhizal symbiosis (divergence between fungi and plants, origin of AMF of the order Glomeromycota, first terrestrial plants and AM, and angiosperm plants). The suggested number of fungal species is around 1.5 million (Hawksworth, 2001).

2.2.2 Arbuscular mycorrhiza identification

2.2.2.1 Phenotype

Based on the phenotype identification, AM fungi could be classified by their forming structure in the living plant root. The fungal structure forming in the plant root included i) hyphae, ii) arbuscule, iii) vesicle, and iv) spore, which are able to detect by staining and observe under microscope (Figure 2.3, 2.4). AM fungal hyphae are non-septate and spread within cortex cells. The morphological types of AM fungal hyphae have been divided into two groups, the Paris and Arum types, which are named after the two plants (*Paris quadrifolia* and *Arum maculatum*) were first described around 100 years ago (Karandashov and Bucher, (2005). The “Paris” showed coiling form which hyphae spread by forming coil within the cells because there are no continuous longitudinal air spaces. Whereas, the “Arum” showed linear form which hyphae proliferate in the plant cortex by growing longitudinally between host cell (intercellular space). The AM fungal hyphae in connection with their host plant, can penetrate deep into the soil more than that of non-AM colonized plant roots. This fungal hyphae could adsorp nutrients from soil and transfer into plant cells through the specific structures called arbuscule (Figure 2.5a-b).

The arbuscule looks like the tree structure presented inside the plant cell. Arbuscule has specific membrane transporters including phosphate-, amino acid-, urea-, ammonium-, and nitrate-transporters, which can transfer more nutrients in the soil to plant cell. At the same time, the carbon compounds derived from plant photosynthesis were

supplied to AM fungi in form of sucrose and fructose. Arbuscules start to form approximately 2 days after penetration into the root (Tripathi, 2008).

Mycorrhizal vesicles structure (Figure 2.6) is the energy storage structure of fungi formed in root cortex. The vesicles contain lipid and cytoplasm. The intraradical vesicles develop from the tip of hyphae or from lateral brunches within the cells or intercellular spaces of living root. This structure frequently enlarges the volume in plant cells. It is known that large numbers of vesicles may be formed in roots at the end of the growing stage and maintained for the long period of time. The vesicle formation usually occurs after the senescence of arbuscules, indicating that the fungus may require to accumulate the energy from host. The cytological features of mature vesicles presence of lipid bodies and many nuclei. However, not all genera of AM fungi form intraradical vesicles, but some of them could form extraradical vesicles. For example, two genera of *Gigaspora* and *Scutellospora* form extraradical vesicles in the extraradical mycelium. It was initiated as lateral branches that rapidly expand into globose structures with varying in color and with ornamented walls. Inside the extraradical vesicle stores the large amount of lipid, vacuolar inclusions, and multinucleate.

In case of extraradical mycelium (ERM), it was difficult to study *in situ* because of the heterogeneous nature of soil and the ability to locate the hyphae forming mycelium network. Studies of this structure could be examined using light microscopy. An extraradical hyphae network were described as dimorphic, having with coarse, angular branching hyphae, and also fine hyphae.

Lastly, the AM fungal spore which is the structure that was used to characterize classify their specie. The characteristic of spore size, color, wall layers, and features of the subtending hypha were used to classify species of arbuscular mycorrhizal fungi see: International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (INVAM) at http://invam.caf.wvu.edu/. The mature spores of AM fungi vary in the number

of wall layers, the pigment, and the impermeable. Wall layers are also of varying composition including polysaccharides, lipids, proteins and chitin. Chitin is presented in one or more layers and often exists in a complex arrangement of fibrils. The cytological features showed that there were numerous of storage lipid bodies and nuclei in the AM fungal spore. Amount of lipid, vacuolar inclusions, and multinucleate. The germinated under experimental conditions in the absence of plants both in vitro and in soil. However, the rate of germination can be increased by host root exudates (Prasad, K. 2017). AM fungal spores germinate given suitable conditions of the soil matrix, temperature, carbon dioxide concentration, pH, and phosphorus concentration.

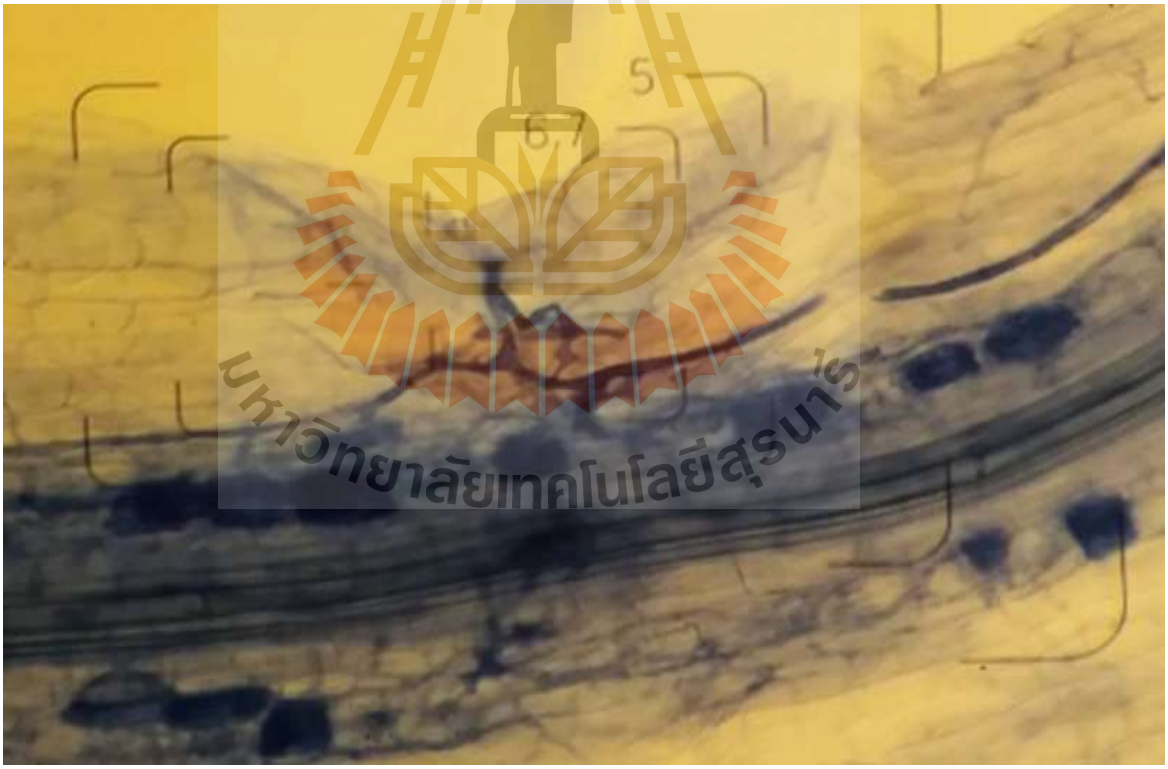

Figure 2.3 *Lotus japonicus* WT Gifu S-34 root colonized by *Rhizophagus irregularris* stained with tryphan blue observed under stereo microscope 4X Zoom by compound microscope Olympus series model: B061.

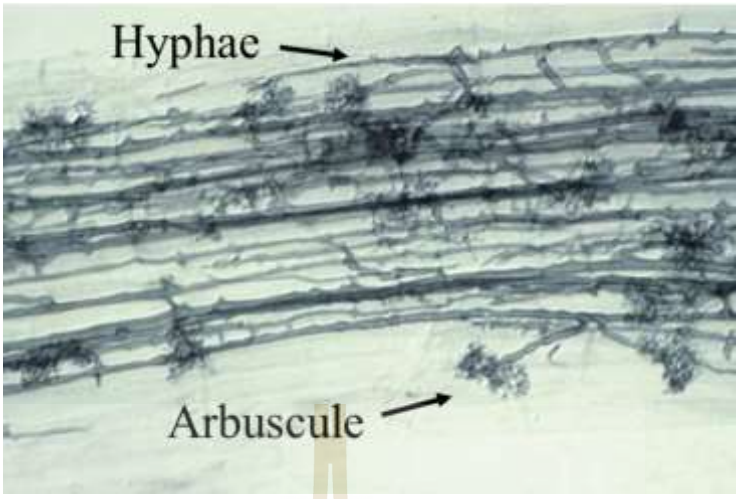

Figure 2.4 Longitudinal growth of AM (*Glomus versiforme*) hyphae along a cortex air channels in a leek root. Hyphae and arbuscule structures are indicated by arrows. (from http://mycorrhizas.info/vam.html)

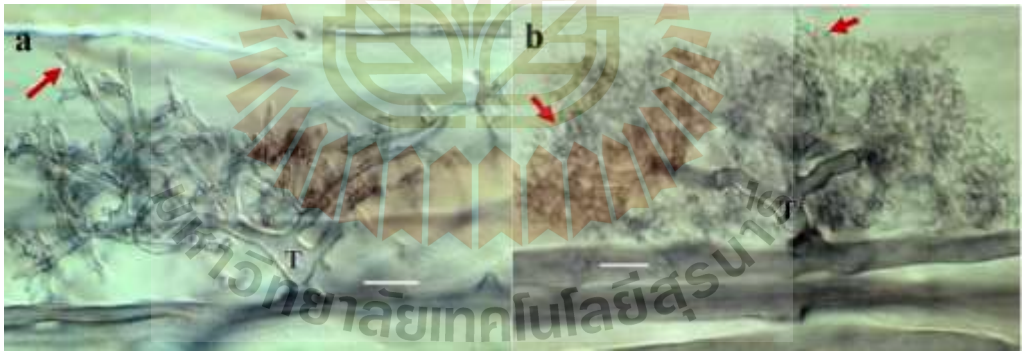

Figure 2.5 (a) Developing arbuscule of *Glomus mosseae* in a root cell with fine branch hyphae (arrows). (b) The trunk (T) of this arbuscule branched from an intercellular hyphae. Mature arbuscule of *Glomus* showing trunk (T) and numerous fine branch hyphae are indicated by arrows. (http://mycorrhizas.info/vam.html)

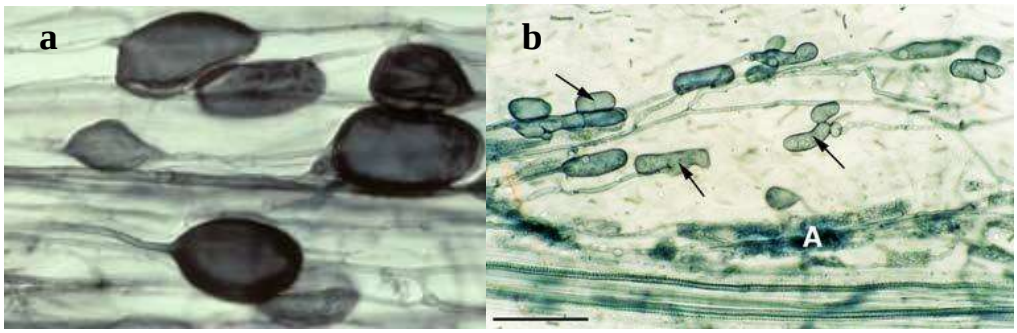

Figure 2.6 (a) Vesicles (V) produced by a *Glomus* species in a leek root. This root also contains many intercellular hyphae (scale bar 50 μm). (b) Lobed vesicles of an *Acaulospora* species in a clover root. Arrows = vesicles, A = arbuscules (scale bar 100 μm). (http://mycorrhizas.info/vam.html)

2.2.2.2 Genotype

After AM fungi have been described by means of morphological feature of spores. A set of PCR primers was design to facilitate specific amplification AM fungi, using genotypic identification was started in 2000 until now. Molecular techniques have evolved, different marker regions have been used to characterize AMF communities such as small subunit rRNA (SSU rRNA), Internal transcribed spacer (ITS), large subunit (LSU), and 5.8S rRNA (Figure 2.7) each with their strengths and weaknesses (Hart et al., 2015). SSU rRNA gene is region most often targeted because primer pairs exist that amplify most known AMF families. ITS region is one of the universal for fungi (Schoch et al., 2012). And AMF specific primers exist for ITS (Krüger et al., 2009), However, this region is less frequency used in AM fungi surveys study, due to poor amplification of AM fungi with general fungal ITS primers (Tedersoo et al., 2018).

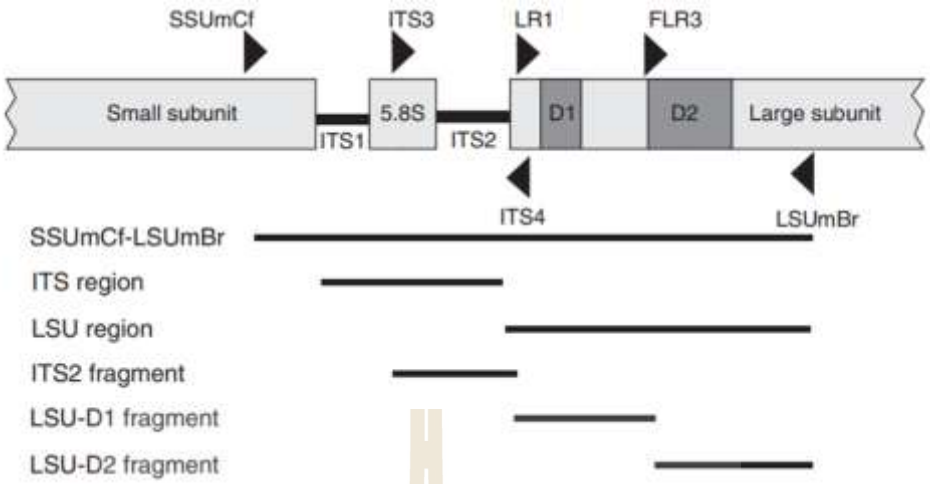

Figure 2.7 Schematic representation of the nuclear ribosomal DNA regions studied. Triangles indicate positions of priming sites that were used as borders for in silico analyses of the fragments. Lines indicate the fragments analysed.

A small subunit rRNA (SSU rRNA) gene was used for AMF-specific primers in study because it is less variable than ITS the variation of AMF SSU rRNA gene was showed in figure 2.7. Result from grasss house and field sampling show similar ecological responses, but different in phylogenetic structure and family-level abundances when detected by SSU and ITS markers (Lekberg et al., 2018). Morover, LSU nuclear ribosomal DNA also used for AM fungal identification AM colonized in *Medicago* species (Pivato et al., 2007).

