Diversity and Biological function of Indigenous Arbuscular Mycorrhizal Fungi in Rice

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ชื่อผู้เขียน ขจรพงศ์ นพกาศ

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บทคัดย่อ

ข้าวและเชื้อราอาร์บัสคูลาร์ไมคอร์ไรซา อาศัยอยู่ร่วมกันในสภาวะพึ่งพาอาศัย โดยเชื้อราอาร์บัสคูลาร์ไมคอร์ไรซาช่วยเพิ่มประสิทธิภาพในการลำเลียงแร่ธาตุ มากไปกว่านั้นเชื้อราอาร์บัสคูลาร์ไมคอร์ไรซายังช่วยเพิ่มฟอสฟอรัสที่ใช้ประโยชน์ในดินได้ โดยการหลั่งเอนไซม์แอซิดฟอสฟาเตส ในนาข้าวที่มีน้ำท่วมขังทำให้การอาศัยอยู่ร่วมกันของข้าวและเชื้อราอาร์บัสคูลาร์ไมคอร์ไรซาลดน้อยลง แต่อย่างไรก็ตามยังพบเชื้อราอาร์บัสคูลาร์ไมคอร์ไรซาบางสกุลที่สามารถตรวจพบได้ในรากข้าวภายใต้สภาวะน้ำท่วมขัง เพื่อที่จะตรวจสอบบทบาทของน้ำท่วมขังต่อความหลากหลากหลายของเชื้อราอาร์บัสคูลาร์ไมคอร์ไรซาในนาข้าว จึงได้ทำการทดลองปลูกข้าวสังข์หยดเมืองพัทลุงในสภาวะน้ำท่วมขัง และสภาวะที่ไม่มีน้ำท่วมขังในดินออร์แกนิกที่เก็บมาจากนาข้าวท้องถิ่น จังหวัดพัทลุง จากผลการศึกษาที่ได้หาลำดับเบส DNA บริเวณ SSU-ITS-LSU ของไรโบโซม พบว่ามีเชื้อราอาร์บัสคูลาร์ไมคอร์ไรซาทั้งหมด 21 OTU โดยมี *Acaulospora morrowiae* เป็นชนิดเด่น เพื่อที่จะตรวจสอบบทบาทของเชื้อราอาร์บัสคูลาร์ไมคอร์ไรซาชนิดเด่นดังกล่าว จึงได้ทำการทดลองไปเลี้ยงร่วมกับข้าว 3 สายพันธุ์ คือ ข้าวสังข์หยดเมืองพัทลุง ข้าวไร่ดอกข่าเมืองพังงา และ Nipponbare ในกระถางขนาด 0.8 ลิตร ที่บรรจุทรายและcompost โดยเติมสารละลายที่มีแบคทีเรียที่ผ่านการกรองจากดิน ระยะเวลาในการปลูกทั้งหมดคือ 6 สัปดาห์ ผลการศึกษาพบว่ามีเชื้อราอาร์บัสคูลาร์ไมคอร์ไรซาชนิด *Acaulospora morrowiae* 60-70 %ในรากข้าวแต่ละสาย

พันธุ์ โดย *Acaulospora morrowiae* ทำให้ข้าวมีการเจริญเติบที่ต่ำลง แต่ประสิทธิภาพการสังเคราะห์ด้วยแสงไม่มีการเปลี่ยนแปลง ส่วนบทบาทของ *Acaulospora morrowiae* ในดิน พบว่าช่วยเพิ่มฟอสฟอรัสที่ใช้ประโยชน์ได้โดยการหลั่งเอนไซม์แอซิสฟอสฟาเตส พวกเราได้คาดการว่า *Acaulospora morrowiae* จะถูกจำกัดการอาศัยอยู่ร่วมกันกับข้าวในระหว่างการปลูกข้าวในสภาวะน้ำขัง แต่อย่างไรก็ตาม *Acaulospora morrowiae* น่าจะเข้าไปอาศัยอยู่ร่วมกับรากของวัชพืช และช่วยหลั่งหลั่งเอนไซม์แอซิสฟอสฟาเตสเพื่อเพิ่มฟอสฟอรัสที่ใช้ประโยชน์ได้ ทำให้ดินในนาข้าวมีฟอสฟอรัสเพิ่มขึ้นสำหรับการปลูกข้าวในฤดูปลูกข้าวถัดไป

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Abstract

Rice forms a symbiotic interaction with arbuscular mycorrhizal fungi (AMF). AMF provides rice with the improvement of nutrient acquisition through the AMF uptake pathway. Moreover, AMF enhances phosphorus availability by the release of acid phosphatase. In lowland rice paddies, AMF symbiosis deteriorated by flooding. However, some AMF genera were preferentially detected in rice roots under flooded conditions. To examine the influence of flooding on AMF diversity in lowland rice paddies, Sangyod Muang Phatthalung (SMP) rice seedlings were cultivated in organic rice paddy soil under non-flooded and flooded conditions. Based on the SSU-ITS-LSU ribosomal DNA sequences, there were 21 AMF operation taxonomic units (OTU) in the rice roots. *Acaulospora* was the dominant AMF genus in the flooded roots. Later, *Acaulospora* spores were isolated and propagated by trap culture. Based on their SSU-ITS-LSU sequenes, the cultured *Acaulospora* spores were *Acaulospora morrowiae*. To characterize the symbiotic function of the dominant *Acaulospora morrowiae* in rice, a lowland *indica* SMP rice, an upland *indica* Rai Dawk Kha Phangna (DK), and a lowland *japonica* Nipponbare rice were grown in 0.8-L pots containing sterile sand and compost, recolonized with a native microbial filtrate in the greenhouse for 6 weeks. The result showed that 60-70 % of the rice roots were colonized by *A. morrowiae*. The

inoculation of *A*. *morrowiae* suppressed rice growth but did not change the efficiency of photosynthesis. In soil, *A. morrowiae* increased P availability via the higher activity of acid phosphatase. We anticipated that the growth suppression due to the *A. morrowiae* might be mitigated by the limitation of AMF colonization during rice cultivation due to flooding. However, the *Acaulospora* fungi might form a symbiosis with weedy plants in dry seasons, indirectly improving soil P for rice growth in the next cultivation seasons

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My sincere gratitude goes to my family and my friends for their support. I would like to extend my appreciation to my friends that has been there supporting me, cheering me up and teaching me. I love you all.

Khachonphong Nopphakat

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	ABBREVIATION	
1	AMF	Arbuscular mycorrhizal fungi
2	OUT	Operational taxonomic unit
3	SMP	Sangyod Muang Phatthalung
4	DK	Dawk Kha
5	P	Phosphorus
6	N	Nitrogen
7	MAPK	Mitogen-activated protein kinase

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CHAPTER1

Introduction

Arbuscular mycorrhizal fungi (AMF) are one of the most important endophytic fungi on the planet. They form a mutualistic relationship with about 90 % of terrestrial plants. AMF symbiosis enhances host plant to uptake mineral nutrient especially phosphorus (P). Moreover, AMF improve host plant against root pathogen. Lowland rice ecosystem is reported to be poor of nutrient since flooded condition can leaching and runoff of nutrients. To overcome this problem, rice forms symbiosis with AMF to deal with nutrient deficiencies in soil. AMF prevent loss of nutrient in flooded soil condition. Rice is one of the most important food sources for human. It is mostly cultivated in irrigated lowland. Flooding remarkably reduces AMF colonization and AMF diversity since it accelerates of the degradation of root cortical cell and form aerenchyma. However, some AMF genera are predominantly detected in rice root under flooded condition.

In our previous study, we examined the effect of flooding on AMF community composition in Sangyod Muang Phatthalung (SMP) rice root. Flooding shaped the AMF community in the SMP roots. The question in present study is does flooding affect AMF diversity in SMP roots. We hypothesize that flooding may decrease AMF colonization and AMF diversity. Here, we aimed to investigate the effect of flooding on AMF diversity and to characterize symbiotic function of AMF in rice. Although native AMF are present in several lowland, knowledge of a symbiotic function of indigenous AMF has been poorly understood.

We used the Krüger primers to detect 1.5kb SSU-ITS-LSU region in the SMP roots grown in organic rice paddy soils under non-flooded and flooded conditions. Subsequencely, a neighbor joining tree were constructed to identify AMF species. Shannon's diversity index was carried out to compare AMF diversity between the two different soil water conditions. To propagate the AMF fungi, we performed trap culture using maize as a host plant. Later, we identified cultured AMF spores by analyzing a phylotaxonomy constructed from the 1.5kb SSU-ITS-LSU region of the cultured AMF spores. Then, we inoculated the cultured AMF fungi in tree rice cultivars, i.e. a model *japonica* lowland Nipponbare, SMP, an *indica* lowland rice and Rai Dawk Kha Muang Phangna (DK), an *indica* upland rice to characterize a symbiotic function of cultured AMF spores.

Findings from this study lead to the understanding of the role of native AMF in rice.

Moreover, the AMF inoculation could be applied in rice paddy to improve soil P availability, which are important to improvement of rice cultivation. In addition, our report presents AMF species in organic lowland rice paddies, which are a valuable natural resource for improvement of rice cultivation.