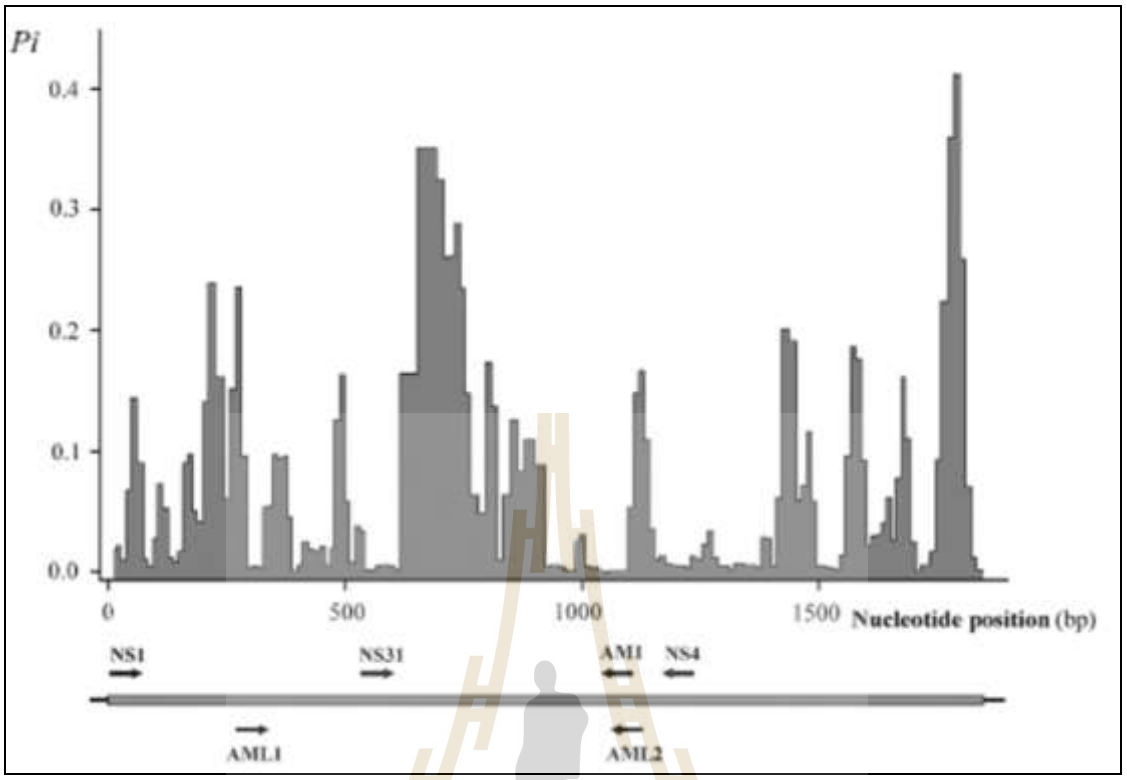

Figure 2.8 Variation of AMF SSU rRNA gene and position of PCR primers. The nucleotide diversity, Pi, is the average number of differences per base between sequences compared pairwise, plotted for 10-base windows (Nei, 1987; equations 10.5 or 10.6). An alignment of 92 published AMF sequences was used. Amplified sequence length is about 1100, 795 and 550 bp with primer pairs NS1–NS4, AML1–AML2 and NS31–AM1, respectively.

2.2.3 Plant and Arbuscular Mycorrhizal Fungi interaction

Symbiosis starts with plant root exudate **(**strigolactones**)** was released from plant root an AM fungi then induce specific signal formed by AM called MYC factor**.** Pre-symbiosis development of AM fungi including with three stages of development**.** The first is spore germination, host recognition, hyphal growth and appressorium formation**.** After establishing of AM fungi in plant root, the symbiosis interaction of nutrient transformation

between plant and AM fungal was occurred. More details of the plant and arbuscular mycorrhizal fungi interaction are described bellowed.

2.2.3.1 Initial plant-AM fungi interactions and signaling

The interaction is initiated by the plant signal called "strigolactones" which is a group of carotenoid-based phytohormones. These hormones regulate several aspects of plant development (Akiyama et al., 2005; Lopez-Obando et al., 2015). Strigolactones are secreted into the rhizosphere especially during P-deficient condition (Yoneyama et al., 2007a, 2007b; Kretzschmar et al., 2012). Once the strigolactones were detected by AM fungi, the oxidative metabolism of fungal cell started to activate the hyphal growth and branching to contact on plant roots (Akiyama and Hayashi, 2006; Besserer et al., 2006, 2008). However, strigolactones may not be the only signal molecules during this stage of interaction. Sunflower and black oat (Kim et al., 2014; Uneno et al., 2014) produced carlactones, which have been reported to promote AM hyphal branching (Mori et al., 2016) and may also contribute to pre-symbiotic signaling along with strigolactones. Nadal et al. (2017) has also been reported that the NOPE1 transport protein which transported a plant-derived N-acetylglucosamine (GlcNAc)-based molecule, was also important for the establishment of symbiosis between AM fungi and maize or rice.

On AM fungal site, the cell secreted at least two types of oligosaccharides, mycorrhizal-lipochitooligosaccharides (Myc-LCOs) and short-chain chitooligosaccharides (COs), which were named as chitotetraose (CO4) and chitopentaose (CO_5) (Figure 2.9) (Genre et al., 2013). Although these signals contain β-1–4-linked GlcNAc backbone, the COs are made of simple repeats, while the LCOs are modified with oleic or palmitic acid (Maillet et al., 2011). Both Myc-LCOs and COs are closely related to the well-known signals: (i) the Nod factors of rhizobia (Nod-LCOs), and (ii) the potent microbe-associated molecular patterns (MAMPs) chitoheptaose (CO_7) and chitooctaose (CO_8), which are recognized by the plant and lead to pattern-triggered immunity (PTI) to prevent the

infection of plant pathogen **(**Cao et al**.**, 2014**).** In legumes, the common responses to Myc-LCOs and Nod-LCOs included root hair branching, perinuclear calcium spiking, AM promotion, and lateral root induction and lead to organogenesis of the nodule **(**Oldroyd 2013**).** However, development of AM symbiosis does not change in plant root morphology, but there have been reported to increase in lateral root formation in response to AM fungal inoculation **(**Gutjahr et al**.**, 2019**).**

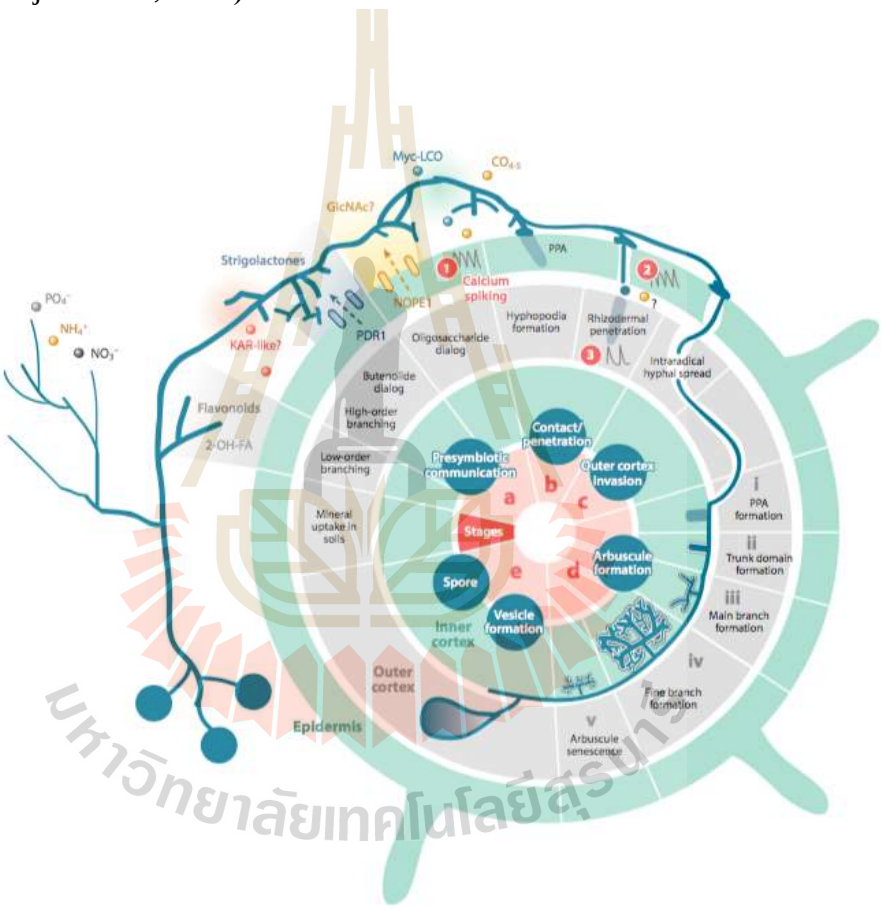

Figure 2.9 Life cycle of AM symbiosis **(**Choi et al**.**, 2018**).**

2.2.3.2 Hyphopodia formation and penetration of hyphae

AM fungal hyphae could form an attachment structures, called hyphopodia, on the surface of the root epidermis. This hyphopodia differentiation was suggested to the result from plant contact recognition. Then, the invasion of plant cell by the fungal hyphae was started from the AM fungi formed a tube-like intracellular infection structure called the prepenetration apparatus (PPA), which requires symbiosis-specific gene

expression (Genre et al., 2008). The PPA is formed as an invaginated plasma membrane supported by an extensive network of endoplasmic reticulum (ER) and cytoskeleton material. Then, the plant cell wall was soften at the contact site and assumed to allow AM fungal hyphae to expand in the apoplastic tunnel (Paniske, 2000). During this stage, the common symbiosis signaling pathway (CSP) has been described in similar manner as legume nodule organogenesis. Establishment of AM symbiosis composed of three steps of plant signaling cascade as shown in Figure 2.10. First step is perception. This is a step that the receptive plant perceives the presence of AM fungi through the detection of Myc-LCOs and COs by LysM receptor-like kinases (*Os*CERK1, *Sl*LYK10, *Lj*NFR1, *Mt*LYK3), and the receptor-like kinase SYMRK/DMI2. Second step is transmission. At this step, the enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase interacts with DMI2 in plant to generate mevalonate and the Ca^{2+} spiking for initiation the downstream transcriptional response (Venkateshwaran et al., 2015). Mevalonate acts as a secondary messenger transmit to the nucleus via interaction with K^+ channel CASTOR and POLLUX/ DMI1. Third step is Transcription. At this stage, a calcium and calmodulin-dependent kinase CCaMK act as a master decoder to activate the transcriptional response. In the presence of calcium, Ca^{2+}-calmodulin associates with CCaMK induces the phosphorylation of the CCaMK substrate protein, CYCLOPS. The the phosphorylated CYCLOPS forms a complex with CCaMK, which acts in concert with GIBBERELLIC-ACID IN-SENSITIVE, REPRESSOR (GRAS) of GAI, and SCARECROW domain regulatory protein DELLA to induce the expression of a down- stream regulator(s) such as RAM1 that are necessary to accommodate the fungal symbiont.

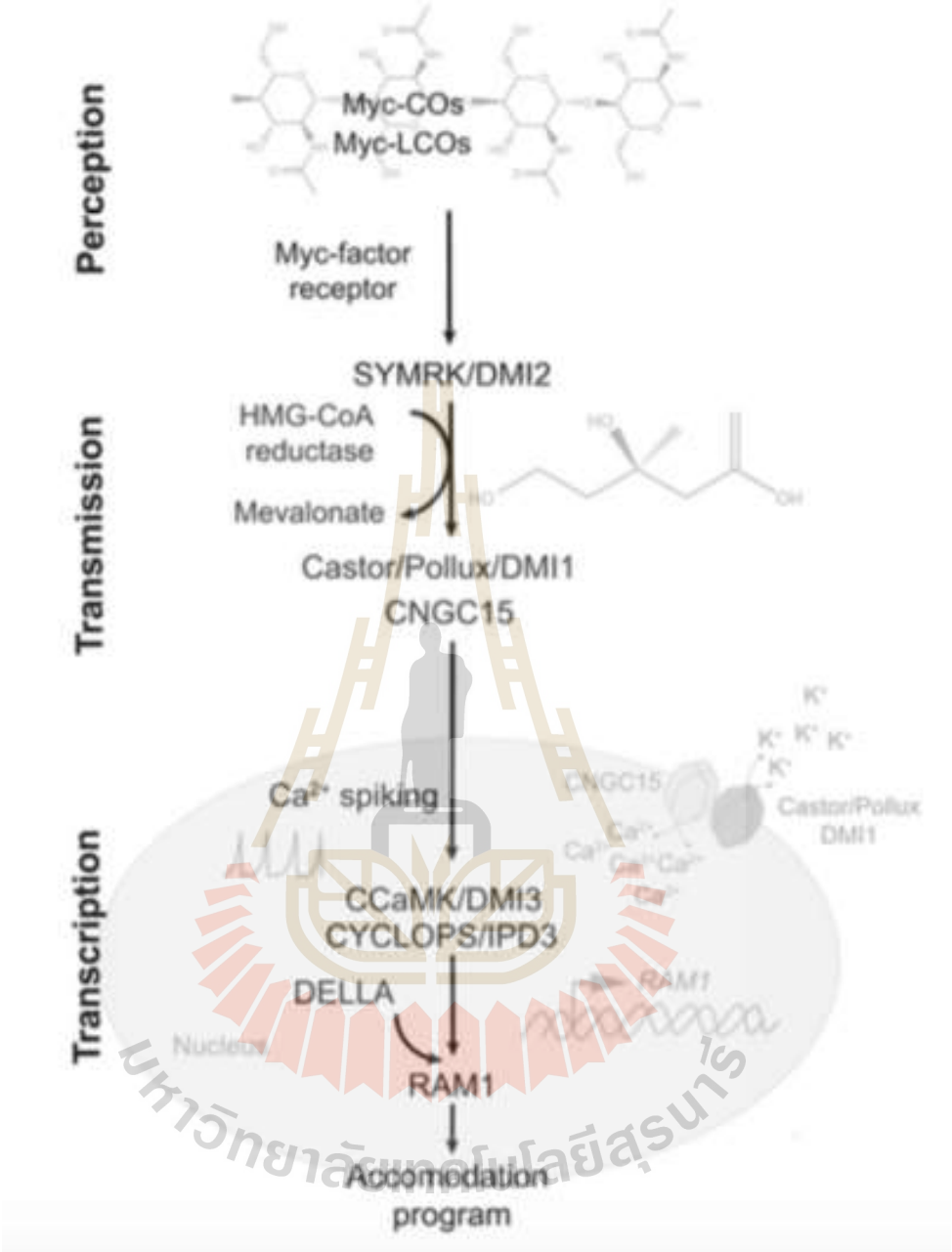

Figure 2.10 Establishment of AM Symbiosis (MacLean et al., 2017).

2.2.3.3 Arbuscule formation and nutrient exchange

Once the AM fungal hyphae reach the inner cortex, AMF develop highly branched, tree-shaped feeding structures called arbuscules. The formation of arbuscules composed of several stages including (i) Rhizodermal penetration, (ii) Outer

cortex penetration, (iii) PPA formation, (iv) Trunk formation, (v) Main branch formation, (vi) Fine branch formation, and (vii) maintenance degradation. The transcription factors and the induced genes expression in plant cell were shown in Figure 2.11. At this organelle, AMF deliver Pi and nitrogen to the plant in exchange for organic carbon. The extraradical mycelium of AMF acquires inorganic Pi from the soil (Harrison and VanBuuren, 1995) and converts it into polyphosphates for storage and transport to the intraradical mycelium within the vacuolar compartment (Solaiman et al., 1999). Then, hydrolysis of polyphosphates to inorganic Pi form allow phosphate to be available to plants. Plant can regulate the P-uptake from AMF by hormone controlling, such as increase the release of SLs to activate AM colonization under P-deficient condition (Liu et al., 2011), while high levels of Pi in plant cell stimulated the expression of gibberellin biosynthetic genes, leading to DELLA degradation and subsequent reduction of arbuscule development (Floss et al., 2013). Similarly, AMF could also transfer inorganic forms of nitrogen into the host plant through the specific transporter protein such as ammonium transporter (Govindarajulu et al., 2005). Plant also monitor the fungal delivery of Pi and nitrogen to continuously support the maintenance of the AM symbiosis. Thus, these nutrients could be signals to control the symbiosis interaction. On the other hand, it has been suggested that the main form of carbon delivered to fungi from plant is glucose (Shachar-Hill et al., 1995). Glucose is taken up by a high-affinity fungal monosaccharide transporter and then converted into glycogen and trehalose before export through the mycelia (Helber et al., 2011). Since the majority of fungal hyphae, vesicles, and spores contained lipids, it has been proposed that carbohydrates are converted into lipids and accumulate in these organelles (Trepanier et al., 2005). Therefore, it is interesting to investigate whether lipid metabolism plays an important role in this relationship.