1.Literature review

1.1 Biology of arbuscular mycorrhizal fungi

1.1.1 Description of AMF symbiosis

Arbuscular mycorrhizal fungi (AMF) belong to the phylum Glomeromycota. AMF form majority a mutualistic relationship with the root system of terrestrial plants in both natural and agricultural ecosystem (Dodd, 2000) and this relationship has existed for over 400 million years (Smith & Read,2008). The relationship is beneficial to both host plants and fungus, AMF promote plant growth and plants feed lipid and sugar in exchange. Approximately 95% of terrestrial plants including angiosperm, gymnosperm, pteridophytes, gametophytes can associate with AMF (Read et al., 2000). Arbuscule and vesicle are part of AMF hyphae structure, and they are established in the inner cortex of root plant system. AMF can improve soil nutrient acquisition to plants especially phosphorus and nitrogen (Nouri et al., 2014). They also play crucial roles in plants to tolerate enhanced water stress (Pavithra & Yapa, 2018) and provide them resistance against soil pathogens (Elsen et al., 2008). Moreover, AMF have been reported to promote plant growth under environmental stresses, such as salt stress, water stress (Huang et al., 2020) and heavy metal stress (Weissenhorn et al., 1995)

1.1.2 AMF spore propagation using traditional trap culture assay

Trapping method is very important to maintain the health of AMF spores. The suitable host plants for AMF culture are described in International Vesicular Arbuscular Mycorrhiza (INVAM) as AMF host plants should be compatible with greenhouse

climates, such as growth in a potting medium, UV radiation, and temperature. The host plants should be able to adapt to a wide range of soils varying in nutrient properties and soil physical structure. The host plants should have a moderate rate of root growth is moderate so that complete infiltration of a pot culture takes at least 8 to 12 weeks. The branching of roots should be extensive and usually is colonized by AMF. They should be high photosynthetic efficiency with a high P requirement (metabolism) such as maize, wheat, and sorghum. They should be resistant to a wide range of insect pests, and pathogens including soil-borne nematodes (INVAM).

1.1.3 AMF symbiosis

Under phosphate deficiency in plants, strigolactone, which is a plant hormone that synthesized and released by the plant roots into its surrounding environment which then stimulates symbiosis between plant and AMF (Figure1). Strigolactone induces hyphopodium branching of the AMF spore since it stimulates nuclear division, enlargement of mitochondria, and rapid increase in ATP content in AMF hyphae leading to hyphae proliferation, whereas AMF in turn releases a signal molecule known as chitooligosaccharides from the hyphopodium to induce host plants which allow AMF establishment in the cortical cell. Chitooligosaccharide stimulates expression of a suite of plant genes, calcium spiking in rhizodermal cells, starch accumulation in roots, and lateral root formation before colonization (Gutjahr and Parniske, 2013)

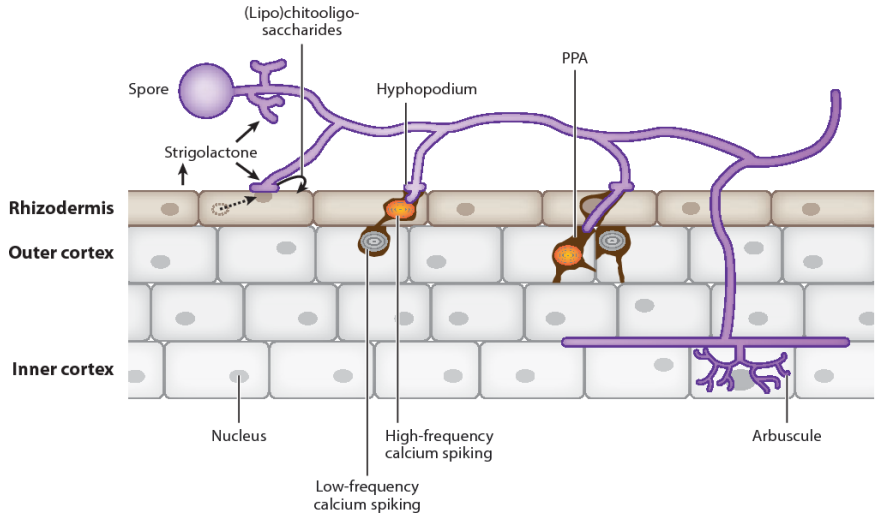

Figure 1 AMF development in a host plant. Stingolactone is a plant hormone, which produced and released by a plant root. This chemical induces hyphopodium branching of AMF. Lipochitoologosaccharide is a singnal molecule exudated by AMF allows the penetration of hyphae into root cortical cells. Subsequently, the mutualistic relationship between a host plant and AMF takes place when arbuscule is formed in the root inner cortex (Gutjahr and Parniske, 2013)

1.2 Diversity composition of arbuscular mycorrhizal fungi in a wetland ecosystem

1.2.1 AMF symbiosis in the wetland ecosystem

A wetland ecosystem is a transition habitat between aquatic and terrestrial ecosystems, which has either seasonally flooding or permanently flooding, low oxygen, and extremely high or low concentration of available nutrients in the soil. AMF requires oxygen to survive, and many species of wetland plants have been described as non-mycorrhiza (Khan 1974; Mosse et al. 1981; Anderson et al. 1984; Mejstrik 1984). However, numerous previous studies have reported that AMF forms a symbiosis with

many species of plants in different wetland ecosystems such as swamps (Bâ & Rivera-Ocasio, 2015), saltwater marsh (Wilde et al., 2009), freshwater marsh (Miller, 2000), bog (Bohrer et al., 2004) and mangrove ecosystem (Wang et al., 2010). AMF plays important roles, such as improving biomass production and nutrient acquisition in wetland plants (Wolfe et al., 2007). In addition, they play a role in the composition and diversity of wetland plants (Wolfe et al., 2006)

1.2.2 Flooding strongly inhibits AMF symbiosis

Flooding is a detrimental factor, which can decrease the diversity of AMF based on phylotype richness, Shannon's index, and phylotype composition (Wang et al., 2011) because AMF fail to germinate new hyphae to the root plant under flooding condition. Flooding is decrease AMF colonization via increasing aerenchyma of the inner cortex cell in the root plant (Vallino et al., 2014). However, AMF can function under flooding conditions because the two AMF marker genes including *Gint*PT and *Gint*AMT2 involved in Pi transport and NH_4^+ retrieving, respectively are expressed very similarly compared between dry and flooding conditions, this evidence strongly indicates that flooding does not influence AMF viability but affects AMF colonization (Vallino et al., 2014).

1.2.3 Fungal microbiome in lowland rice paddy

Rice paddy field is threatened by water regimes during rice cultivation time, where AMF diversity and colonization are reported to decrease. However, a previous study found that AMF can colonize at 11% to 40% within rice seedlings. The phylogenetic analysis revealed that the *Acaulospora* and *Glomus* are the dominant

genera found in the paddy field (Watanarojanaporn et al., 2013). In the natural colonization, rice was colonized by AMF, with a root colonization rate of 1.8% to 61.4% (Bernaola et al., 2018). Although flooding reduced the colonization rate in a rice paddy, the remaining AMF in root is sufficient to uptake nutrient since the AMF contribute the mineral nutrient to rice (Vallino et al., 2014). Moreover, flooding reduces fungal diversity in lowland rice roots compared to upland rice root. In upland rice, roots are reported to increase in some fungi families (Pang et al., 2020).

1.3 Role of arbuscular mycorrhizal fungi in agroecosystem

1.3.1 AMF associated with plant to overcome nutrient depletion in soil

The main problem factor in an agricultural area is the depletion of nutrients especially phosphorus and nitrogen in the soil. This problem limits agricultural production in the world. To overcome this problem, plants form a symbiosis with AMF in the root system. AMF plays an important role in phosphorus mobilization and uptake from the soil into rice root (*Oryza sativa* L.) through hyphae and improves plant growth under flooded and non-flooded conditions (Hajiboland et al., 2009). AMF increase nitrogen content by 64.8% in shoot and 41.29% in the root of AM-upland rice compared with a non-colonized plant by using a pot assay cultivated in a greenhouse (Xiao et al., 2010). At the level of gene expression, AMF has been reported to increase stress-response genes in plants. Reactive oxygen species homeostasis and oxidative stress battling genes are higher in AMF-plants compared with non-AMF-plants (Fan & Liu, 2011). AMF improves plant growth under drought stress by increasing the level of gene expression of the mitogen-activated protein kinase (*MAPK*) pathway, which

involves drought stress resistance (Huang et al., 2020). Furthermore, AMF enhances phosphate uptake from the soil into rice roots by driving the gene expression of the phosphate transporter gene (*Os*PT11) in rice plants (Chen et al., 2013a).

Unavailable phosphorus (P) such as iron phosphate, aluminum phosphate, and calcium phosphate are also limiting factors for plant growth because plants cannot uptake unavailable P from the soil. Acid phosphatase biosynthesis and exudation from root plants under P starvation can hydrolyze unavailable P forms into Pi, which is made readily available for plant roots or soil microorganisms (Nannipieri et al., 2011). The acid phosphatase activity is increased by the associated AMF (Y. Liu et al., 2021), which instead can promote plant production. Many studies reported that the most abundant form of soil organic phosphorus is phytase, which can increase P nutrition of AMF-plant resulting in increased plant biomass (Wang et al., 2017)

1.3.2 AMF-specific gene expression in rice

Knowledge about specifically expressed genes of arbuscular mycorrhizal fungi (AMF) in rice was elucidated by Gutjahr et al., (2008). They selected 18 rice genes that are specifically associated with AMF species (*Glomus intraradices*). They performed RT-PCR to investigate the level of gene expression of AM-specific genes at different times i.e.3,5,7 and 9 weeks after inoculum with *G. intraradices* compare with mock treatment (non-inoculation). They demonstrated that all 18 AM-specific genes were expressed at different times after inoculation. Among 18 AM-specific genes, four genes including AM1, AM2, AM3, and AM11 were expressed early three weeks after inoculation. Other genes were expressed at 7 weeks after inoculation. In mock treatment

all the 18 AM-specific genes were undetectable. The result indicated that AMF is the strong driving force in AM-specific gene expression in rice plants.