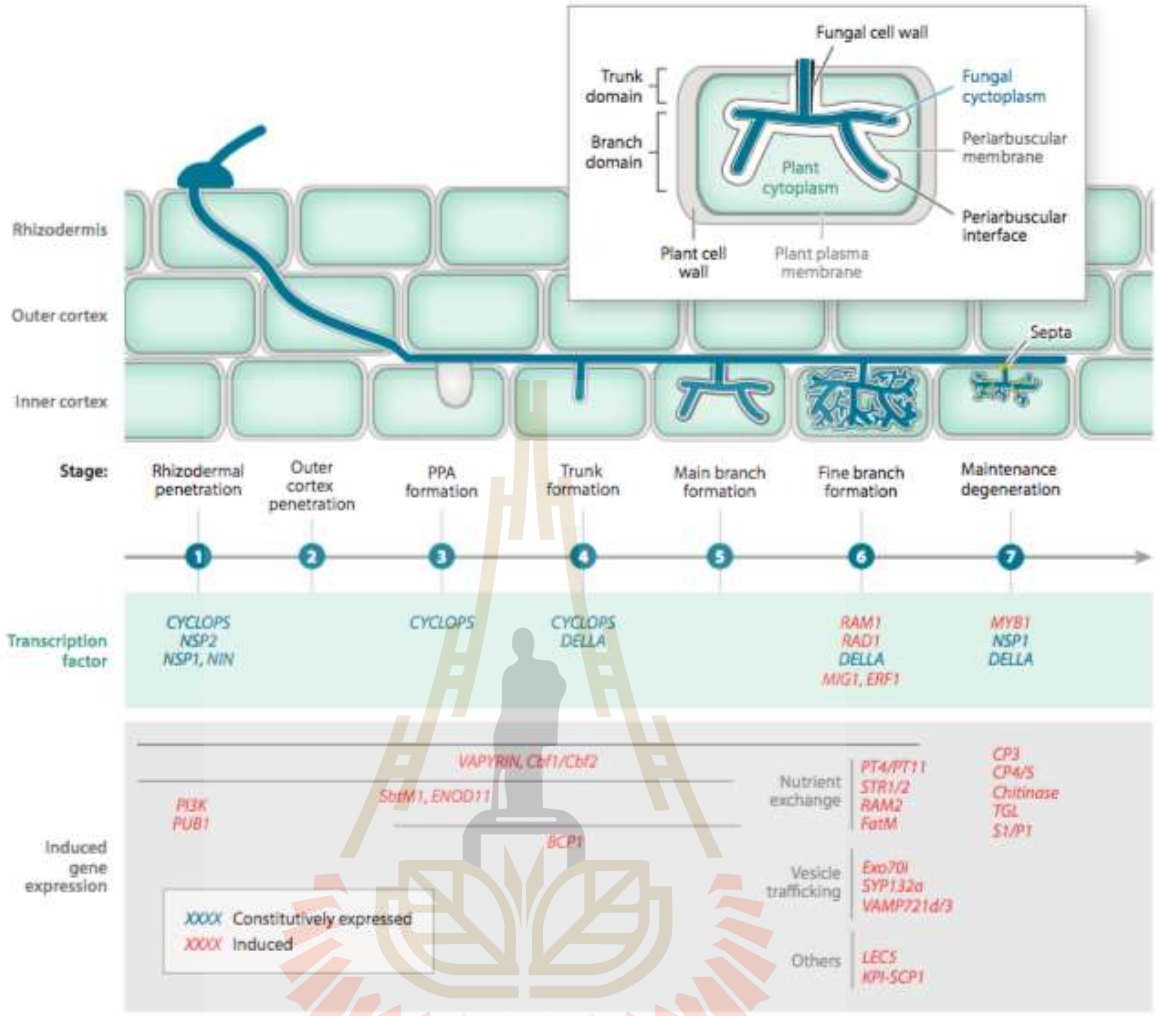

Figure 2.11 Transcriptional regulation of intraradical fungal colonization. Intracellular fungal colonization and arbuscule development are regulated by gene activation and governed by a network of transcription factors (Choi et al., 2018).

2.2.4 Role of Arbuscular mycorrhiozal fungi and their applications

The sustainable of soil-plant ecosystem can be occurred when the utilization of mineral resources is balanced through the biological efficient cycling**.** For example, nutrients are not rapidly used by plants community, or too much nutrients were not added up from the agricultural chemical fertilization**.** Sustainability of ecosystem is the way that important for management the ecosystem**.** AM fungi constitute a key functional group of

soil microbe that can contribute to the crop production and ecosystem sustainability. Not only through the improvement of plant nutrient or non-labile phosphorus for plant uptake, but also preventing erosion and reduce plant stress (Smith et al., 2009). More details of AMF applications are described below.

2.2.4.1 Agricultural application

Poor soil fertility, particularly the availability of nitrogen and phosphorus, is the most limiting to increase crop yields. AM fungus is one of the essential components in soil and can be found in normal cropping systems, especially in sustainable crop practices (Gianinazzi and Schüepp, 1994). AM fungi play an important role in the food security and simultaneous since increase in demand of phosphate is necessary to feed the growing population (Rodriguez and Sanders, 2015). Growth promotion or quality improvement of vegetable crops by AM inoculum is controlled by biotic interactions and also by the abiotic properties of the soil. Moreover, the organic matter and mineral fertilization were revealed to control the impact of AM fungi on vegetable crops (Linderman and Davis, 2004). The combinations with other fertilizers or other biofertilizers also increased the efficiency of AM inoculation. Brown and Carr (1984) revealed that dual inoculation AMF and *Azotobacter* spp. produced the larger lettuce plants than using inoculum alone. Sreenivasa (1994) studied the response of pepper plants to the inoculation of AMF and organic amendments. It was found that all organic amendments increased the proliferation of AM fungi. Moreover, the inoculation of *Glomus macrocarpum* in conjunction with some of the organic amendments tested improved the growth and yield of pepper. In Northeastern of Thailand, AM fungi had been found to have high diversity associated in rubber tree and cassava plant root (Herrmann et al., 2012). Oil palm (*Elaeis guineensis* Jacq.) also had been studied and found high diversity of AMF in this plant (Kaonongbua, W. 2018). AM fungi had an effect on crop plant growth included carrot, only weak effects on the growth promotion was found in red pepper and leek (Kim et al., 2017).

2.2.4.2 Arbuscular mycorrhiza and restoration of forest and degraded land

There are abundant scientific evidences to demonstrate that AMF significantly improve soil attributes, increase above and belowground biodiversity, significantly improve tree/shrub seedlings survival, growth and establishment on moisture and nutrient stressed soils. AMF have also been shown to drive plant succession and may prevent invasion by alien species. Therefore, the successful restoration of infective AMF can potentially improve the restoration success of degraded lands (Asmelash et al., 2016). Brundrett and Abbott (2002) proposed that AMF could have several abilities that contribute to ecological restoration, such as improved plant fitness (survival, growth and reproduction), nutrient uptake and accumulation, tolerance of biotic and abiotic stresses, and altering plant community structure.

AMF can improve soil aggregation and increase organic matter in the soil due to its ability to produce a lot of hyphae in the soil matrix that holds the soil particles together to form soil aggregates (Augé, 2004). Moreover, the dead AMF hyphae produce glomalin which increase both soil aggregation and stability (Barea et al., 2002). Moreover, AMF have been reported to increases plants resistance to pathogens. Gianinazzi et al. (2010) proposed that AMF can be as a 'health insurance' of plants may be due to the synergistic interaction of AMF with plant growth promoting rhizobacteria (PGPR). PGPR are very well known to have a role in plant pathogen inhibition (Figueiredo et al., 2010).

Interestingly, AMF have been proposed to increase tree/shrub seedlings growth, productivity, field survival and establishment on degraded lands. In general, it was known that inoculation of AMF resulted in significantly increase plants growth and productivity (Lekberg and Koide, 2005). However, Huante et al. (2012) reported that AMF inoculation has also significant effect on seedlings growth and most significantly slow growing tree species. Similarly, Kapulnik et al. (2010) found that the AMF nursery

inoculation enhance the seedlings field establishment and growth of *Olea europaea* L. Besides, they could observe AMF inoculation improved seedlings field performance significantly and most importantly for the first 2.5 years from transplanting. This effect could benefit to the forest restoration. AMF could also potentially influence plant community structure by affecting richness or evenness of coexisting plants (Brundrett and Abbott, 2002). Since not many AMF have been described to form association with 80% of terrestrial plants (Lee et al., 2013), it could be indicated that AMF have no host specificness. Moreover, forming of mycorrhizal networks have been shown to facilitate regeneration of new seedlings, alter species interactions, and change the dynamics of plant communities therefore, thus increasing plant diversity (Simard and Austin, 2010). AMF do not only influence the plant community but also the microorganism community. AMF hyphae and root litter are the most abundant carbon source in the soil, thus increased supply of energy for soil microbes (Brundrett and Abbott, 2002). AMF hyphae also exudes some compounds that may stimulate some microorganisms or also inhibit some others (Herman et al., 2012). Hence, AMF may increase the diversity and abundance of microorganisms that are beneficial to plants' growth and health. Based on these data, AMF have high potential to be used for restoration of degraded land or forest in order to increase the richness of plant as well as other microbial community.

2.2.4.3 Arbuscular mycorrhizal Fungi and bioremediation

Increase the usage of agrochemicals resulted in destroy not only soil fertility, but also enhance some unwanted elements **(**e.g. Cd**)** into soils**.** Accumulation of metals and metalloids from contaminated sources of fertilizers or chemical pesticide in agricultural soil raise the problem of food safety issues and potential of health risk**.** AM fungi have been reported to remediate some of metals contaminated in the soil**.** Since AMF were found in the soil of most ecosystems as well as in polluted soils, the heavy metal taken up through the mycelium to plant can be used as a phytoremediation strategy to

reduce the metal contamination in the soil. However, some mycorrhizal plants show enhanced heavy metal uptake and root-to-shoot transport (phytoextraction) while other AM fungi contribute to heavy metal immobilization within the soil (phytostabilization). The efficiency of AMF colonization on clean-up of contaminated soils depends on the plant–fungus–heavy metal combinations and is influenced by soil conditions (Gohre and Paszkowski 2006). Rivera-Becerril et al. (2002) reported that G. intradices could alleviate the symptoms of Cd toxicity in pea (P. sativum). For As, the competition of P and As transportation to plant facilitate the lower uptake of As once the AM fungi could uptake P through the hyphae efficiently (Knudson et al. 2003).

Besides the remediation of metals in the soil, AM fungi have been shown to biodegrade on the polyaromatic hydrocarbon (PAH) compounds. Joner and Leyval (2003) found lower accumulation rates in soil contaminated with a PAH at level of 2000 mg/kg when ryegrass and white clove were colonized by AMF. Similarly, Liu et al. (2004) found that the AMF can increase the degradation rates of benzo [a] pyrene in soils planted with alfalfa. However, PAH concentration of 100 mg/kg was found to have negative effects on degradation rate. The examples of AMF on bioremediation were summarized in Table 2.4

2.2.4.4 Golf course application

One of the major factors in a turfgrass rating system is the visual quality. Maintaining good grass color is a vital component of turfgrass management, and the use of microbial inoculants in turfgrass management programs may be a useful tool to increase visual appeal, root zone microbial activity, and turfgrass drought tolerance. Several studies of the mycorrhizal relationship with plants are available in the literatures. Study of Wu et al. in 2011 has found that the above ground dry weight of Bermuda grass was significantly increase when colonized with AM fungi. Moreover, the fungal species diversity in the metal-contaminated soils was significantly high in Bermuda grass. On the other hand, spore was significantly lower in uncontaminated soil (Wu et al., 2010).

Turfgrass are mostly grown under high maintenance conditions**. (**Ali Nikbakht et al**.**, 2014**).** Therefore, the application of AM fungi may facilitate the availability of nutrients to plant**.** Luckily, most grass species in environments could form a beneficial association with AMF, thus some area of golf course has already had AM fungal colonization on turf grass even no inoculation**.** Mycorrhizal fungi are presented in soil as spores, hyphae **(**filaments**)**, while forms special symbiosis structure **(**arbuscules**)** inside the plant cell**.** These structures increase surface area for exchanges of carbon source to the fungus and nutrients for the grass**.** Thus, mycorrhizal fungi hyphae that surround the root plant provide a variety of benefits for the grass plant**.** This network of hyphae could obtain 15 major plant nutrients as well as water, and transport these materials back to the grass root system**.** AM are significantly important for uptake of nutrients that do not readily available in the soil such as phosphorous and many of the micro-nutrients**.** Moreover, the network also binds soil particles together, improves soil porosity and the movement of air and water within the soil**.**

Table 2.4 Application of arbuscular mycorrhiza on contaminated soils **(**modified from Das et al**.**, 2017**)**

Plant species	Mycorrhizal inoculum	Contamination type	References
Pisum sativam (Pea)	*Glomus intradices*	Alkaline clay loam laden with Cd (100 mg/kg)	Rivera-Becerril et al. (2002)
Lolium perenne (Perennial ryegrass)	*Glomus mosseae*	PAH-contaminated industrial soils (400–2000 mg/kg)	Joner and Leyval (2003)
Trifolium repens (White clover)	*Glomus mosseae*	PAH-contaminated industrial soils (400–2000 mg/kg)	Joner and Leyval (2003)
	Glomus mosseae	Innert soil less substrate spiked with Cd	Vivas et al. (2003)
Zea mays (Maize)	*Glomus caledonium*	Calcareous sandy soil and loam soil, spiked with Zn (0–600 mg/kg)	Chen et al. (2004)
		Quartz sand spiked with Cu and Cd	Liao et al. (2003)
	Glomus intraradices	Sand spiked with Pb	Malcova et al. (2003)
	Glomus etunicatum	Soil spiked with atrazine	Huang et al. (2009)
Medicago sativa (Alfalfa)	*Glomus caledonium*	Soil spiked with benzo[a]pyrene (0–100 mg/kg)	Liu et al. (2004)
Alnus glutinosa (Common alder)	*Glomus intraradices*	Soil from an acetylene and polyvinylchloride factory	Oliveria et al. (2005)
Pinus sylvestris (Scots pine)	*Paxillus involutus* (ECM)	Growth substrate spiked with Cd (0–100 mg/kg)	Kim et al. (2004)
Leymus cinereus (Basin wild rye)	Site specific AM	Silica sand spiked with arsenic	Knudson et al. (2003)

2.3 Plant Growth Promoting Rhizobacteria

Plant Growth Promoting Rhizobaceteria (PGPR) belong to a beneficial and various group of microorganisms that can be found in the rhizosphere around plant root or within root tissues. They have ability to stimulate plant growth and development and yield (Beneduzi et al., 2012; Ahemad and Kibret, 2014). Several mechanisms by which PGPR stimulate plant growth involved in the availability of nutrients originating from genetic

processes through difference ways, divided into direct and indirect mechanisms. The direct mechanisms is direcly promote plant growth. For example, in biological nitrogen fixation that enhanced N by Free-living bacteria (non-symbiotic) including cyanobacteria. A further is symbiosis bacteria including with rhizobium that forms symbiosis with leguminous plant. *Frankia*, associate with actinorhizal plants and phosphorus solubilization that released by bacteria through enzyme production such as phytase, phosphatases. *Pseudomonas, Bacillus, Rhizobium, Burkholderia,* and *Enterobacter* are known well as a powerful phosphorus solubilizing bacteria (Mohammadi, 2012). In addition, PGPR act the mode of function in stress alleviation through the modulation of 1-aminocyclopropane-1-carboxylate ACC deaminase expression, and production of phytohormones for example, Indole-3- acetic acid or IAA and siderophores as it is involved in various main of biological processes, such as photosynthesis, respiration, chlorophyll biosynthesis (Kobayashi and Nishizawa, 2012). An another mode of action is indirect mechanism, the present of PGPR, induces plant defense system to protect against pathogens such as releasing siderophores production of tomato plants from *Pythium* by *Pseudomonas aeruginosa* 7NSK2 (Buysens et al., 1996). Therefore, PGPR play an important role in agriculture for plant growth and yield. Moreover, it could maintained soil nutrients status which reduced the chemical and fertilizers input and it cost-effective technology in sustainable agriculture.

2.4 Burmuda grass

The high quality of production and maintenance of fine turfgrass surfaces for golf putting greens is a high cost and labour-intensive business (Adams and Gibbs, 1994). Prior to the mid-1940s, golf courses used common Burmuda grass (Arizona common) from tee-box to green. This grass provides a course textured uneven, and thin putting

surface. The common Burmuda grass seeds were not certified because of seed from a tall, rapid growing Giant Burmuda grass (*Cynodon dactylon aridus*) was contaminated, until 1963 in an attempt to reduce the contamination. In 1947, the first recorded release of an improved vegetative established Burmuda grass that provide by the U.S. Golf Association (USGA) cooperate with the USDA. During the 1950s and 1960s, Dr. Glen W. Burton with USDA in Georgia released several interspecific hybrids, including Tiflawn, Tifgreen, Tifway, and Tifdwarf (McCarty and Miller, 2002). Tifgreen (or Tifton 328) is a hybrid between a fine-textureed *C. dactylon* and *C. transvaalensis*. It is a medium dark green Bermuda grass with high shoot density, fine texture, soft leaves, and low growth habit. Tifgreen does not tolerate a long-term mowing height less than 0.5 cm and it susceptible to sting nematodes and spring dead spot. This cultivar was released in 1956. Tifdwarf, this cultivar is definded as a natural mutant from Tifgreen that was found in the latitudes approximately 32˚ north and south of equator. Tifdwarf resembles Tifgreen except for its shorter leaves and internodes. It develops a very dark green turf with high shoot density and slow growth habit. FloraDwarf was relased from the University of Florida in 1995. It was selected from a previously planted Tifgreen Burmuda grass golf green located in Hawaii but is genetically different from Tifgreen and growth characteristics appear similar to Tifdwarf except for its much finer leaf blade, denser stand, shorter intermodal spacing, and extreamly low growth habit. TifEagle is one of the popularity grass species in golf green that is the third generation of Bermuda grass varieties which is developed exclusively for golf greens at the Coastal Plains Experiment Station in Tifton GA. Therefore, Tifdwarf was replaced Tifgreen 328 as the standard for putting greens over the last three decades, while TifEagle is assured to set an even higher standard for the 21st century. Based on careful evaluation at university research stations since 1988, and beginning in 1996 at golf course locations from Florida to the desert Southwest, TifEagle has proven to be a premier putting

surface. TifEagle also recovers more quickly from mechanical injury, has better color, and is extremely cold-hardy, drought-tolerant and disease-resistant (Hanna and Elsner, 1999).

CHAPTER III

MATERIALS AND METHODS

3.1 Soil samples and source of collection

Soil samples were obtained from four golf courses in Thailand including (i) Toscana valley golf course, Nakhon Ratchasima, Thailand (14°30'25.8"N 101°30'12.3"E), (ii) Pattana golf club and resort Chon Buri, Thailand (13°05'12.6"N 101°08'41.2"E), (iii) Suranaree golf club, Nakhon Ratchasima, Thailand (14°57'36.5"N 102°05'50.9"E) and (iv) Tiger golf club, Nakhon Ratchasima, Thailand (14°56'28.5"N 102°04'11.7"E). The rhizospheric soil samples were randomly collected by soils sampling tube (Figure 3.1). The rhizospheric soils were collected around 15 cm deep from soil surface on each golf course by removing shoots, while the remaining roots in the soil were chopped and mixed thoroughly before using in further experiments. (The soil chemical properties were shown in appendix I)

Figure 3.1 Soils sampling tube (25 mm in diameter) tool for rhizospheric soil sampling

3.2 Observation of AM re-colonization by trap culture technique

Each rhizospheric soil sample was mixed 1:1 (v/v) with autoclaved coarse sand and transferred into a 15 cm (diameter) plastic pot. Seeds of Bermuda grass (purchased from Westar seeds international Inc. El Centro, California) were planted into the soil around 80-100 seeds/pot. Plants were grown in a light room under the controlled environment condition following, 28 ± 2˚C with 16 h light/ 8 h dark cycle at light intensities 300 μE/m^2S and with 50% humidity for four months to allow plant growth and re-colonization by indigenous AM fungi in each soil sample. Fertilization is kept to a minimum level with ½ Hoagland's solution (Hoagland and Arnon, 1938) containing 50 μM of Phosphate (Pi), and being applied only when plants show signs of phosphorus (P) deficiency (purpling of leaf sheaths), or nitrogen deficiency (chlorosis of young leaves) (West Virginia University, 2017a). The re-colonization ability of AM fungi presented in each soil sample was observed by determination of AM root colonization efficiency.

3.3 Determination of root colonization efficiency

To assess the grass root colonized by indigenous AM fungi from each location, grass roots were washed with tap water to remove soil and cut into 1 cm pieces. The root samples were bleached with 10% (w/v) of KOH and incubated at 90˚C for 10 min. Then, the solution was removed and rinsed 3-4 times with deionized water (DI). After removing the water, 2% (v/v) of HCl solution was added and incubated at room temperature for 5 min. The solution was discarded and stained roots with 0.05% (w/v) Trypan blue in lacto glycerol by incubating at 90˚C for 10 min. Then, the root samples were de-stained with 50% (v/v) glycerol. The mycorrhizal colonization efficiency was determined in three replicates by followed the estimation of AMF colonization method (Trovelot, 1986). For frequency of mycorrhiza in the root system (F%), intensity of the mycorrhizal colonization

in the root system (M%), intensity of the mycorrhizal colonization in the root fragments (m%), arbuscule abundance in the root system (A%) and arbuscule abundance in mycorrhizal parts of root fragments (a%) using the MYCOCALC computer program https://www2.dijon.inra. The soil sample showing an ability of indigenous AM fungi re-colonization on grass root was used for further experiments.

3.4 Isolation of AM fungal spore from rhizospheric soils

The AM spores were collected by wet sieving and following by 60% (w/v) sucrose gradient centrifugation method (Dandan and Zhiwei, 2007). The process was started by re-suspending a 100 g of soil sample in 200 ml of tap water and stirred for 10 min. The suspension was allowed to settle for 10 sec and then the fraction between 38 µm and 500 µm were collected using Sieve Shaker model EML 200 Premium Remote machine (OMEGA SCIENTIFIC (THAILAND) Co., LTD) (Figure 3.2.)

The collected fraction was transferred into beaker and re-suspended again in 200 ml of tap water to repeat the procedure. The samples on sieve were washed using a fine stream tap water to transfer the samples from sieve into 50 ml centrifuge tubes and centrifuged for 10 min at 6000 x g to remove floating organic debris from rhizospheric soil. Then, the sample was re-suspended in 60% (w/v) sucrose for 8 min. After shaking vigorously to separate the spores from denser soil particles, the supernatant of sucrose solution was poured through a 38 µm sieve, and the trapped spores were washed with tap water to remove the sucrose. Then, the collected spores were rinsed into a small petri dish for further characterization.

Figure 3.2 Sieve Shaker model EML 200 Premium Remote machine (OMEGA SCIENTIFIC (THAILAND) Co., LTD) for mycorrhiza spore isolation from soil.

3.5 Spore morphology characterization

AM fungal spores isolated from each soil sample were grouped according to their morphological characteristics (West Virginia University, 2017b (http://fungi.invam. wvu. edu/the-fungi/species-descriptions.html) and determined the population in each group by counting under a stereo microscope. Criteria for morphological spore characterization were mainly based on spore size, spore color, and wall structure. The number of AM fungal spore in each population was counted from 20 g soil sample in triplicates. The AM fungal isolates showing high number of population were used to confirm their genus by small subunit ribosomal ribonucleic acid (SSU rRNA) gene sequencing method.

3.6 DNA extraction and sequencing

DNA from single AM spore was extracted by crushing spore in a PCR tube using a needle, and used directly as template for PCR. Partial SSU rRNA (18S rRNA) gene fragments were amplified using nested PCR with the universal eukaryotic primers NS1 (5′-GTA GTC ATA TGC TTG GTC TC-3′) and NS4 (5′-CTT CCG TCA ATT TCC TTT AAG -3′). The first PCR product (1,100 bp) as follows: initial denaturation at 94 ˚C for 3 min, followed by 30 cycles at 94 ˚C for 30 s, 40 ˚C for 1 min, 72 ˚C for 1 min, followed by a final extension period at 72 ˚C for 10 min. The diluted PCR product was used as DNA template in second PCR reaction to perform 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′) which gave a product size of 795 bp (Lee et al., 2008). as follows: 3 min initial denaturation at 94 ˚C, followed by 30 cycles of 1 min denaturation at 94 ˚C, 1 min primer annealing at 50 ˚C and 1 min extension at 72 ˚C, followed by a final extension period of 10 min at 72 ˚C. For comparison, PCR products derived from each AM fungal isolate were directly cloned into the pTG-19 cloning vector (Vivantis, USA) and transformed into *Escherichia coli* DH5α (using as the competent cell). The positive transformed cells were screened by X-gal (white colonies were selected only) and inserted clone was examined using M13f, (5′-GTA AAA CGA CGG CCA GT-3′), and M13r primers (5′-AGG AAA CAG CTA TGA CCA T-3′) PCR amplification. The insert DNA in plasmid was extracted followed by Sambrook et al., 1989. The sequenced by Macrogen Inc. The DNA sequence was analyzed by GENDOC program. All sequences were submitted to a BLAST search using the GenBank database (http://www.ncbi.nlm.nih.gov).

3.7 Phylogenetic tree construction

DNA sequences obtained from this study were aligned using Molecular Evolutionary Genetics Analysis version 6.0 (MEGA6) along with the representative 16 AM fungal sequences from GenBank. The alignment of SSU rRNA (18S rRNA) gene dataset was trimmed to the primer terminal ends (*c.*800 bp). Neighbour-joining (NJ) was constructed using MEGA6. Distances for the NJ tree were computed using the Kimura 2-parameter model with 1000 bootstraps. (Lee et al., 2008). A consensus phylogenetic tree was computed using *Archaeospora trappei* (accession no.Y17634.3), *Paraglomus brasilianum* (accession no. AJ301862.1), *Appendicispora fennica* (accession no. AM26819.4) and *Archaeospora leptoticha* (accession no. AJ301861.1) sequences as out group.

3.8 Propagation of selected AM fungi

Different plants including Bermuda grass (*Cynodon dactylon*), maize (*Zea mays*), sorghum (*Sorghum* sp.), and *Lotus japonicus* were used as host plant for testing AM fungal spore propagation. Fifty spores of selected AM fungus were mixed with 600 g of sterilized coarse sand: vermiculite (1:1) mixture and transferred into a 15-cm (diameter) plastic pot (50 spores/pot). Seeds were surface sterilized using 3% (v/v) sodium hypochlorite (for Bermuda grass 30 min, maize 10 min, sorghum 10 min, and *Lotus japonicus* 10 min) and germinated on 0.85% (w/v) water agar. Seedlings were planted in a pot (100 seeds for Bermuda grass, and 1 seed for maize, *Lotus japonicas* and sorghum). Plants were grown in a greenhouse for four months to allow the colonization and spore production, except *Lotus japonicas* was grown in growth chamber to maintain the temperature at 28˚c ± 2˚c. Fertilization is kept to a minimum level as described above. The number of AM fungal spore produced in each host plant was observed by counting under stereo microscope after separation the spore from soil as method described above. The spore number was calculated per 100 g soil in triplicate samples.

3.9 Evaluation the potential of isolated indigenous AM fungi on grass growth promotion

To observe the potential of the selected indigenous AM fungi on Bermuda grass growth promotion, 2.5 g of sterilized grass seeds was sown on plastic tray containing sterilized sand. Seedlings at 10 days after germination were transferred into plastic bag containing acidic clay soil pH3.29 (Soil chemical property shown in appendix 1) (bag size 7 cm in diameter with 500 g soil/bag). Then, each isolated indigenous AM fungus was inoculated into the soil at rate of 50 spores/bag to compare with non-inoculated plant. This experiment was performed under greenhouse condition in triplicates for each treatment. Plants were watered with tap water twice a day until 45 days after AM fungal inoculation. The data of plant height, root length, chlorophyll content, shoot fresh weight, shoot dry weight, and phosphorus (P) content in the shoot were collected. The chlorophyll content was measured by SPAD 502DL Plus chlorophyll meter (Spectrum Technologies, *Inc.*). The P content in plant was determined according to the standard method (Barton 1948). The colonization efficiency (F%, M%, m%, A%, and a%) of AM fungus on plant roots was also determined using the method as described above.

3.10 Effectiveness of the commercial AM fungal strain and PGPR co-inoculation for grass growth promotion

3.10.1 Determination the effect of commercial AM fungal strain on grass growth promotion

The commercial AM fungal strain (*Rhizophagus irregularis*) was used in this experiment. To test whether *R. irregularis* could promote Bermuda grass growth, the experiment was performed in both neutral (pH6.5) and acidic (pH4.5) soil conditions under laboratory (in a light room under the controlled environment condition following, 28 ± 2˚C

with 16 h light/ 8 h dark cycle at light intensities 300 μE/m^2S and with 50% humidity). Fifty seeds of Bermuda grass were sterilized with 70% (v/v) ethanol for 5 min and followed by 3% (v/v) sodium hypochlorite for 90 min (Stephenson, 1942), and rinsed with sterilized water for 10 times. Then, Bermuda grass seeds were sawn into nursery tray size 2.3×2.3 cm^2 containing sterilized peat moss and watered with sterilized water by mist spray. At 3-5 days after seed germination, fifty spores of *R. irrigularis* (Product from Premier Tech Biotechnologies Mycorise® ASP Arbuscular Mycorrhizal Fungal Inoculant obtained from Prof. Dr. Katsuharu Saito, Shinshu University) were inoculated into nursery tray containing grass seedling. Then, plants at 2 weeks after planting were transferred into new pot containing sterilized the mixture (1:1) of sand and 4 mm gravel. Plants were watered twice a week with ½ Hoagland's solution (Hoagland and Arnon, 1938) with 100 μM of Pi (ml/tray). The pH of plant nutrient solution was adjusted with NaOH or H_2SO_4 to obtain the pH level of 6.5 and 4.5 before watering. The data of plant dry weight and chlorophyll contents (measured by chlorophyll meter (Mc, and Jarvis, 2011)) were collected at one month after AM fungal spore inoculation.