1.3.3 Nutrient loss prevention

Although many agricultural ecosystems are added with chemical fertilizer, which contains all the macronutrients for plants such as phosphorus, nitrogen, potassium, and other micronutrients. However, the nutrient in the soil can be lost from agricultural fields via runoff and leaching especially P loss in paddy soil because plants can uptake just 10%-20% of these fertilizers during growing seasons (Cordell et al., 2009). This problem reduces sustainable rice production and leads to environmental issues, such as eutrophication, groundwater pollution, and loss of aquatic biodiversity. Plants have a crucial role in reducing P loss in soil. In addition, AMF association in an agricultural ecosystem is a common way to alleviate P loss from paddy soil because it can cut down P loss via leaching and runoff, the P loss in the mycorrhizal rice line was 10% less than with the control (non-mycorrhizal rice). Therefore, AMF symbiosis might facilitate a sustainable rice production field (S. Zhang et al., 2020). Moreover, other nutrients, such as nitrogen, potassium, and zinc can be lost via leaching and runoff processes. AMF can promote the host plant to take up nutrients from the soil into the root because they increase the nutrient interception zone in the root plant. Therefore, AMF can reduce nutrient loss from soil (Cavagnaro et al.,2015)

1.4. Identification of arbuscular mycorrhizal fungi

1.4.1 Identification of AMF based on a molecular approach

The fundamental issue of AMF identification and classification limited the study of AMF diversity and communities in both agricultural and natural ecosystems. Many AMF species have been described based on the spore morphology approach following the International Vesicular Arbuscular Mycorrhiza (INVAM) culture collection. However, the great variation of AMF spore morphology in the same species might be due to the wrong identification and classification. The molecular analysis provides potential identification of the AMF growing in plant roots and spores present in the soil, independent of morphological criteria. Several primer sets have been used for the identification of AMF, such as SSU primer, Redecker primer, LSU primer, ITS2 primer, and Kruger primer. However, the Kruger primer is the best primer to study AMF diversity (Kohout et al., 2014a). The target of Kruger primer is composed of the nuclear small subunit of ribosomal DNA (SSU), internal transcribed spacers (ITS region), and large subunit of ribosomal DNA (LSU). The Kruger primer has been used for amplification by polymerase chain reaction (PCR), and the SSU-ITS-LSU fragment allows phylogenetic analysis at the species level because the region (SSU-ITS-LSU) is specific and conserved in all the AMF families (Kruger et al., 2009).

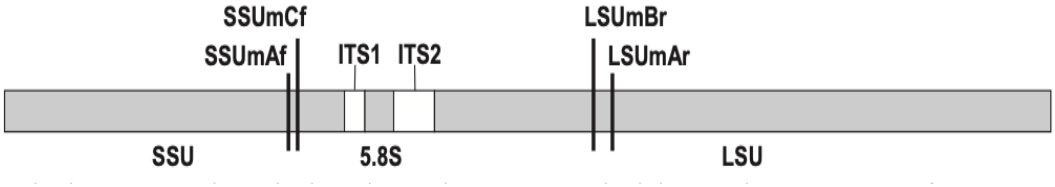

Figure 2 The SSU-ITS-LSU region of arbuscular mycorrhizal fungi. The primer pairs used in the study are composed of the SSUmCf (mixture SSUmAf1-2), and SSUmCf (mixture SSUmCf1-3) as a forward primers, and LSUmAr (mixture LSUmAr1-4) or LSUmBr (mixture LSUmBr 1-5) as a reverse primers. SSU represents a small subunit of ribosomal DNA, ITS represents internal transcribed spacer and LSU represents a large subunit of ribosomal DNA (Kruger et al., 2009).

1.4.2 Identification of AMF based on spore morphological approach

The International Vesicular Arbuscular Mycorrhiza (INVAM) is very important to assist researchers in mycorrhizal science, especially in AMF spore morphology identification. Knowledge about AMF diversity has considerably increased due to the use of DNA-based identification and continued to progress in morphological identification (Kruger et al., 2009). New species of AMF have been described every year through AMF single-species culturing and concomitant morphological and phylogenetical analysis. The important spore characteristic including spore ornament, number of spore walls, spore sessile, and sporiferous saccule have been used to identify at the species level.

Research questions

1. Does flooding affect AMF diversity?

2. Does AMF inoculation affect rice growth performance and soil P availability?

Research objectives

1. To examine the influence of flooding on AMF diversity

2. To characterize the symbiotic function of AMF in rice

CHAPTER2

Methodology

2.1 Determination of AMF in rice roots

2.1.1 Soil sampling and experimental design for determination of AMF diversity

In our previous study, organic paddies soil was collected randomly from organic rice paddies of the Phatthalung Rice Research Center, Phatthalung, Thailand. Some of the organic paddies soil was adjacent to a rhizosphere of SMP from previous rice cultivation. For soil properties analysis, we determine total N, total P, available P, Total K, soil PH, and electroconductivity. Subsequently, we grown SMP rice seedlings in a pot containing 1.5 kg of the organic paddies soil under non-flooded and flooded conditions for six weeks in an open greenhouse condition (n = 6 biological replicates) (Klinnawee et al., 2021).

2.1.2 Plant material

SMP rice seedlings was obtained from the Phatthalung Rice Research Center, Phatthalung, Thailand. SMP is a lowland *indica* rice verity, which a protected geographical indication status grown in Phatthalung province in Southern Thailand. The unique geographical origin of the SMP was reason for selection as a model to determine the diversity of indigenous AMF and their symbiotic function. Moreover, we considered other two rice verities, included DK, an upland *indica* rice verity along with model rice Nipponbare, a lowland *japonica* rice verity to test the biological function

between plant and AMF. The experimental design to test a symbiotic function of AMF and the three rice cultivars was described in topic 2.3.1.

2.1.3. Genomic DNA extraction from SMP rice root

SMP roots sample was weighed at 100 mg and ground into fine powder in liquid nitrogen. The tissue was added with 750 µl of extraction buffer containing 200 mM Tris-HCl pH 8.5, 50 mM EDTA, 0.5 M NaCl, 1% SDS and 0.1% B-mercaptoethanol. The lysate was incubated at 65°C for 20 min. The mixture was added with 250 µl of 5M potassium acetate pH6. Before incubating the lysate on ice for 20 min it was vortexed for a few min. Most proteins and polysaccharides were removed as a complex with the insoluble potassium dodecyl sulfate precipitate. Subsequently, the sample was centrifuged at 11,000 rpm for 10 min. The supernatant was transferred into a new microcentrifuge tube containing 700 µl of isopropanol and then incubated on ice for 30 min. The tube was centrifuged at 11,000 rpm for 5 min. The supernatant was gently discarded by pipetting since the DNA pellet is transparent because of isopropanol DNA precipitation. The pellet was washed with 1 ml of 70% ethanol and centrifuged at 11,000 rpm for 5 min. After removing the ethanol, the DNA pellet was air-dried and resuspended in 25 µl of deionized water. The quantity and quality of DNA samples were determined at 260/280 nm with Nanodrop (Thermo Fisher Scientific, USA).

2.1.4 Amplification of the 1.5 kb SSU-ITS-LSU region of AMF rDNA

The 1.5 kb SSU-ITS-LSU region of AMF rDNA was amplified by nested PCR using the Kruger primers (Kruger et al., 2009b). The primary PCR was performed in a 25 µl reaction containing 50 ng of root gDNA spore,1xPhusion HF buffer, 0.2 mM dNTPs mixture, 0.5 µM of the forward (SSUmAf) and reverse primers (LSUmAr), and Phusion high-fidelity DNA polymerase (Thermo Scientific, USA). The primary PCR condition was programmed as follows: initial denaturation at 98ºC for 30 s followed by 35 cycles of a 3-step PCR including denaturation at 98ºC for 10 sec, annealing at 60ºC for 30 sec, and extension at 72ºC for 1 min. The reaction was completed with a final extension at 72ºC for 10 min. The PCR product was diluted at 1:50 and used as the DNA template in the secondary PCR. For the secondary PCR, the reaction was performed in a 25µl reaction containing 1X ViBuffer S, 0.1 mM dNTPs mix, 0.4 µM each of forward (SSUmCf) and reverse primers (LSUmBr), and 1 unit of Tag DNA polymerase (Vivantis, Malaysia). The secondary PCR was carried out under the following conditions: 2 min initial denaturation at 94°C, 30 cycles of 30 s denaturation at 94°C, 30 s annealing at 60°C, 2 min elongation at 72°C, and 7 min final elongation at 72°C. PCR products were loaded onto 1.5% agarose gels and electrophoresis was performed at 100V for 30 min and the DNA was visualized under UV light.