3.10.2 Determination the effectiveness of commercial AM fungus and PGPR co-inoculation on Bermuda grass growth promotion

The experiment was performed under greenhouse condition in triplicates. Sterilized of Bermuda grass seeds were sowed on plastic tray size 11x23x2 $inch^3$ contained the mixture (1:1) of sterilized sand and vermiculite, and watered with tab water twice a day. Then, grass seedling at 10 days were used for testing in the soil condition. Two soil conditions were used in this experiment. The acidic soil (pH3.29) and neutral soil (the same acidic soil and adjusted pH to 6.5) were prepared in the plastic tray as described above. Before transferring grass seedling into the soil, the *R. irrigularis* around 2,600 spores/tray were inoculated into the soil. Then, the 100 ml culture of selected PGPR (*Pseudomonas* sp. SUT19) at concentration of 10^7 cells/ml dissolved in DI water were co-inoculated on plant

seedling. The data of plant biomass, mycorrhizal root colonization and chlorophyll content were collected at 45 days after AM and PGPR co-inoculation.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Re-colonization efficiency in root of Bermuda grass by indigenous AM fungi isolated from different golf courses

Based on the hypothesis that the effective indigenous AM fungi can be found in the rhizospheric soil of turf grass in the golf course, soil samples from different golf courses were collected and evaluated the re-colonization efficiency of isolated indigenous AM fungi soil. Bermuda grass (*C. dactylon*) was used as host plant for trap culture test. The result indicated that soil sample from all four golf courses contained indigenous AM fungi which could re-colonize Bermuda grass roots, but with the differences in colonization abilities (Table 4.1). The indigenous soil AM fungi from Toscana valley golf club showed highest frequency of mycorrhiza presented in the root system (F%) of Burmuda grass. Whereas, the highest intensity of the mycorrhizal colonization in the root system (M%) and in the root fragments (m%) were detected when using soil sampling from Pattana golf club, and the highest value of arbuscule abundance in the root system (A%) and in the root fragments (a%) were detected from Suranaree golf course soil sample. The high level of colonization and arbuscule abundance indicates the potential of fungi to colonize the root and exchange nutrients with plant. Differences in re-colonization efficiency of AM on Bermuda grass could be due to their host-fungus specificity (Leake et al., 2004). Host plant species may have an important role in AM development, sporulation, diversity and also patterns of fungal species composition in the rhizosperic soil. In this experiment, Toscana golf club and Pattana golf club used Bermuda grass as a turf grass, while Zoysia grass

(*Zoysia* spp.) and Paspalum grass (*Paspalum* sp.) were used as turf grass in Suranaree golf club and Tiger golf club, respectively. It could be implied that different grass species causes an effect on specific signal between plant-indigenous AM interaction (Gadkar et al., 2001) and influence on the re-colonization with Bermuda grass. Nevertheless, these results reveal that soil sample collected from each soil sample contains indigenous AM fungi which could re-colonize Bermuda grass and has potential to be selected as biofertilizer for application in the golf course.

Table 4.1 Re-colonization ability on Bermuda grass by AM fungi presented in each soil sample.

Sampling site	**F%**	**M%**	**m%**	**A%**	**a%**
Suranaree golf course	53.33^{c}	2.03^{a}	3.01^{a}	0.62^{a}	30.33^{a}
Tiger golf course	30.00^{d}	0.43^{c}	1.44^{b}	0.10^{b}	22.31^{b}
Toscana valley golf course	80.00^{a}	1.07^{b}	1.33^{b}	0.03^{c}	2.50^{d}
Pattana golf club and resort	66.67^{b}	2.57^{a}	3.85^{a}	0.24^{a}	9.22^{c}

Note; F%, Frequency of mycorrhiza in the root system; M%, Intensity of the mycorrhizal colonization in the root system; m%, Intensity of the mycorrhizal colonization in the root fragments; A%, Arbuscule abundance in the root system; a%, Arbuscule abundance in mycorrhizal parts of root fragments. Data represents mean of three replicates. Different letters in the same column indicate statistical difference by the Duncan test ($\alpha = 0.05$).

4.2 Isolation and identification of AM fungi from rhizospheric soil

To evaluate the diversity and to isolate the AM fungi that has high potential to colonize grass root, the AM fungal spores in each golf course were isolated from rhizospheric soil by wet-sieving technique. Indigenous AM fungal spores presented in each rhizospheric soil were grouped and counted the spore number in each group based on spore morphological characteristics. The isolated indigenous AM fungal spore named Tos1, Tos2, and Tiger1 were found in high population (more than 5 spores/g soil) (Figure 4.1a-d). Based on spore morphology index (http://fungi.invam.wvu.edu/the-fungi/species-descriptions.html) Tos1 may be classified as *Claroideoglomus lamellosum* (*REF No.*ON393) with cream to pale yellow color, globose to subglobose shape, and size distribution of spores was 80-140 µm (average 104 µm). Tos2 may be classified as *Claroideoglomus etunicatum* (REF No.NE108A) with orange to red brown color, globose to subglobose shape, and size distribution of spores was 60-160 µm (average 129 µm). Tos3 may be classified as *Acaulospora foveata (*REF No. BR861*) with* red-orange to dark red-brown color (immature spores initially are cream-colored and gradually acquire an orange tint as the spore wall begins to differentiate), globose to subglobose shape (sometimes irregular), and size distribution of spores was 240-360 µm (average 289 µm). Tos4 may be classified as *Cetraspora pellucida* (REF No.FL966) with hyaline/white color in most recently formed spores to yellow-brown in older spores, globose to subglobose shape, and size distribution of spores was 120-240 µm (average 189 µm). Tos5 may be classified as *Gigaspora gigantea* (REF No.MA435A) with bright greenish yellow to bright yellow green color, globose to subglobose shape (rarely irregular), and size distribution of spore was 240-400 µm (average 324 µm).

The number of spores were found at 8, 6, 3, 2 and 1 spores/g soil for Tos1 to Tos5, respectively (Fig. 4.1 a). AM fungi spore from Suranaree golf club, Pattana golf club which were labeled as Sur 3 and PT 1, respectively showed the same morphology with spore

Tos3. Moreover, Sur1 may be classified as *Claroideoglomus luteum* (REF No.SA112) which spore color: pale yellow (0-0-20-0 to dark yellow with a brownish tint (0-10-60-0). Spores have a thin “halo” when all layers are present. shape: globose to subglobose usually, while Sur2 may be classified as *Acaulospora kentinensis* (REF No.TW112) spore color: Orange-brown (0-20-60-0) to dark orange-brown (0-10-60-0), with many between these two extremes (0-40-100-0). Contents of juvenile spores are dense, so spores appear much more opaque than at maturity, shape: globose to subglobose, more rarely ellipsoid or irregular and Sur4 may be classified as *Rhizophagus fasciculatus* (unknown REF No.) which spore color: pale yellow to pale yellow-brown, shape: globose, subglobose. Wall structure: two layers are clearly discernible which are continuous with L1 and L2 of the spore wall. L3 of the spore wall also may continue into the hypha, but more often appears to curve and form a septum-like structure. Lastly, Tig1 may be classified as *Scutellospora dipurpurascens* (REF No.WV932), while Tig 2 could not be identified clearly from the reference.

The diversity of AM fungi population may cause from soil type, soil pH, organic compound in the soil, and climate parameters. The AM communities from different soil pH (neutral and acidic) were changed after pH manipulation (Kawahara et al., 2017). The diversity and specie richness of AM were subsequent reduced (measured by Shannon–Wiener index) when the nitrogen was enriched. The nitrogen enrichment also reduces spore abundance (Egerton-Warburton and Allen, 2000).

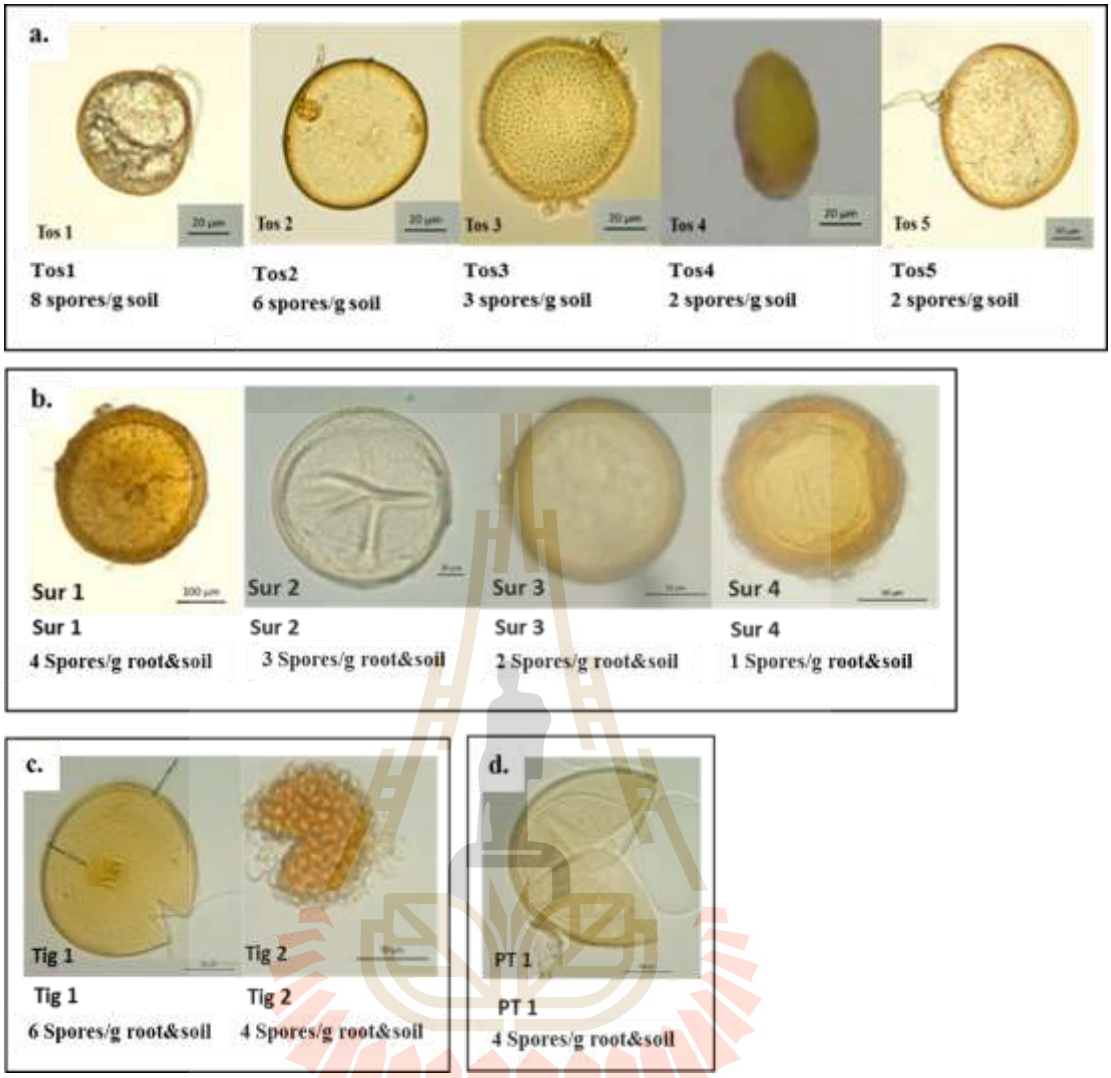

Figure 4.1 Spore morphology of AM fungi isolated from; a) Toscana valley golf course (Tos1, Tos2, Tos3, Tos4, and Tos5), b) Suranaree golf club (Sur 1, Sur 2, Sur 3, Sur 4), c) Tiger golf club (Tig 1, Tig 2,) and d) Pattana golf club and resort (PT1). Spores observed were under light microscope with objective lens 40x. The average spore number of each AM was indicated.

The top three dominant population, Tos1, Tos2, and Tos3 were collected for identification by sequencing the 18S rRNA gene. Partial SSU rRNA (18S rRNA) gene fragments were amplified using nested PCR with the universal eukaryotic primers NS1 and NS4. The result of PCR products size of 1,100 bp were presented in Figure 4.2. Then, the 2 nd PCR was amplified with AML1and AML2 primers using the first PCR product as a DNA

template and the DNA product size of 795 bp was obtained (Figure. 4.3). Each DNA fragment of T1, T2 and T3 derived from the 2nd PCR were inserted into pTG19-T cloning vector before transformed into *E. coli* DH5α. The colony of *E. coli* DH5α contained plasmid of pTG19-T+DNA fragment were selected on LB agar containing Ampicillin, X-Gal and IPTG white colony were selected and sequencing.

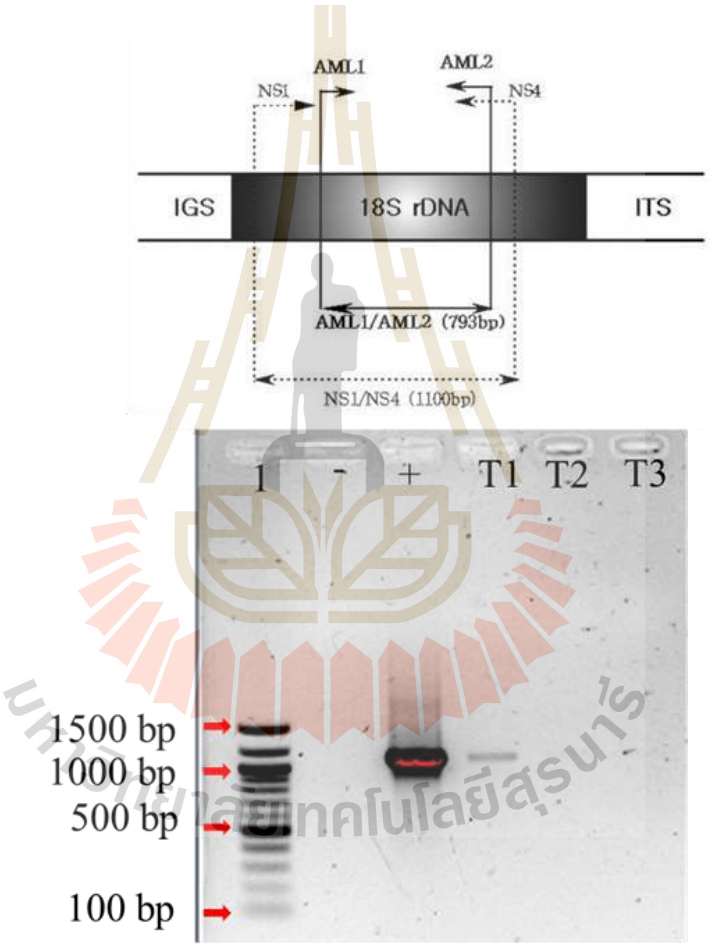

Figure 4.2 18S ribosomal DNA gene with annealing sites of primer pairs, NS1/NS4 and AML1/AML2 and their approximate DNA lengths; Partial SSU rRNA (18S rRNA) gene fragments (1 st PCR) by NS1, NS4 primer product size 1,100 bp. Lane1: 100 bp DNA marker; (-): negative control; (+): positive control (*Lotus japonicas*); T1: Isolate 1 Toscana spore (Tos1); T2: Isolate 2 Toscana spore (Tos2); T3: Isolate 3 Toscana spore (Tos3)

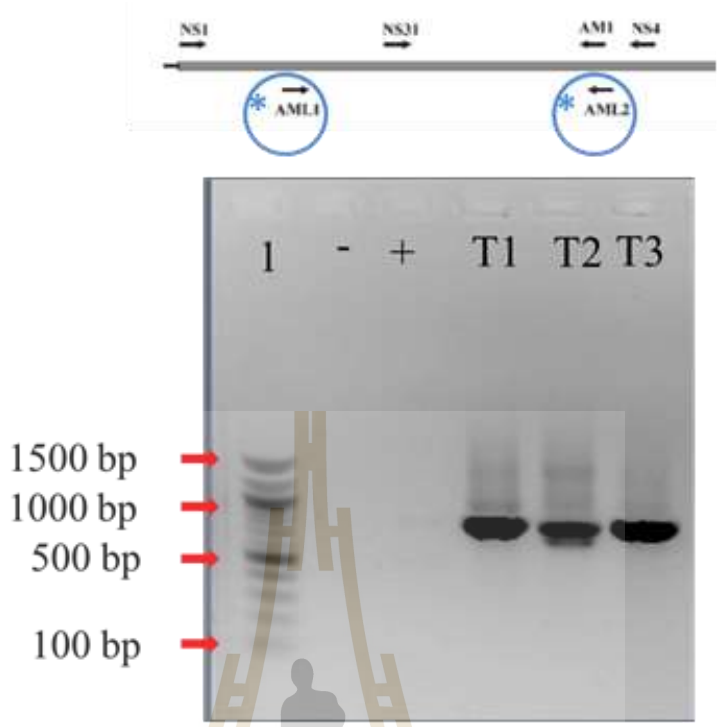

Figure 4.3 Partial SSU rRNA (18S rRNA) gene fragments (2 nd PCR) by AML1, AML2 primer product size 795 bp. Lane1: 100 bp DNA marker; (-): negative control; (+): positive control (*Lotus japonicas*); T1: Isolate 1 Toscana spore (Tos1); T2: Isolate 2 Toscana spore (Tos2); T3: Isolate 3 Toscana spore (Tos3)

BLAST search analysis showed 96-98% similarity to sequences belonging to the phylum Glomeromycota. The phylogenetic tree analysis on 18S rRNA gene of these three dominant isolates was constructed along with the representative 16 AM fungal sequences from GenBank. The result showed that the spore of AM isolates Tos1 and Tos2 were belonging to Claroidioglomus group, and Tos3 was located in Acaulospora group with 95% of similarity (Figure 4.4). This result confirmed the identification of AM by morphological observation at the level of genus. However, the specie of these AM isolates could not be verified due to the PCR product of 18S rRNA gene sequences in this study was only 795 bp long. The longer fragment of gene should be amplified for further identification in the level of AM species.