Table 1 The Krüger primers used in this study for amplification of the SSU-ITS-LSU region of AMF rDNA

Primer	Nucleotide sequence
SSUmAf1	TGGGTAATCTTTTGAAACTTYA
SSUmAf2	TGGGTAATCTTRTGAAACTTCA
SSUmCf1	TCGCTCTTCAACGAGGAATC
SSUmCf2	TATTGTTCTTCAACGAGGAATC
SSUmCf3	TATTGCTCTTNAACGAGGAATC
LSUmAr1	GCTCACACTCAAATCTATCAAA
LSUmAr2	GCTCTAACTCAATTCTATCGAT
LSUmAr3	TGCTCTTACTCAAATCTATCAAA
LSUmAr4	GCTCTTACTCAAACCTATCGA
LSUmBr1	DAACACTCGCATATATGTTAGA
LSUmBr2	AACACTCGCACACATGTTAGA
LSUmBr3	AACACTCGCATACATGTTAGA
LSUmBr4	AAACACTCGCACATATGTTAGA
LSUmBr5	AACACTCGCATATATGCTAGA

2.1.3 Cloning

2.1.3.1. Competent cell preparation

In this study, *E. coli* cells strain top 10 was selected for the preparation of competent cells. Based on the $CaCl_2$ method (Sambrook et al.,1989), frozen glycerol stocks of each bacterial strain were streaked on Luria-Bertani (LB) agar plate and incubated at 37 ºC 16-17 h. Then, a single colony was incubated into a tube containing 5 ml of LB medium and grown overnight at 37 ºC with shaking. A small volume of

overnight *E. coli* culture (500 µl) was aliquoted into a new 250 ml flask containing 50 ml of LB medium and continuously shaken at 37 ºC for 3-4 h. During culturing, the bacterial cell suspension was checked the $O.D._{600}$ values every 1-2 h until it reached 0.3 to 0.5 which indicated that *E. coli* cells were still in the logarithmic growth. Afterward, *E. coli* cells were chilled on ice for 20 min and collected by centrifugation at 3,000xg for 10 min at 4 ºC, the remaining pellet was gently resuspended in ice-cold 0.085 M $CaCl_2$ containing 15% glycerol and quickly aliquoted into sterile 1.5 ml microcentrifuge tube (50 µl each). All *E. coli* competent cell tubes were frozen in liquid nitrogen before storing at -80 ºC.

2.1.3.2 DNA extraction from agarose gel

The PCR product with the expected size was purified using a QIAquick gel purification kit (Qiagen, Hilden, Germany) following manufacturer' instructions. The concentration and purity of expected PCR product was quantified at $O.D._{260/280}$ by nanodrop (Ds-11, USA)

2.1.3.3 Ligation

PCR products with the expected size product from the same treatment (n=6) were pooled into a single sample. The pooled PCR product was purified from 1% agarose gel with the QIAquick gel purification kit (Qiagen, Germany) following manufacturer' instructions. The reaction was carried out in a total volume of 10 µl containing purified DNA, 5 µl 2X Rapid ligation buffer, 50 ng of pGEM-T Easy vector and 3 Wiess units of T4 DNA ligase (Promega, USA). The reaction was mixed and

incubated for 17 hours at 16 °C. The ligation reaction was preserved at -20 °C until further use for transformation.

2.1.3.4 Plasmid transformation

Plasmids were transformed into a *E. coli* top10 using the heat-shock method and incubated at 37 °C for 17 hours. The transformants were grown in the LB medium with 100ml/l of ampicillins. Colonies containing the genuine construct were verified by the bacterial colony PCR using the M13-forward and reverse primers. The positive transformants were cultured in the LB broth with 100ml/l of ampicillins and incubated with sharking incubator at 200 rpm at 37 °C for 17 hours.

2.1.3.5 Plasmid extraction and sequencing

Plasmid DNA from *E. coli* culture cells were extracted using the Presto^{TM} Mini Plasmid kit (Geneaid, Taiwan) following manufacturer' instructions. The plasmids were sequenced by Sanger's sequencing using the T7 and SP6 primers.

2.1.4 Phylotaxonomic analysis

2.1.4.1 AMF sequence alignment

The 5′ and 3′ SSU-ITS-LSU sequences were manually assembled into the completed 1.5 kb SSU-ITS-LSU sequence using in BioEdit software (RRID: SCR_007361). All the sequences were blasted in the NCBI and MaarjAM databases to identify AMF species. We aligned the AMF sequences using ClustalW implemented in the MEGA version10.

2.1.4.2 Operational taxonomic units clustering

120 AMF sequences were clustered into different operational taxonomic units (OTUs) at a 97% identity threshold (Edgar, 2010) using adegenet and kmer packages (Jombart, 2008; Wilkinson, 2018) in the statistical software R (version 3.6.3).

2.1.4.3 Rarefaction analysis

We constructed rarefaction curves and extrapolation by the vegan package (Oksanen et al., 2019) in R to determine whether the obtained clones sufficiently represented Glomeromycotean diversity in the rice root endosphere under the non-flooded and flooded conditions.

2.1.4.4 Construction of phylogenetic trees

The 21 AMF representative OTUs from our study 20 AMF reference genes from NCBI and MaarjAM database sharing sequence similarity with our AMF OTUs at least 90 %, and two sequences of *Chaetomium globosum* and *Myrothecium* sp. using as outgroup from Genbank (http://www.ncbi.nlm.nih.gov) were used to construct a phylogenetic tree. A neighbor-joining tree (Kimura 2 parameters, 1000 replications) was constructed using the MEGA software version 10 (Kumar et al., 2018).

2.1.4.5 Analysis of AMF diversity in rice roots

The Shannon's (H) diversity index was calculated as a measure of AMF diversity in each treatment from the formula: $H' = -\sum_{i=1}^{n} pi \ln pi$ where *pi* is the

proportion of sequences belonging to each OUT relative to the total number of sequences. AMF genetic evenness was calculated by dividing Shannon's diversity index H by the natural logarithm of species richness (total number of OTUs) ln(s), using Microsoft Excel.

2.2 Identification of culturable AMF spores

2.2.1 Preparation of trap culture

2.2.1.1 Soil preparation

In a pot 20 cm in height and 24 cm in diameter was contained with soil collected from the organic paddy field at the Phattalung Rice Research Center, Phatthalung, Thailand. The original organic paddy soil was mixed at 1:3 (w/w) with sterile sand to be used for a trap culture using maize as the host plant. AMF spores were isolated by the wet sieving and decanting method (Gerdemann & Nicolson, 1963) and used to obtain a monospore culture. For the monospore culture, a total number of three hundred of *Acaulospora* spores were isolated based on their spore morphology appearance (Figure 3) and inoculated in four maize plants in a mixture of sterile sand and compost. The compost used in this study was ordered from Tippgarden, Hatyai, Songkhla, Thailand under the trademark of Dinthip (ดินทิพพ์) at a ratio of 1:1 (w/w) in a 1-l pot at 12 cm in height and 14 cm in diameter. The culture was recolonized with 100 ml of a native microbial wash, prepared from 50 g of the native paddy soil suspended in 1 l of distilled water, and filtered 3 times with Whatman no.1 paper. The trap culture was fertilized with 0.5X Hoagland's solution twice a week and watered daily with tap

water for 10 weeks and later air-dried for two weeks in a greenhouse to complete the trap culture.

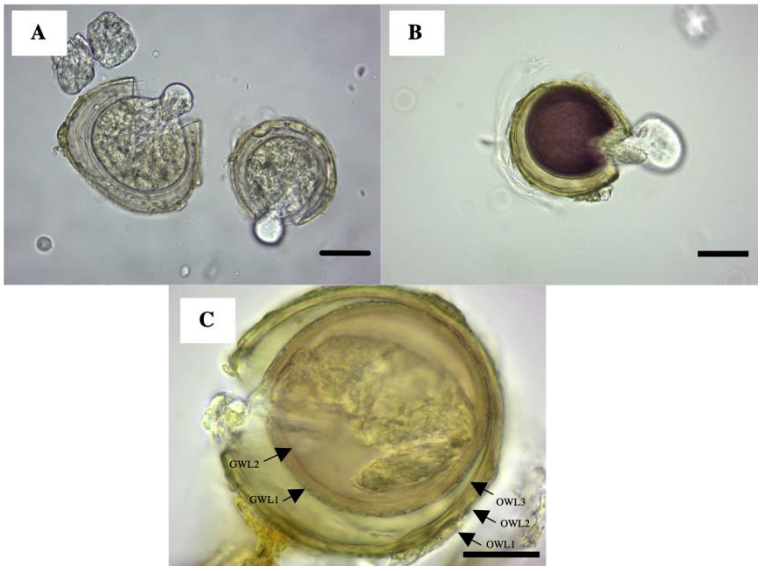

Figure 3 Spore morphology of *Acaulospora* fungi. The *Acaulospora* spore mounted in PVLG reagent without Melzer's reagent (A) and with Melzer's reagent. The germination wall of spore was stained dark red-purple in Melzer's reagent. Scale bar is 30 µm (B). The *Acaulospora* spore mounted in PVLG reagent, tri-outer wall layer1, 2, and 3, and bi-germination wall1, and 2 was visualized under a bright field microscope. Scale bar is 20 µm (C).

2.2.1.2 Propagation of AMF spores

A total of 300 *Acaulospora* spores were isolated and inoculated in maize seedlings in a 1-L pot containing sterile sand and compost at the ratio of 1:1 (w/w). The trap culture was fertilized with 0.5X Hoagland's solution twice a week and watered

daily with tap water for ten weeks and later air-dried for two weeks in a greenhouse to complete the trap culture.