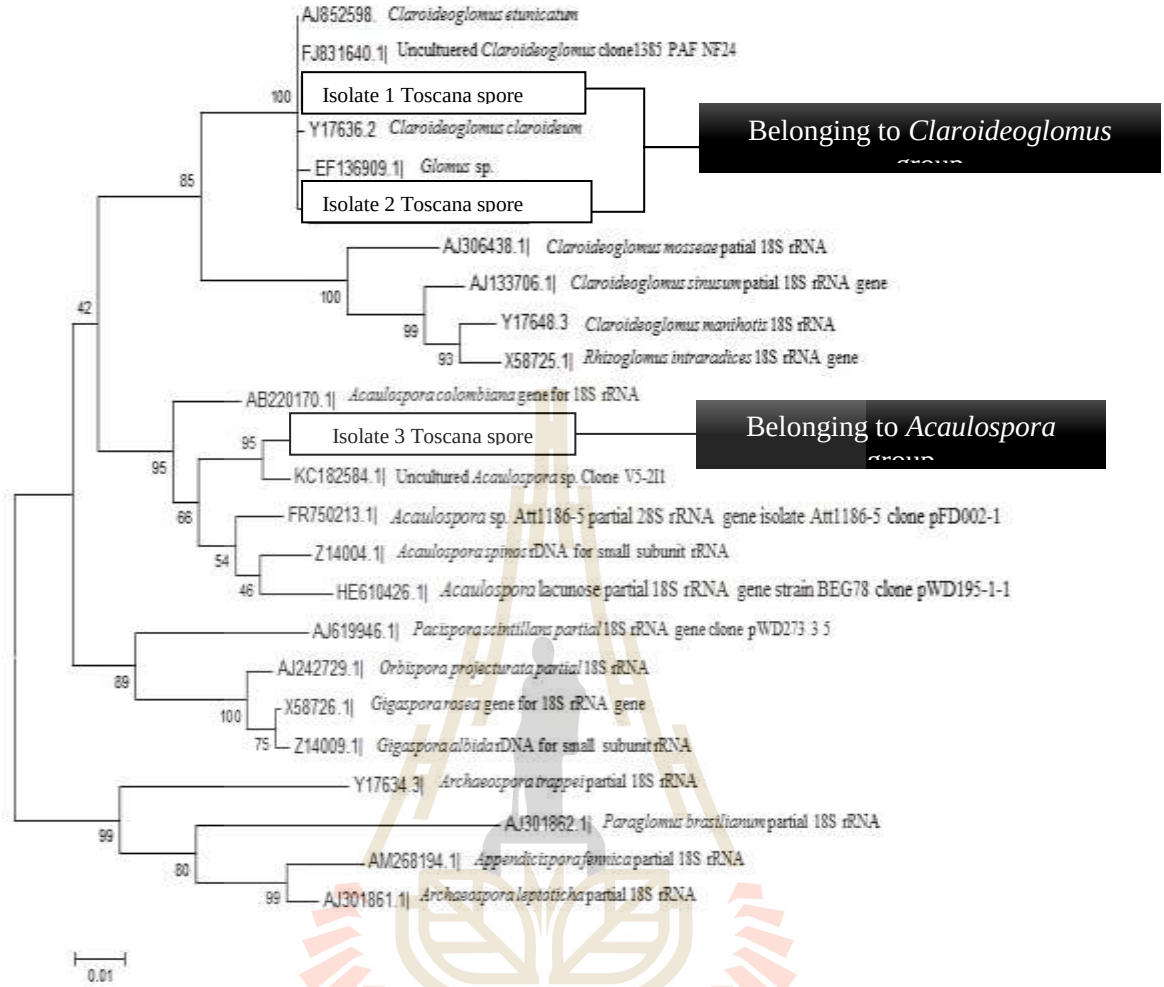

Figure 4.4 Phylogenetic tree based on sequences of 18S rRNA genes showing classification of the arbuscular mycorrhiza fungi isolated from soil sample. Bootstrap values are expressed as percentages of 1,000 replications. The bar represents 1 estimated substitution per 100 nucleotide positions.

4.3 AM fungal spore propagation

To continue testing the grass growth promoting property of these indigenous AM fungi, the selected AM fungal spores must be propagated before used in the next experiment. Based on the hypothesis that the most dominant specie could have higher ability to colonize plant root and support plant growth due to the host plant preference selection, the most dominant AM fungal species form Toscana valley golf course,

Suranaree golf course, Pattana golf course, and Tiger golf course were selected. The spore isolate of Tos1, Sur1, PT1, and Tig1 were selected for propagation in different host plants including Bermuda grass (*C. dactylon*), maize (*Zea mays*), *Lotus japonicus* and Sorghum (*Sorghum bicolor* (L.) Moench) to compare the host preference for the purpose of spore production in greenhouse. The result showed that Tos1 was significantly produced in high spore number when using maize and sorghum as a host plant (Figure 4.5a), while the spore of PT1 and Sur1 were significantly produced in higher number when using grass and sorghum as host plant (Figure 4.5b and c). For Tig1, high spore number production was showed only when grass plant was used for propagation (Figure 4.5d). The result in this experiment indicates that each AM fungal isolate has different host preference for spore propagation. However, it seems that the common preference host plants were grass and sorghum (Figure 4.5). It has been reported that the monocotyledon host plant could have high percentage of AM root colonization due to the density of root system (Rahim, Jais and Hassan, 2016). However, different ability of spore production may be due to the preference of AM fungus and host plant. Since AM fungi use strigolactones as signal molecules in the initial communication with host plants for symbiosis (Yoneyama et al., 2008), it may be possible that different plant hosts released different types or concentrations of strigolactones and affected the symbiosis interaction and lead to affect the spore production. Unfortunately, in this experiment, the AM fungal spore of isolate Tig1 was obtained in very low number. Thus, it could not be used in next experiment.

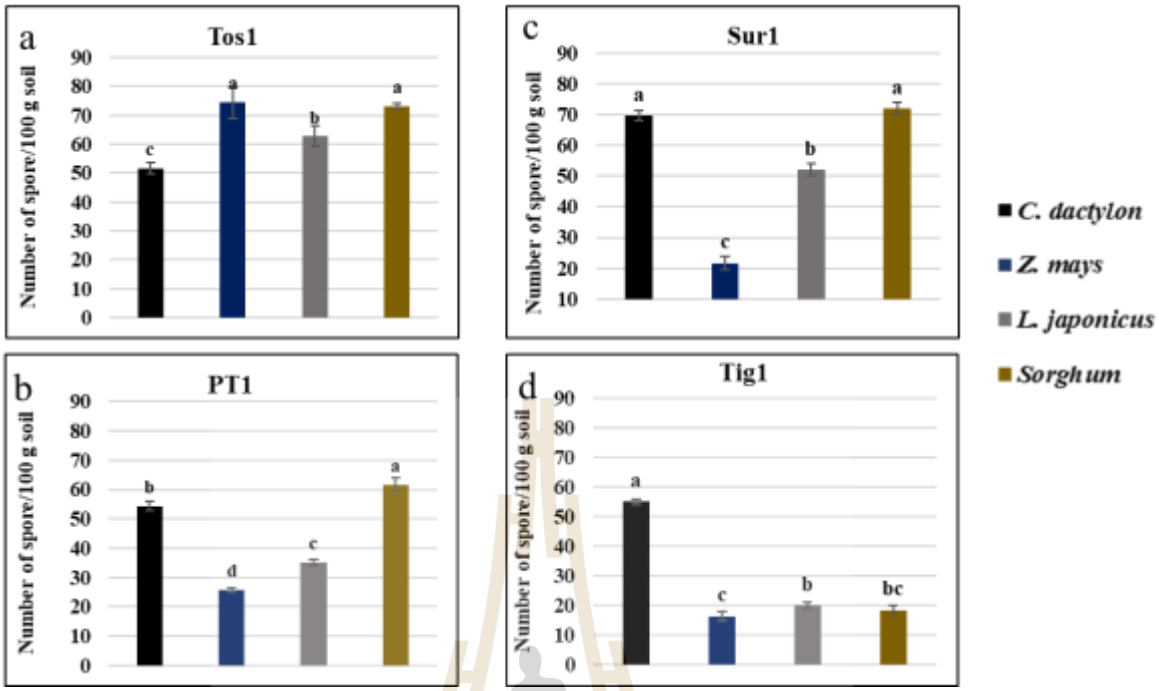

Figure 4.5 Number of AM spore production when propagated with different host plants growing under greenhouse condition. Black, blue, gray and brown bar where represents spores number of Bermuda grass (*C. dactylon*), maize (*Z. mays*), *L. japonicus*, and sorghum, respectively. Data represents mean of three replicates ± standard error of the mean. Different letters indicate statistical difference by the Tukey HSD range test ($\alpha = 0.05$).

4.4 Evaluation the potential of isolated indigenous AM fungi on grass growth promotion

To study the effect of isolated indigenous AM fungi on grass growth promotion, the strongly acidic soil (pH 3.29) was used in this experiment (the soil chemical properties were shown in appendix I). It was known that several plant nutrients, such as nitrogen (N), phosphorus (P), potassium (K), sulfur (S), calcium (Ca), and magnesium (Mg) are the major deficient nutrients in acidic soil. Since the main function of AM fungi is to make

these nutrients available for plant, the effect of plant growth promotion by AM fungi should be clearly observed in acidic soil. The overall grass growth promotions by AM fungi isolate Sur1, PT1, and Tos1 in comparison with non-inoculated plant were shown in Figure 4.6 and 4.7. The fungi isolate Sur1 and PT1 could promote plant growth in acidic soil greater than those inoculated with isolate Tos1 and non-inoculated plant. The shoot and root of grass inoculated with Sur1 and PT1 were observed to be clearly longer than those of Tos1 and non-inoculated plants (Figure 4.7). The longer of plant root system would provide the better nutrient acquisition from the soil and support plant growth growth under acidic condition. The longer of plant root system may be due to the high colonization of AM fungus in the root. The result of root colonization efficiency was confirmed in Table 4.2.

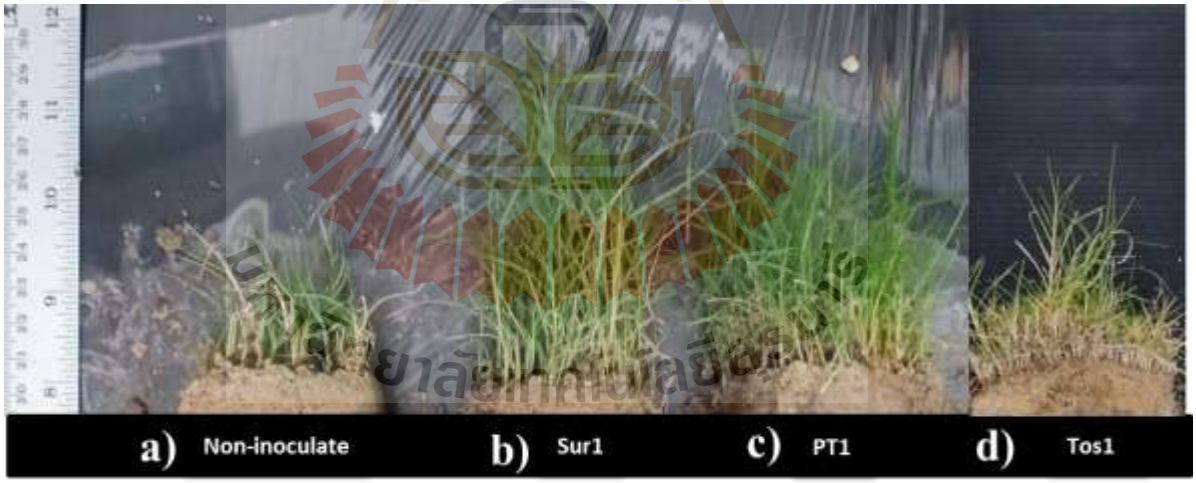

Figure 4.6 Plant growth under acidic soil condition (pH3.29) in green house at 45 days after AM fungal spore inoculation; a) non-inoculation (Control), and plant inoculated with AM fungi spores b) isolate Sur1, c) isolate PT1, and d) isolate Tos1, respectively.

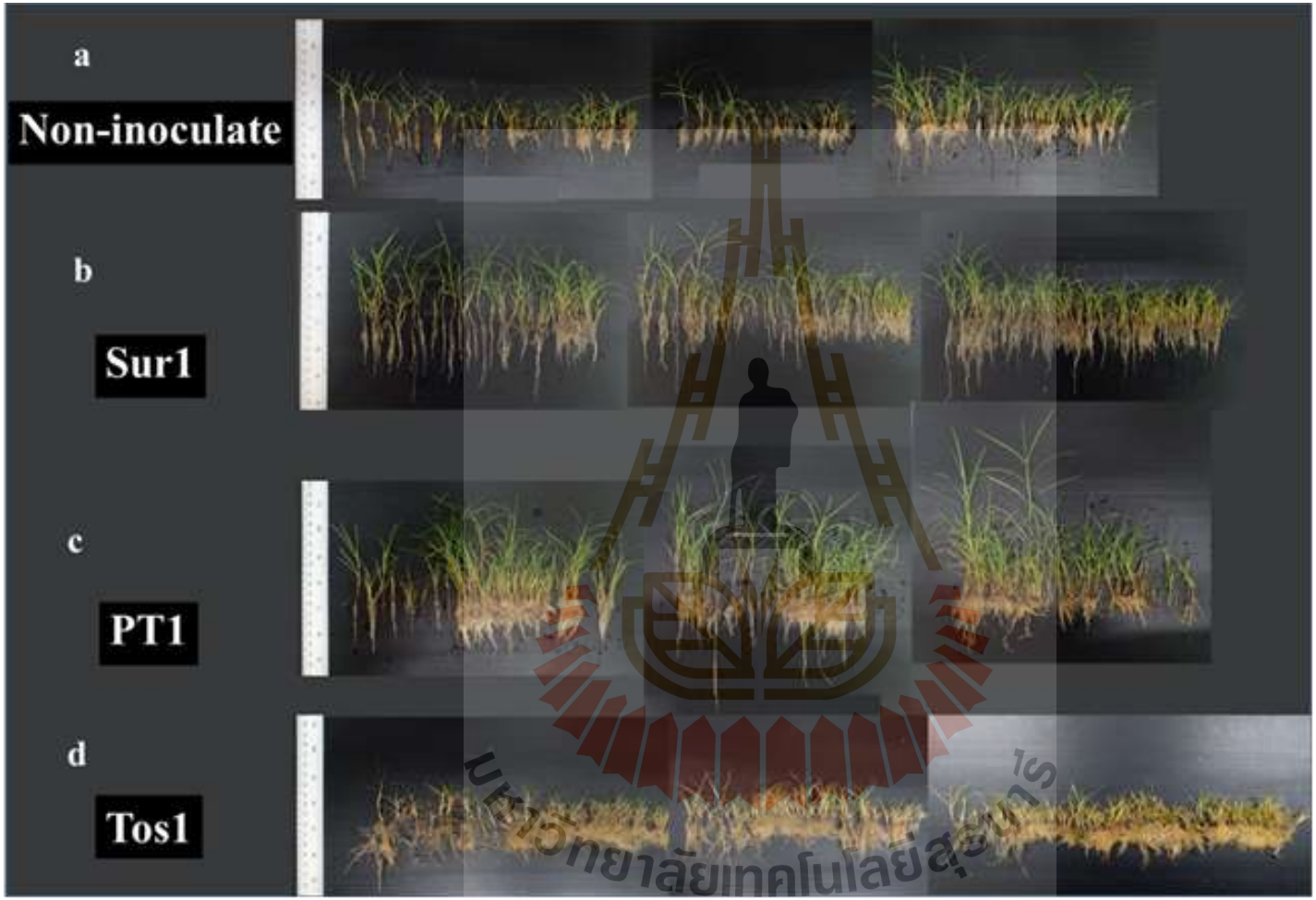

Figure 4.7 Shoot and root of grass grown under acidic soil condition (pH3.29) in green house at 45 days after AM fungal spore inoculation; a) non-inoculation (Control), and plant inoculated with AM fungi spores of; b) isolate Sur1; c) isolate PT1; and d) isolate Tos1, respectively in triplicate sampling pots.

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The results of shoot fresh weight, shoot dry weight, shoot biomass, phosphorus and chlorophyll contents were shown in Figure 4.8. In this experiment, all the root parts were collected for determination the colonization efficiency (Table 4.2), thus the root fresh weight, root dry weight, and root biomass could not be recorded. However, the data from the shoot parts showed that Bermuda grass inoculated with all isolates of AM fungi could promote plant growth (shoot fresh and dry weight and biomass) significantly greater than that of non-inoculated plant. The best AM fungus on plant growth promotion was the isolate PT1, while no difference in plant growth parameters were observed among plant inoculated with Sur1 or Tos1 (Figure 4.8a-c). However, AM fungus isolate Tos1 tend to promote plant growth less than other AM fungal isolates. For chlorophyll content, grass inoculated with Sur1 and PT1 contained significantly higher chlorophyll content than those of Tos1 and non-inoculated plant (Figure 4.8d). The highest phosphorus content in plant was found in grass inoculated with isolate PT1. Although it was not significantly difference from non-inoculated or inoculated with isolate Sur1, but significant difference was shown when compared with isolate Tos1. However, the phosphorus content detected in this experiment was lower than the data reported by McCrimmon, J. N. (2001), which showed the Bermuda grass in general contain around 0.2-0.5% phosphorus by weight.

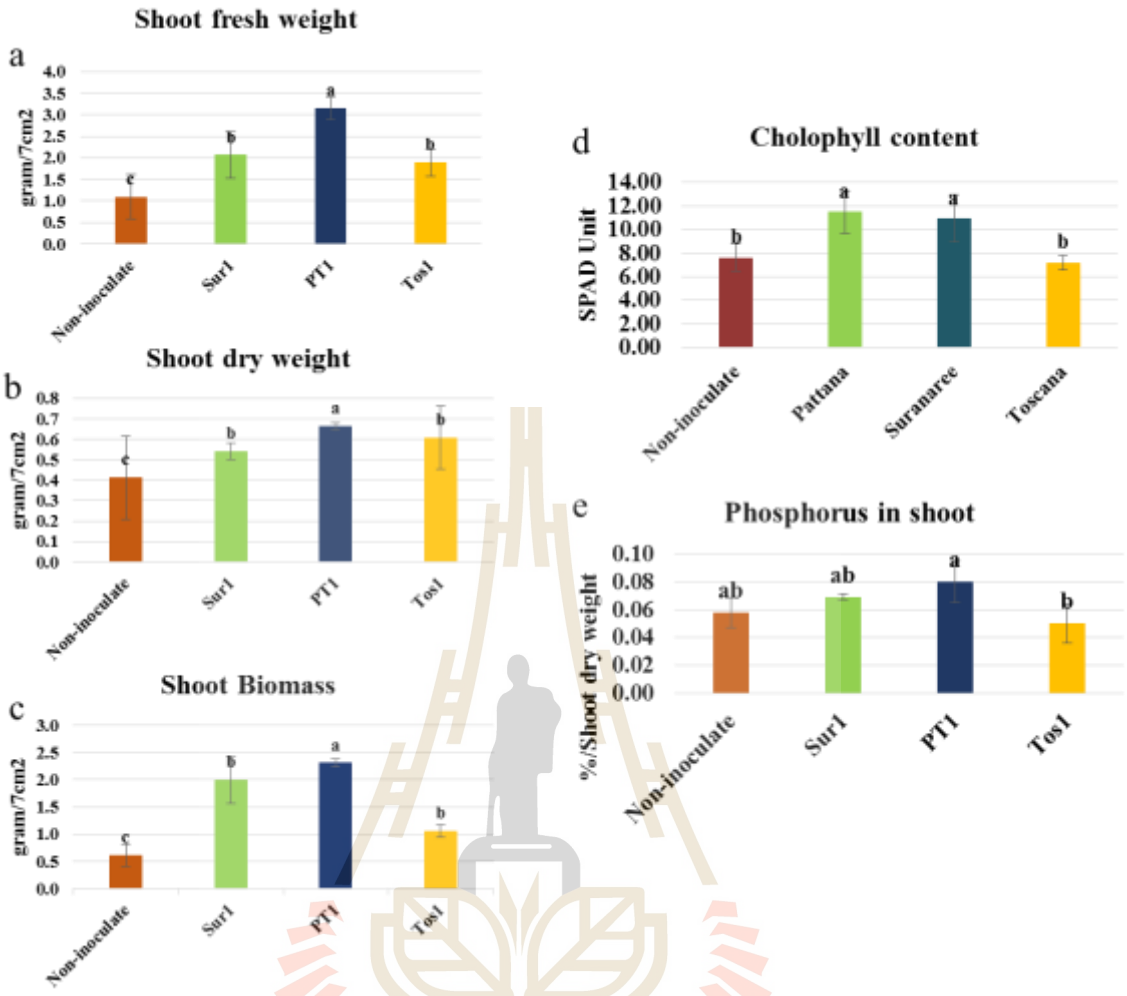

Figure 4.8 Effect of isolated indigenous AM fungi on; a) shoot fresh weight; b) shoot dry weight; c) shoot biomass; d) chlorophyll content; and e) phosphorus content in the grass grown under acidic soil condition (pH3.29) in green house at 45 days after AM fungal spore inoculation.

The colonization efficiency of each AM fungal isolate was also observed and the results were demonstrated in Table 4.2. AM fungus isolate Sur1 and PT1 tended to have high root colonization ability (F%, M%, and m%) with high arbuscule abundance (A% and a%), while these abilities were lower in grass root inoculated with the isolate Tos1. These data revealed that the plant growth promotion ability by AM fungus under strong acidic soil condition may be due to the ability of AM fungus to colonize and exchange the plant nutrient through the abundance of arbusbule in the plant cell (Rouphael et al., 2015).

Although this thesis lack of strong evidence to show the function of AM fungi on facilitating nutrients available for plant, the physiological properties of plant growth and colonization ability may support the potential of using indigenous AM fungal isolate Sur1 and PT1 as biofertilizer for Bermuda grass in the golf course.

Table 4.2 Colonization efficiency of different AM fungal isolates on Bermuda grass root grown in acidic soil (pH3.29) condition

Treatment	F%	M%	m%	A%	a%
Sur1	51.2	7.86	15.25	0.89	11.38
PT1	62.11	9.74	15.68	0.81	8.32
Tos1	32	0.52	1.63	0.01	1.15

Note; F%, Frequency of mycorrhiza in the root system; M%, Intensity of the mycorrhizal colonization in the root system; m%, Intensity of the mycorrhizal colonization in the root fragments; A%, Arbuscule abundance in the root system; a%, Arbuscule abundance in mycorrhizal parts of root fragments. Data calculated by Mycocal program

4.5 Effectiveness of AM fungus and PGPR co-inoculation for grass growth promotion

To evaluate the efficiency of plant growth promotion by the combination of AM fungus with PGPR, the commercial AM specie of *Rhizophagus irregularis* and *Pseudomonas* sp. SUT19 were used in this experiment. Firstly, the Bermuda grass growth promotion by the commercial strain of *R. irregularis* was observed in laboratory using the mixture of sand and gravel (1:1) as planting material and watering by Hoagland solution with low available P content. The result showed that *R. irregularis* could significantly

increase Bermuda grass dry weight greater than non-inoculated plant and *R. irregularis* could promote plant biomass around 1.9 folds and1.6 fold when compared with control under pH 4.5 and pH 6.5 conditions, respectively (Figure 4.9a and b). The chlorophyll content in plant leaves was also significantly increased when inoculated with *R. irregularis*.

Plant inoculated with *R. irregularis* contained chlorophyll content around 5 folds and 3.1 folds when compared with control under pH 4.5 and pH 6.5 conditions, respectively (Figure 4.9c**).** The overall plant phenotypes grew on pH4.5 and 6.5 were shown in Figure 4.9d and Figure 4.9e**.** It seems that the overall growth of Bermuda grass was affected from acid condition. The external pH causes an effect on plant growth and nutrient absorption response due to unavailable of some plant nutrients, such as N, P, K, Ca, Mg, Mo and S (Ruan et al., 2007; www.pda.org.uk). However, the significant increase of plant biomass or chlorophyll content was influenced by inoculation of AM fungi. Colonization of plant roots by AM would increase more possibility of nutrients acquisition through the fungal mycelia (Rillig and Mummey, 2006). Moreover, it has been reported that AM colonization on plant root could increase mineral content accumulation in plant under acidic condition (Clark and Zeto, 2000). Therefore, the access of unavailable nutrients under acidic condition would be increased when plant was colonized by AM. However, this efficiency is depended on the AM specie and its host plant (Clark, 1997). This result indicated that the commercial AM fungus of *R. irregularis* can be used as biofertilizer to support Bermuda grass growth as well.

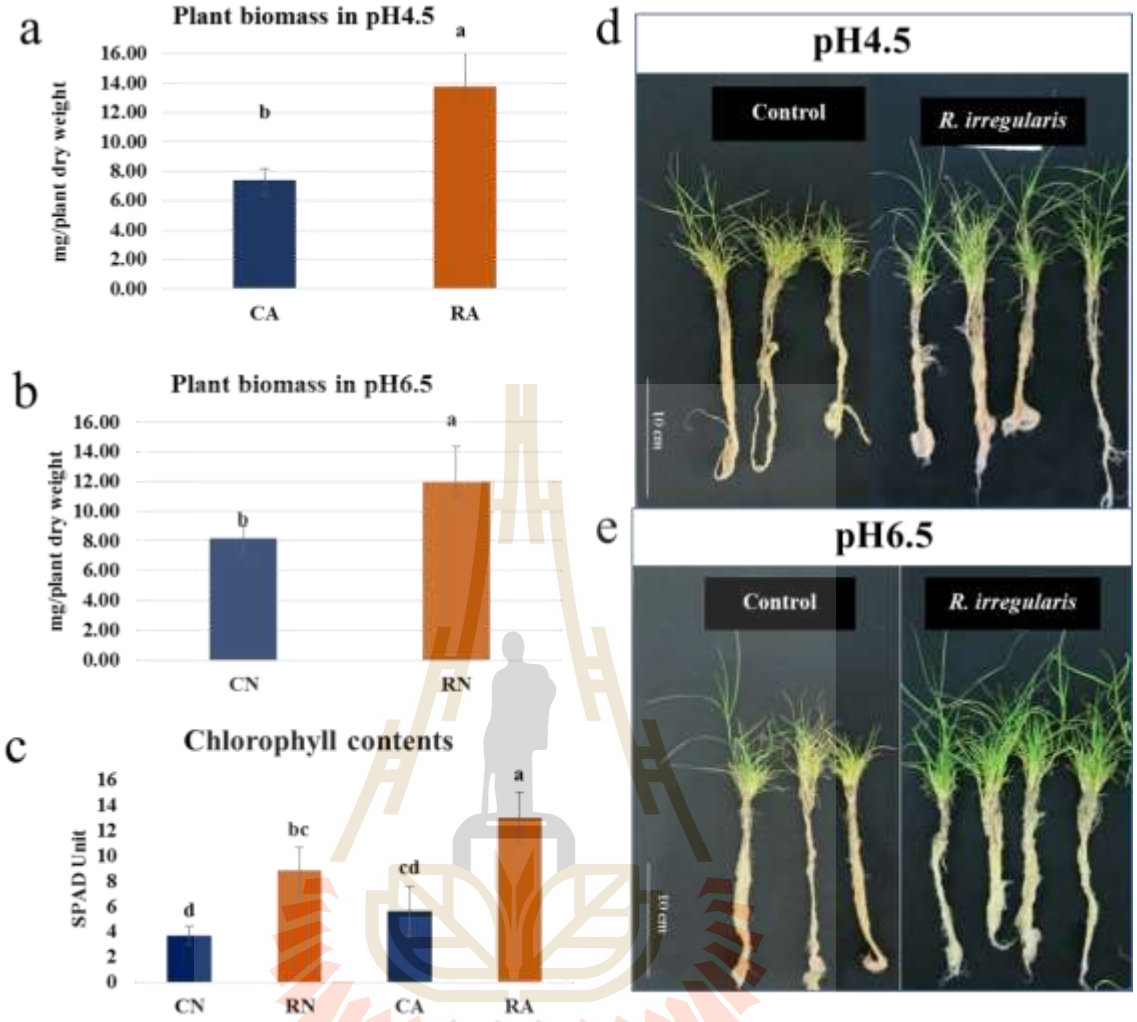

Figure 4.9 Effect of *R. irregularis* inoculation on (a) plant biomass in pH4.5, (b) plant biomass in pH6.5, (c) chlorophyll content of Bermuda grass growing under different pH, (d-e) plant growth under pH4.5 and pH6.5 condition, respectively. CN, non-inoculated plant grew under neutral pH; RN, *R. irregularis* inoculated plant grew under neutral condition; CA, non-inoculated plant grew under acidic pH; RA, *R. irregularis* inoculated plant grew under acidic condition.

Therefore, *R. irregularis* spore was used for co-inoculation with PGPR to observe the synergistic effect on Bermuda grass growth promotion under both neutral soil (pH6.5) and acidic soil (pH 3.29) in the greenhouse conditions.