2.2.1.3 AMF spore extraction from soil

AMF spores were isolated using modified wet sieving and decanting method (Gerdemann and Nicholson, 1963). Approximately 50 g of fresh soil sample was wet sieved using two different sizes of sieve (500 microns and 38 microns) to isolate the AMF spores from the larger soil particles and soil debris. The remains collected by the smaller sieve (38 microns) were collected in a 50 ml falcon tube. Then distilled water was added to the falcon tube making it to a volume of 30 ml and centrifuged at 9,000 rpm for 10 min. The supernatant was discarded carefully, and the tube was then added with 30 ml of 70% sucrose and centrifuged again at 9,000 rpm for 10 min. The supernatant was used for decanting through filter paper (Whatman no.1) and the remaining pellet was discarded. The spores trapped on the filter paper were collected in a petri dish by washing the filter paper with distilled water. The petri dish was placed under a stereomicroscope to observe and isolate the AMF spores collected. The AMF spores were transferred to a microcentrifuge tube containing 50% glycerol and stored at 4 ^{o}C for further use.

2.2.1.4 Genomic DNA extraction from AMF spores and phylogenetic analysis

The *Acaulospora* spores were permanently mounted in polyvinyl alcohol-lactic acidglycerol (PVLG) solution with and without Melzer's reagent and visualized under a microscope. Ten healthy spores were crushed in 5 µl of the 1x HF Phusion buffer

(Vivantis, Malaysia) by a pipette tip in a 1.5 ml microcentrifuge tube. Subsequently, the crushed spores were heated at 95 ^{o}C for 5 min and then the sample was stored at -20^{o}C. For the PCR,1 µl of extract from the tube was used as a sample for every 25 µl of master mix solution. The nested PCR conditions and PCR components were performed as previously described. PCR products were loaded onto 1% agarose gels and electrophoresis was performed at 100V for 30 min before examined under UV light. The PCR product was transformed and sequenced as previously described in the topic 2.1.3.4 and 2.1.3.5. Then, a phylogenetic tree was constructed as previously described in the topic 2.1.4.4.

2.3 Examination of the role of *Acaulospora* fungi in rice seedling

2.3.1 Experimental design

Three different rice cultivars comprising a lowland *japonica* Nipponbare, an upland *indica* Rai Dawk Kha Phangna (DK), and a lowland *indica* SMP rice were recruited. Rice seedlings were placed on wet tissue for two days to induce rice seeding germination. Rice seedlings from each cultivar were grown in 1 l pots containing sterile compost and sand mix, recolonized with the microbial wash described above. Each pot contained five plants. The soil was added with and without the *Acaulospora* AMF inoculum containing 1000 spores (n = 5 biological replicates), and five pots containing the non-inoculated soil without rice seedlings were prepared as a blank treatment to analyze the soil P status at the end of the experiment. All the treatments were fertilized

twice a week with 200 ml of 0.5X Hoagland's solution and watered daily with 200 ml of distilled water for 6 weeks.

Analysis of soil properties

Soil from pots in the blank treatment (n = 5 biological replicates) was collected to analyze soil properties. Soil organic matter was determined by the titration method (Walkley & Black, 1934). Total N was determined by the combustion method using a C/N analyzer CN628 (LECO, Thailand). Total P and available P were determined by the Molybdovanadophosphate method using a spectrophotometer (Prove 300, Merck KGaA, Darmstadt, Germany) (AOAC International, 2016). Total K was determined by the flame photometric method using inductively coupled plasma optical emission spectrometry (ICP-OES) (Avio 500, Perkin Elmer, Waltham, MA, USA) (AOAC International, 2016). Soil pH and electrical conductivity were measured with a conductivity meter (Orion Star A112, Thermo Fisher Scientific, Waltham, MA, USA).

2.3.2 Characterization of AMF function in rice seedling

2.3.2.1 Quantification of root AMF colonization

The AMF colonization is quantified historically using a light microscopy-based method (Tania Ho-Pla´garo et al, 2020). For trypan blue staining, the rhizosphere is carefully washed with tap water and then cut into 1cm long segments. The roots were then added 1ml of 10% (w/v) KOH at 95ºC for 15 min to make the root transparent and then the root is rinsed with distilled water. The roots were incubated in 1 ml of 1% (v/v)

HCl for 10 min at room temperature. The HCl solution was removed and replaced with 1 ml of trypan blue staining solution containing 0.05% (w/v) Trypan blue, 33% (v/v) lactic acid and 33% (v/v) glycerol. The roots were incubated overnight. The roots were then washed with distilled water and de-stain the roots in 1 ml of 50% glycerol overnight. Glycerol is then replaced the next day and a slide is prepared. Five categories of mycorrhizal structures were quantified from 200 observations of roots under a light microscope at 100 × magnification. The categories included (1) non-colonization, (2) hyphae (H), (3) hyphae and vesicles (H+V), (4) hyphae and arbuscules (H+A), (5) hyphae, vesicles and arbuscules (H+V+A) (Ho-Plágaro et al., 2020). The percentage of colonization and each mycorrhizal structure were calculated.

2.3.2.2 Determination of phosphate content in plant tissue

Plant tissue approximately 10-15 mg of the first mature leaf and 20 mg of root from a rice seeding in each pot were crushed in 600 µ L of 3% (v/v) perchloric acid. Subsequently, the crushed tissue was centrifuged at 11,000 rpm for 5 min. 120 µl of the supernatant is then mixed with 80 µl of $FeSO_4$ and then incubated the reaction at room temperature for 10 min. Finally, the sample is measured to determine the absorbance at 720 nm using a microplate reader (BIOTEX, PowerwaveX). The Pi content in each sample was calculated using a KH_2PO_4 (50–250 nmol/ml range) standard.

2.3.2.3 Determination of plant growth performance

Photosynthetic parameters such as Quantum yield of Photosystem II (Phi2), Ratio of incoming light that is lost via non-regulated processes (PhiNPQ), Ratio of incoming light (excited electrons) that goes towards non-photochemical quenching

(PhiNO), and relative chlorophyll content were measured in the first mature leaf of three rice seedlings in the same pot by the Photosynthesis RIDER protocol using the MultispeQ fluorometer (PhotosynQ, East Lansing, MI, USA) (Kuhlgert et al., 2016). The three rice seedlings were dried at 70 ◦ C for 3 days before measuring shoot and root dry weights.

2.3.2.4 Determination of soil acid phosphatase

One gram of soil was collected from the middle of the pot. The fresh soil was suspended in 4 ml of 5% (v/v) toluene and 0.5 ml of the soil supernatant was mixed with 0.4 mL of 200 mM acetate buffer at pH 5.2 and 0.1 ml of 150 mM PNPP disodium hexahydrate (TCI, Tokyo, Japan). The mixture was incubated for at 37°C for 1 hour, after which the reaction was terminated by addition of 0.5 ml of 0.5 M NaOH and centrifugation for 10 min at 12,000 *g* which also removed soil particles. In controls, the 150 mM PNPP was added only after incubation. The activity in the supernatant was measured spectrophotometrically at 410 nm. The activity of acid phosphatase (μg PNP/g soil/hour) was calculated using 2,4-Dinitrophenol (0, 200, 400, 800, 1200, 1600, 2000 nmol ml^{-1} p-nitrophenol) as standard.

Data Analysis

The data were visualized as bar plot by the ggplot2 package (Wickham, 2016) in R. Significant differences among the means of the treatments were analyzed by Student's t-test and One-way ANOVA following LSD by the agricolae package (Package & Mendiburu, 2021) in R.

CHAPTER 3

Results

3.1 AMF diversity in rice roots

To identify endophytic AMF in rice roots, the rDNA sequences of rice roots grown in non-flooded and flooded conditions were amplified by Kruger primers and clustered into different OTUs with 97% sequence similarity. 60 clones from the non-flooded and flooded condition were considered for analysis. To determine whether the numbers of clones sufficiently represented AMF diversity in the rice roots, we constructed rarefaction curves and their extrapolations (Figure 3). In the two different gravimetric regimes, rarefaction curves for OTUs reached a plateau. The data suggest that the number of sequences provided full coverage of the AMF diversity**,**

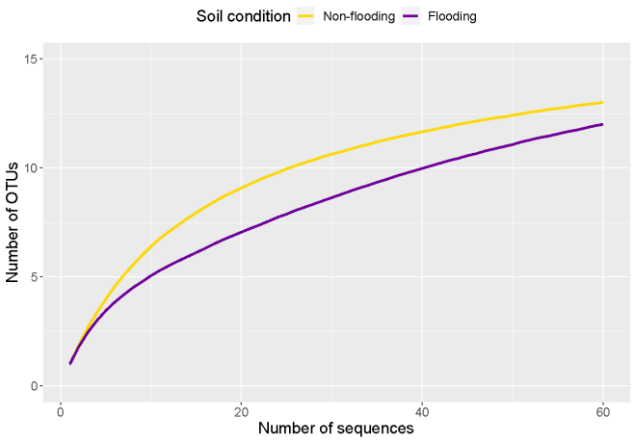

Figure 4 Rarefaction curve analysis. Rarefaction curves were ploted from the SSU-ITS-LSU sequences of Glomaromycota amplified from rice roots grown in flooded and non-flooded conditions.

The total OTU numbers (richness) of the endophytic AMF rDNA were 21 OTUs, 13 OTUs were detected in the non-flooded condition, 12 OTUs in the flooded

condition. The Shannon–Wiener diversity index (H) and AMF rDNA sequence evenness were higher in the non-flooded condition than in the flooded condition (Table 1). This result indicates that flooding reduces the diversity of AMF in rice roots.

Table 2. Diversity indices between the two different conditions

Diversity parameter	Non-flooding	Flooding
Richness S	13	12
Shannon's index H	2.25	1.79
Evenness E	0.87	0.72

The representative OTU sequences were blasted in the NCBI database and these sequences were used for the subsequent phylogenetic analyses (Figure 4). The result revealed the presence of four Glomeromycotean genera in the rice roots, including *Acaulospora*, *Dentiscutata*, *Glomus* and *Paraglomus*. In rice roots grown in the non-flooded condition, the major AMF were *Acaulospora* and *Dentiscutata*, while in the flooded condition, *Acaulospora* was the most dominant genus (Figure 4). Moreover, *Acaulospora* OTU13-14 was relatively more abundant and were found in both the non-flooded and flooded conditions. Thus, they were considered the core AMF in the lowland rice paddy condition.

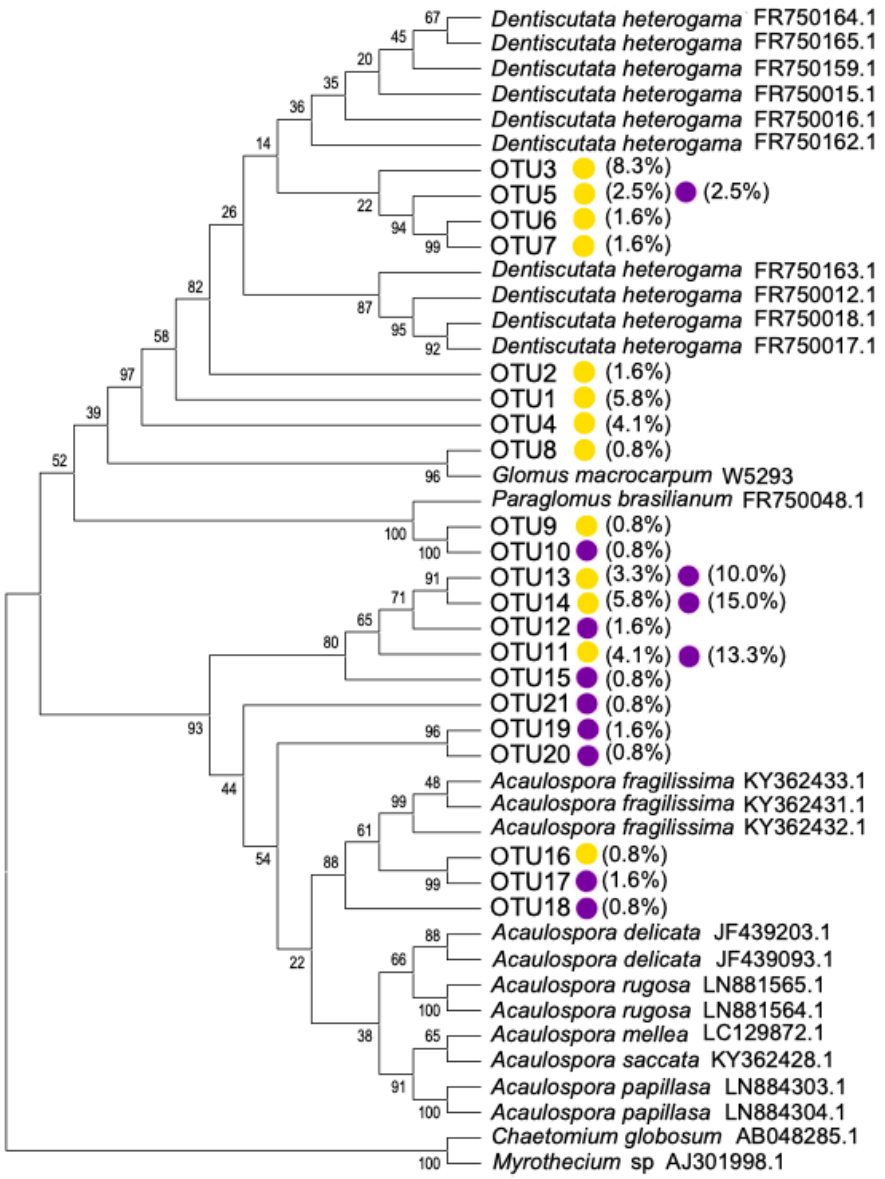

Figure 5 Phylogenetic analysis of the SSU-ITS-LSU in rice root. Neighbor-joining tree (1,000 bootstrap) derived from the representative OTUs and their closest sequence similarity from the Maarjam and NCBI databases were constructed from the SSU-ITS-LSU sequence of AMF colonizing rice roots grown under non-flooded (●) and flooded (●) conditions. GenkBank accession are placed behind AMF species reported in the databases. Relative abundances of representative AMF OTUs are demonstrated in percent with parentheses.

3.2 Propagation of *Acaulospora* spores by trap culture

To propagated AMF spores from paddy soil, the trapping culture method was performed using maize seedling as a host plant. The spore densities in our study relatively range from 500 to 1443 spore per 100 g dry soil. *Acaulospora* spores mounted in PVLG solution with and without Melzer's reagent were visualized under a light microscope. The germination wall of spores was stained dark red-purple in Melzer's Reagent (Figure 4D).

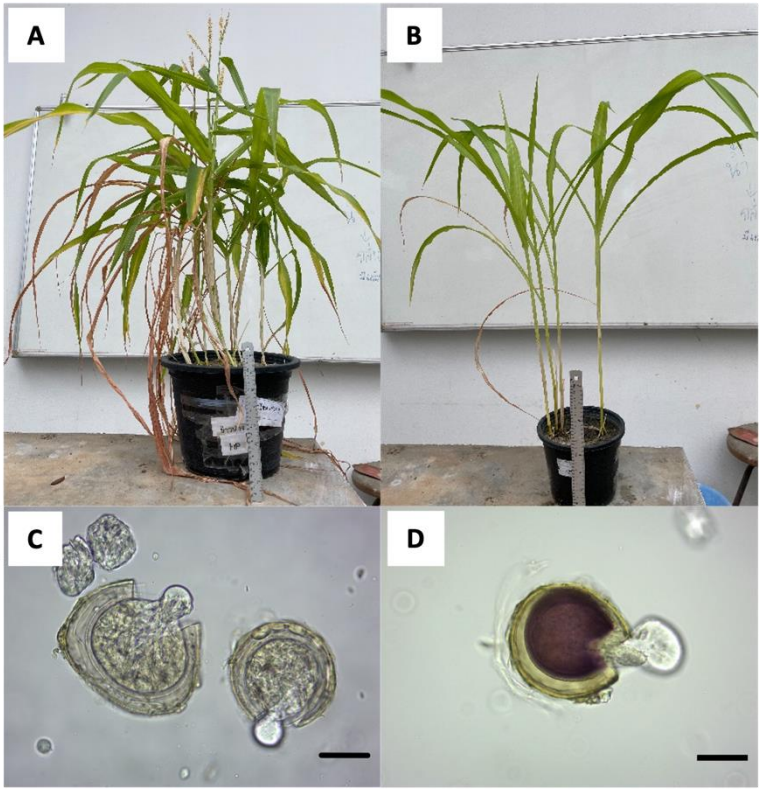

Figure 6 Trap culture of the dominant *Acaulospora* spores. Traditional trap culture was used for maintenance of the indigenous AMF spores. The trap culture using maize as a host plant was prepared from organic rice paddy soil and sterile soil and at a ratio of 1:3 (v/v) in a pot 20 cm in height and 24 cm in diameter (A). Then, *Acaulospora*

spores were collected based on their spore morphology under a stereo microscope for the monoculture using maize as host plant prepared from sterile sand and compost at a ratio 1:1(v/v) in a 1-l pot 12 cm in height and 14 cm in diameter (B). The *Acaulospora* spore morphology after mounted in polyvinyl alcohol-lactic acid-glycerol solution (C) and Melzer's reagent (D), visualized under a light microscope. Scale bars is 30 μm.

3.3 Determination of the diversity of cultured *Acaulospora* spores

To confirm whether the cultured *Acaulospora* spores were the core AMF present in the rice roots, we analyzed the sequence similarity of the SSU-ITS-LSU sequences from spores with the representative *Acaulospora* OTUs. The result demonstrated that eight of the ten spore sequences shared sequence similarity at 98.4–99.4% with OTU13–14, which were the dominant AMF OTUs in the rice roots. The other two spore sequences showed the highest similarity, with OTU14 at 95% (Figure 6). The *Acaulospora* spore sequences were annotated with the NCBI database. They shared the highest identities with *A.delicata* in the range of 93.50–94.29%, *A.mellea* in the range of 90.45–93.58% and *A.rugosa* in the range of 93.58–94.05%.