The result showed that at 45 days after inoculation, the frequency of mycorrhiza in root system (F%) detected in the treatment of pH6AM and pH6AM+19 were 81.22% and 76.60%, respectively, which were significantly higher than that of under acidic soil (Figure 4.10a). This result indicates that soil pH had an effect on mycorrhiza root colonization, while the treatment of co-inoculation AM fungus with PGPR did not enhance AM root colonization. However, the intensity of mycorrhizal colonization in the root system (M%) was significantly increased in the treatment of pH6AM+19 (Figure 4.10b). The intensity of mycorrhizal colonization in the root fragment (m%) was also significantly increased in the treatment of pH6AM+19 and also tended to be increased in the treatment of pH3AM+19 (Figure 4.10c). The arbuscule abundance in the root system (A%) showed highly significant in pH6AM+19 treatment at 3.93%, but no significant difference was observed for the arbuscule abundance in root fragment (a%) among all treatments **(**Figure 4**.**10 d-e**)**. It was noticed that the co-inoculation of *Pseudomonas* sp. SUT19 with AM significantly enhanced the intensity of mycorrhizal colonization under normal condition, while the colonization was low under acidic soil. It could be hypothesized that the plant growth promoting ability of SUT19 may be affected by low pH due to some mechanisms. It has been reported that low pH causes negative effect to *Pseudomonas mandelii* growth (Saleh-Lakha et al., 2009). However, some of bacteria could act as mycorrhiza helper bacteria (MHB) to promote both ectomycorrhiza and arbuscular mycorrhiza by increasing growth and nutrient uptake such as *Pseudomonas fluorescens*, *Bacillus* sp. (Frey-Klett, 2007). MHB can enhance mycorrhizal function, increase mycorrhizal growth, or provide nutrients to the fungus and plant (Johansson 2004). In this study, SUT19 has been reported to promote plant growth through the nitrogen fixing ability, plant hormone (IAA) production, and reduce plant stress due to the activity of ACC deaminase enzyme (Piromyou et al., 2011). Therefore, it is possible that *Pseudomonas* sp. SUT 19 promotes plant growth and leads to support AM fungal colonization in plant roots in this condition.

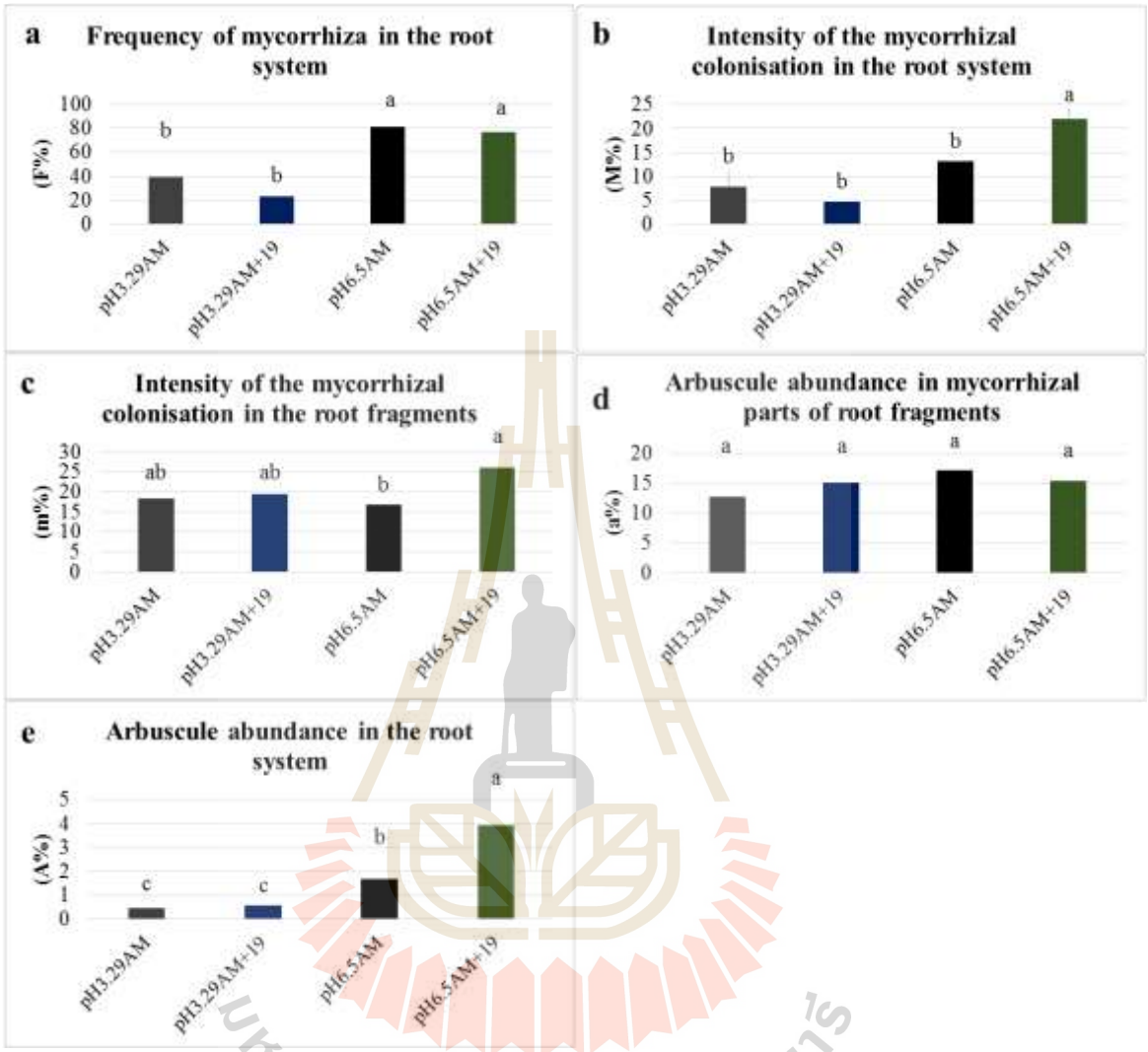

Figure 4.10 Mycorrhiza root colonization in Bermuda grass roots under both neutral and acidic soil conditions (pH6.5, and pH3.29). **(a)** F%, Frequency of mycorrhiza in the root system; **(b)** M%, Intensity of the mycorrhizal colonization in the root system; **(c)** m%, Intensity of the mycorrhizal colonization in the root fragments; **(d)** A%, Arbuscule abundance in the root system; and **(e)** a%, Arbuscule abundance in mycorrhizal parts of root fragments. **(f)** Root length. Data represents mean of three replicates ± standard error of the mean. Normality test by Shapiro-Wilk. Different letters on each bar indicate statistical difference by the Tukey HSD test ($\alpha = 0.05$).

Moreover, the plant physiological data were also recorded. The root length of grass plant was not significantly different under neutral soil condition (pH6.5) (Figure 4.11a). However, the significantly increase of root length under acidic soil condition (pH3.29) was observed when plant are inoculated with AM or PGPR when compared with non-inoculated plant (Figure 4.11a). Plant biomass was significantly increased in pH3AM+19 around 2.2 folds when compared with pH3 control treatment. Under neutral soil pH, the AM and PGPR inoculation did not significantly enhance plant biomass when compared pH6 control treatment (Figure 4.11b). Since the plant nutrients are mostly available for plant absorption under neutral soil condition (Bondy, E. J. 1969), it could be one of the reasons that the supporting benefit from AM or PGPR are dispensable. The chlorophyll content in plant leave was not significantly different among the treatments when tested under pH3.39. However, the significant increase of chlorophyll was found when co-inoculated AM with SUT19. The chlorophyll increased around 1.6 folds when compared with non-inoculation at pH6.5 (Figure 4.11c). In case of phosphorus content, there was no significant difference among the plant grew in the acidic soil at pH3.29. However, the level of phosphorus content increase in the plant grew in neutral pH soil, but there was no effect of the co-inoculation AM with SUT19 on phosphorus content (Figure 4.11d). From this experiment, although the influence of SUT19 co-inoculation did not clearly increase the root length and phosphorus content in the grass, SUT19 may play some roles in synergistic interaction with AM fungus, such as ACC deaminase activity to reduce plant stress and support the overall growth when grew under acidic soil condition (Figure 4.11b). Moreover, the preference of Bermuda grass on PGPR should also be considered to enhance plant growth.

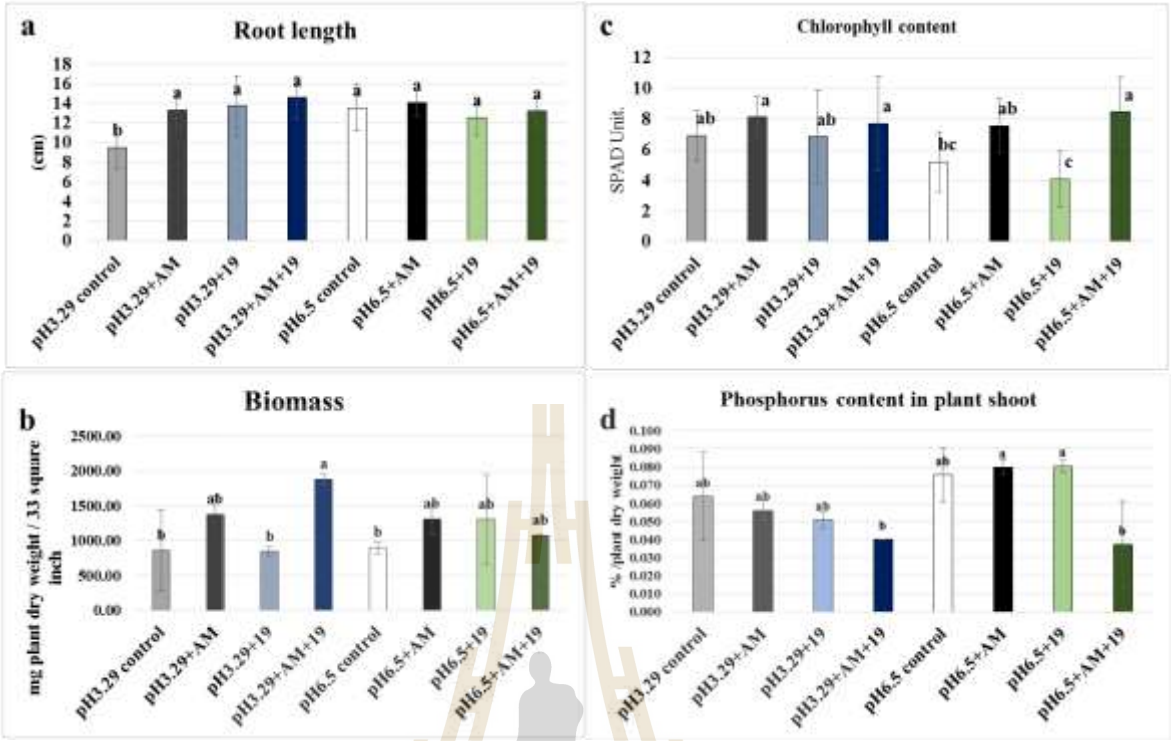

Figure 4.11 The Root length (a.); Plant biomass (b.); Chlorophyll content (c); and phosphorus content in plant leaf (d). Data represent mean of three replicates ± standard error of the mean. Normality test by Shapiro -Wilk. Different letters on each bar indicate statistical difference by the Tukey HSD test (α = 0.05).

CHAPTER V

CONCLUSION

This study revealed that diverse genera and species of AM fungi were presented in the soil collected from different golf courses. The most abundance genera were *Claroideoglomus* and *Acaulospora*. However, the number of populations was different according to the soil source and type of turf grass in each golf course. The isolated indigenous AM fungi have different preferences on host plant for propagation. Bermuda grass and sorghum could promote the propagation of most AM fungal isolates. The indigenous AM fungi isolate Sur1 and PT1 could promote Bermuda grass growth better than non-inoculated plant under acidic condition. Thus, these two isolates have high potential to develop as biofertilizer for application with Bermuda grass in the golf course. Finally, the strategy of co-inoculation AM fungus with PGPR could be used for support the AM colonization efficiency on plant root and the overall growth of Bermuda grass. However, the synergistic effect among AM fungus and PGPR was not clearly observed in this study may be due to the specific preference of Bermuda grass and the AM fungus on PGPR strain.

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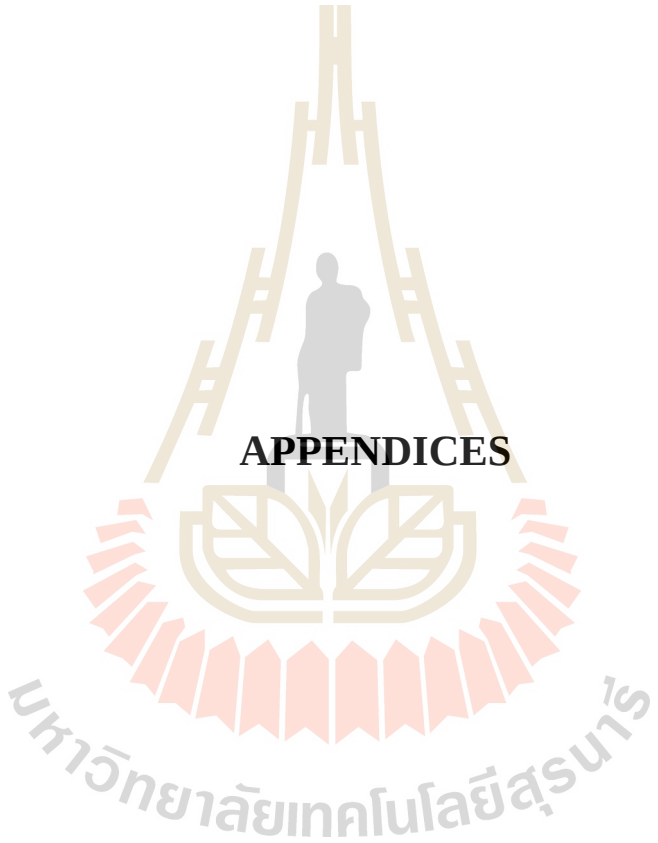
APPENDICES



Appendix I. The soil chemical properties

Sample	EC(dS/m) 1:5	pH 1:5	%OM	P (ppm) available	$\%P_2O_5$	K (ppm)	Ca (ppm)	Mg (ppm)
1. Acid soil	1.2	3.29	2.00	13.8	0.12	132.9	477.9	490.0
2. Toscana	0.27	6.35	3.01	3.0	0.03	178.4	474.6	286.9
3. Tiger	0.28	4.32	0.37	10.8	0.04	64.5	187.3	100.0
4. Pattana	0.41	7.35	2.25	29.6	0.03	776.0	1046.2	242.1
5. Suranaree	0.42	6.80	3.73	10.3	0.10	75.5	1174.1	255.7

Available P (ppm)		Exchangeable (ppm)	
< 10	low	< 60	low
10-15	normal	60-100	normal
>15	high	>100	high

Appendix II. *Pseudomonas* sp. SUT 19 cells culture in LG media under different pH

SUT 19	pH	OD_{600}	Cells numbers
	6.8	0.455	2.3×10^7
	6.0	0.414	-
	5.5	-	-
	5	0.416	2×10^7
	4.5	0.34	2×10^7
	4	0.42	-
	3.5	0.47	1×10^7
	3.3	0.33	-

BIOGRAPHY

Ms. Niramon Lakkason was born on January 18, 1992 in Ubon Ratchatani, Thailand. She graduated with a bachelor degree of Crop Production Technology. From Suranaree University of Technology (SUT) in Year 2015. She continue her Master degree course in School of Biotechnology, Institute of Agricultural Technology, SUT with Assoc. Prof. Dr. Panlada Tittabutr. While studying, she received a Graduate scholarship from SUT OROG to support her tuition and fee. Her research topic was "Selection of arbuscular mycorrhiza for application in Bermuda grass (Cynodon dactylon)". The results from some parts of this study have been presented as poster at the 4th Asian Conference on Plant-Microbe Symbiosis and Nitrogen Fixation, October 16-19, 2016, and oral presentation at the 3rd National and International Research Conference on Science and Technology, Social Science and Humanities, May, 4th, 2017, respectively. She was recognized for the best paper award in this conference.