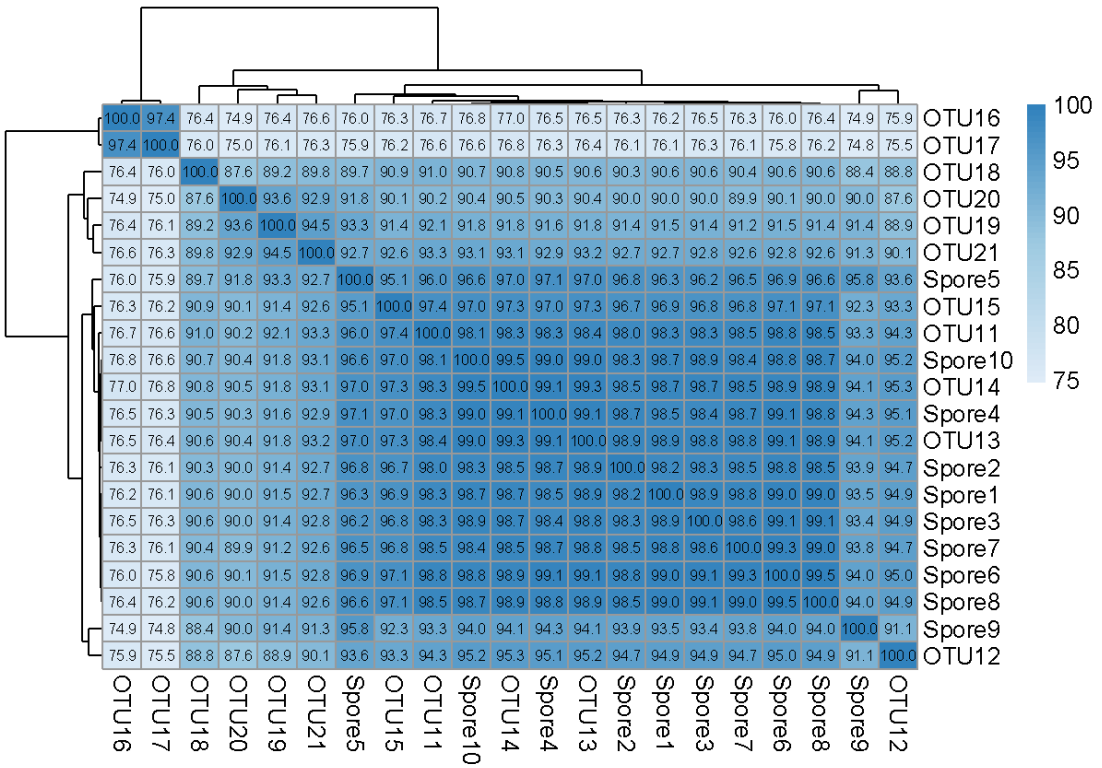

Figure 7 Sequence similarity between the *Acaulospora* OUTs in the SMP rice root and cultured *Acaulospora* spores. The heatmap demonstrate percentage of sequence similarities among SSU-ITS-LSU sequences similarity of *Acaulospora* fungi between *Acaulospora* spore of the representative *Acaulospora* OTUs from the rice roots and *Acaulospora* spore. Dark blues high and low sequence similarities, respectively.

To identify the cultured *Acaulospora* fungi into the species level, we constructed the phylogenetic tree of SSU-ITS-LSU sequences obtained from the cultured *Acaulospora* spores and other *Acaulospora* sequences from NCBI (Figure 7). The results showed that sequence of the cultured *Acaulospora* DNA were in the same clade with the sequence of *Acaulospora morrowiae*. Therefore, we considered that the cultured *Acaulospora* fungi are the as *A. morrowiae*.

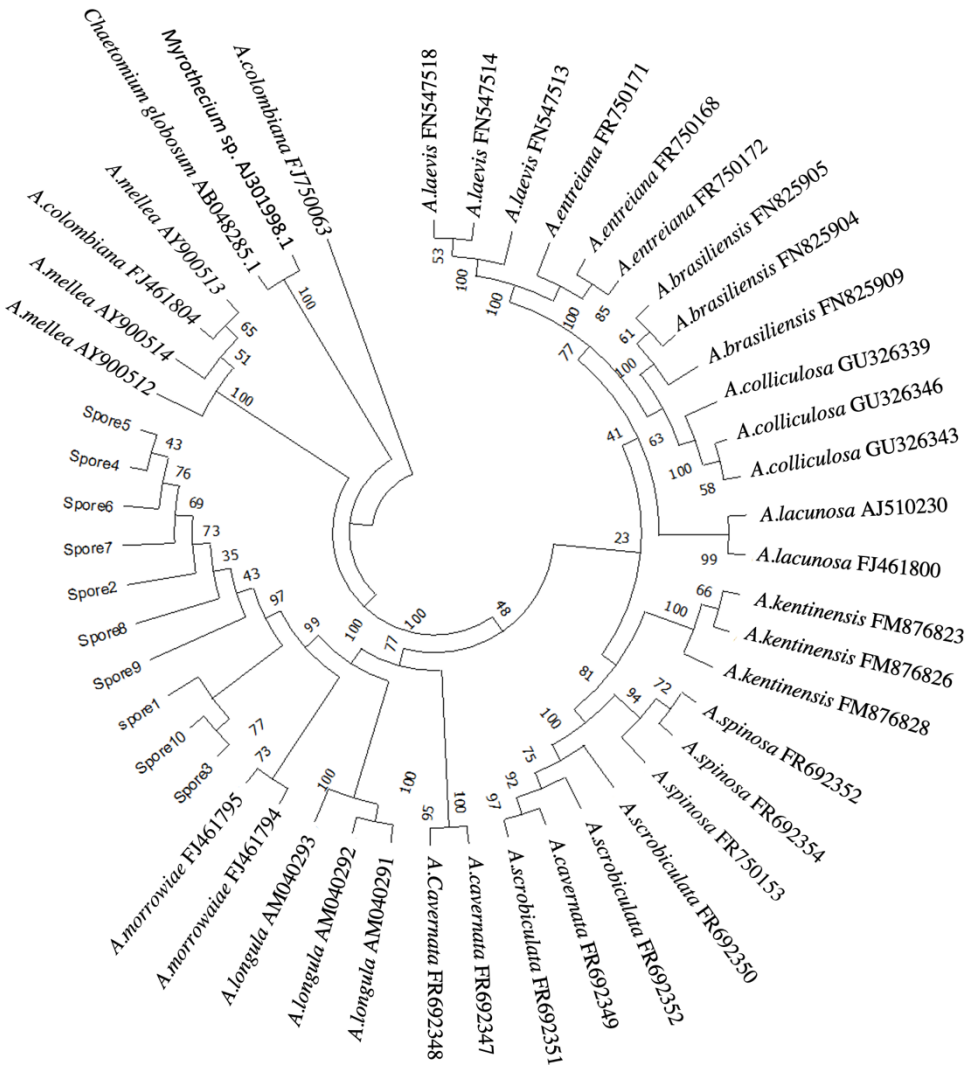

Figure 8 The phylogenetic tree of SSU-ITS-LSU of cultured *Acaulospora* fungi. A neighbor joining tree (kimura 2 parameter, 1000 replicates) was constructed from SSU-ITS-LSU sequences of cultured *Acaulospora* fungi and *Acaulospora* genera sequences from NCBI database. GenBank accession numbers were placed behind AMF species reported in the database.

3.4 The effect of cultured *A. morrowiae* inoculation on plant growth performance.

To investigate the biological function of the cultured *A. morrowiae* in rice, we grew three different rice cultivars including Nipponbare, Sangyod Muang Phathalung (SMP) and Rai Dawk Kha Phangnga (DK) rice seedlings in sterile soil with and without the *A. morrowiae* inoculum for six weeks in a greenhouse. The soil used in this experiment contained 374.96 ppm total nitrogen, 72.52 ppm total P, 37.38 ppm available P, 479.34 ppm total K and 28.48 g/kg organic matter. The soil pH and electrical conductivity were 7.08 and 0.24 ds/m, respectively. Therefore, we considered this growing material a fertile and P-sufficient soil.

To examine the AMF colonization in roots, we collected the 6-week-old rice roots of each rice cultivars and stained with trypan blue staining solution. The results showed that up to 70 % of rice roots of each rice cultivar were colonized with *A. morrowiae* (Figure 6 A). The mycorrhizal structures consisted of arbuscules, vesicles and hyphae were detected in the rice roots of AMF-plants (Figure 6 B), and the mature arbuscules were presence (Figure 6 C). The degree of AMF colonization, i.e.1. Total colonization (TC) 2. Hyphae (H) 3. Hyphae and vesicle (H+V) 4. Hyphae and arbuscule(H+A) 5. Hyphae, vesicle and arbuscule(H+V+A) were not different among the rice cultivars (Figure 8 A). However, the proportion of arbuscule formation in the SMP rice root was the lowest compared with the Nipponbare and DK rice seedlings.

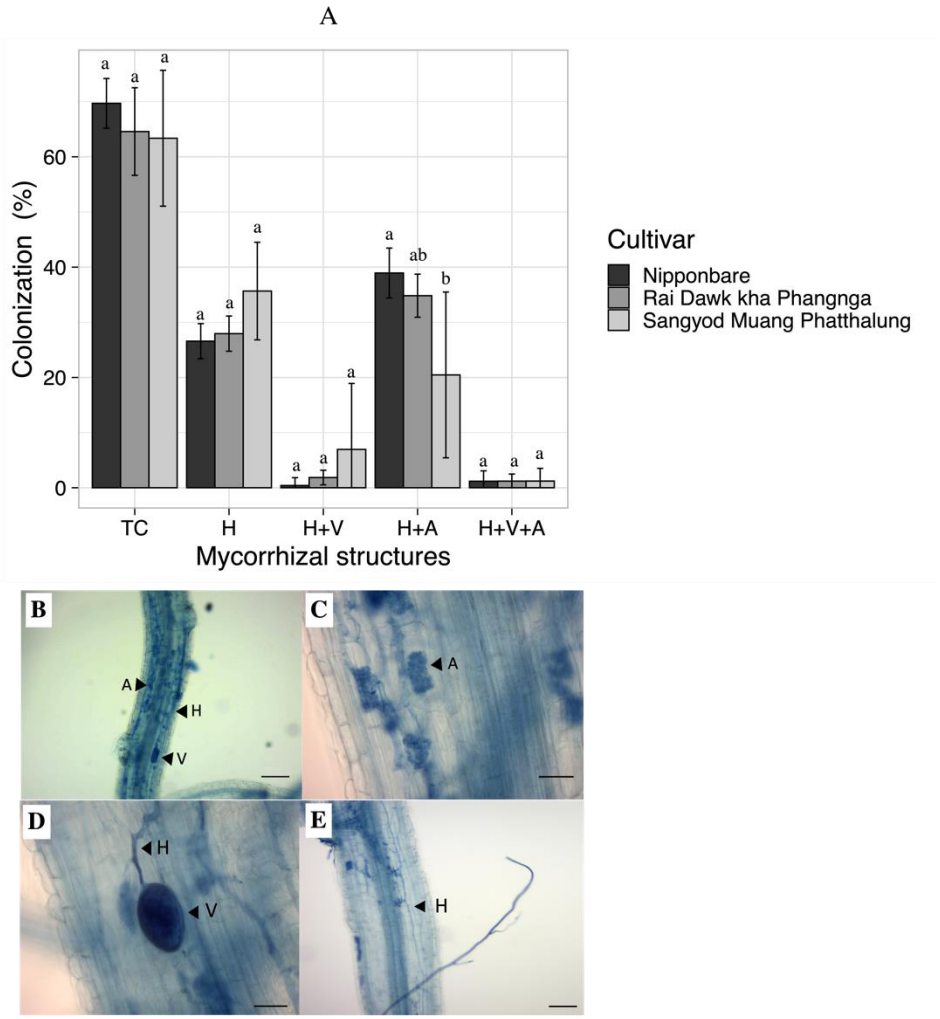

Figure 9 AMF colonization in the roots of host plants. The AMF colonization in rice roots were carried out after growing rice seedlings for 6 weeks. The degree of AMF colonization in the three-rice cultivars, i.e., Nipponbare, SMP, and DK were categorizing included total colonization (TC), hyphae (H), hyphae and vesicle (H+V), hyphae and arbuscule (H+A), hyphae, vesicle and arbuscule (H+V+A). Data correspond to the means of five biological replicates (n = 5). Statistical analysis was performed by One-way ANOVA followed by LSD. Different letters indicate significant differences among the rice cultivars ($p < 0.05$) (A). The mycorrhizal structures (A, V, and H represent arbuscule, vesicle, and hyphae, respectively). Scale bar is 100 µm (B).

The mature arbuscules of *A. morrowiae* in the rice root. Scale bar is 30 μm (C). The vesicle of *A. morrowiae* in the rice root. Scale bar is 30 μm (D). The hyphae of the *A. morrowiae* in the rice root. Scale bar is 100 μm (E).

The AMF inoculation significantly reduced plant height and shoot and root dry weights of Nipponbare and DK whereas it tended to suppress the growth of SMP (Figure 9A-F). To determinate the effect of AMF inoculation on rice P accumulation, we measured shoot and root Pi contents in the non-inoculated and AMF-inoculated rice seedlings. The result showed that the AMF inoculation did not change shoot Pi contents but increased root Pi content in Nipponbare and DK. For SMP, changes in shoot and root Pi contents were not detected in the 6-week-old rice seedlings after the AMF inoculation (Figure 10 A-C)

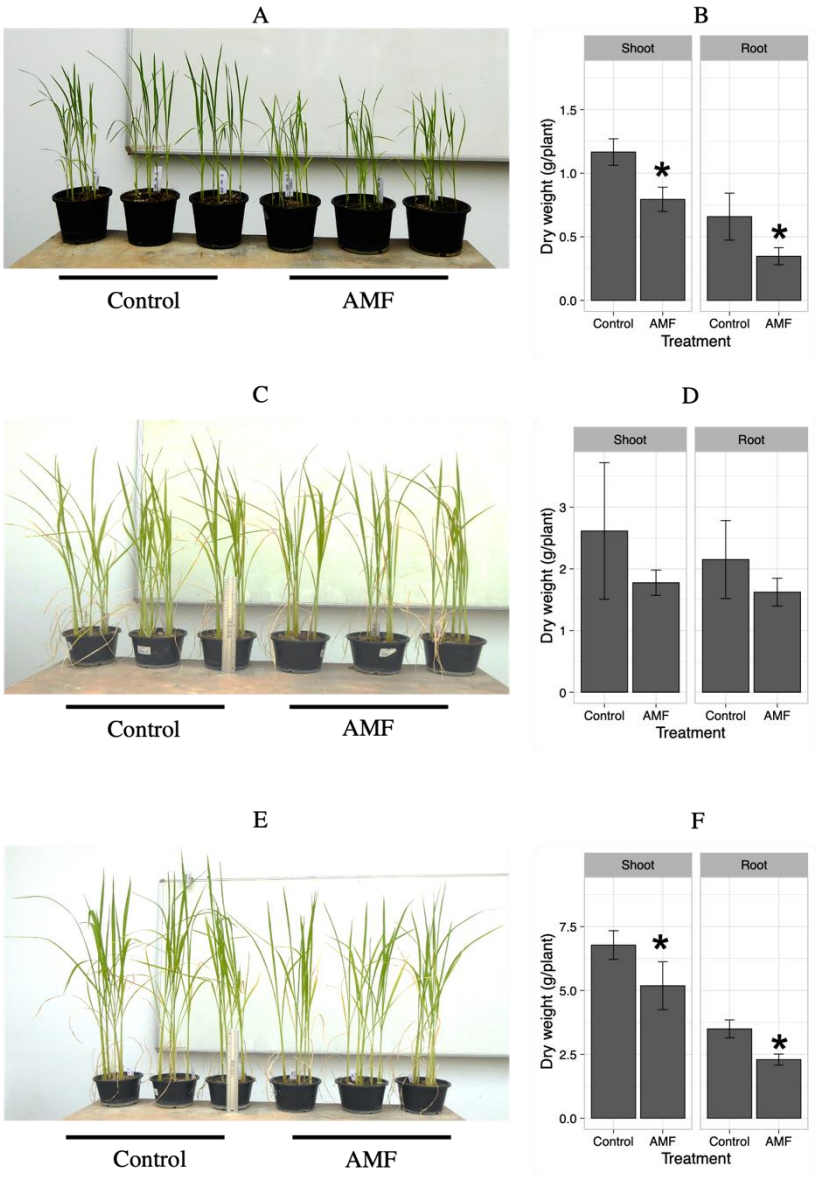

Figure 10 Effect of AMF inoculation on rice growth. Rice seedlings were grown for 6 weeks in sterile soil recolonized with a native microbial filtrate (Control) and with the *A. morrowiae* (AMF). Mycorrhizal growth responses in Nippon bare (A and B), SMP (C and D) and DK (E and F) were visually observed and measured from shoot and root dry weight. Data corresponds to the mean of five biological replicates (n = 5). Statistical analysis was performed by Student's t-test Asterisks indicate significantly differences between the control plants the AMF-treated plant ($p < 0.05$).

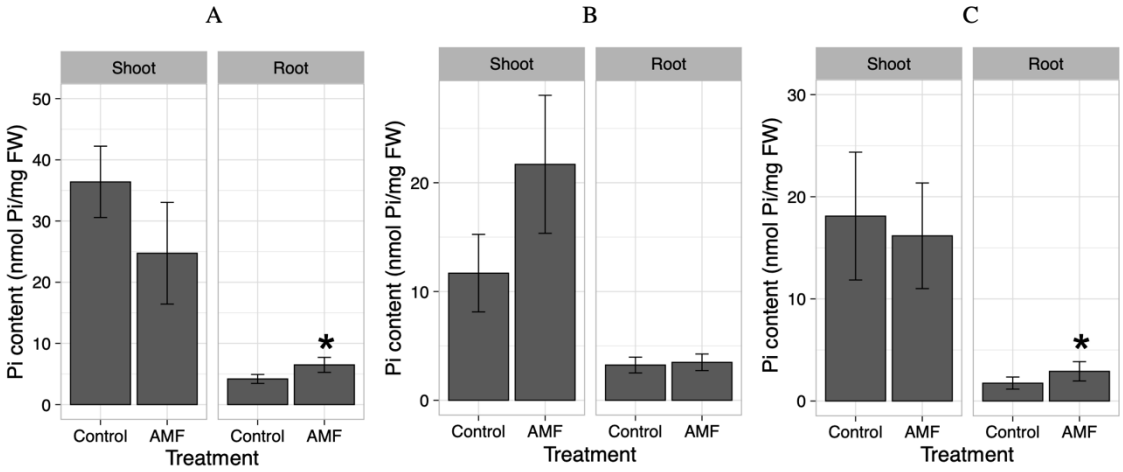

Figure 11 Effect of AMF inoculation on Pi content. Pi contents were measured in shoots and roots of control and AMF-inoculated rice seedling of Nipponbare (A), SMP (B) and DK (C).

Data correspond to the means and SD of five biological replicates (n = 5). Statistical analysis was performed using Student's t-test. Asterisks indicate significant differences between the control and the AMF-treated plants ($p < 0.05$).

We speculate that the growth suppression of rice seedling due to the AMF inoculation is resulted from biotic stress. To evaluate the physiological stress of rice seedlings, the proportion of incoming energy for photosynthesis including Phi2, PhiNO and PhiNPQ between the mycorrhizal and non-mycorrhizal rice seedlings were carried out. The results demonstrated that the AMF inoculation did not change the proportion of Phi2, PhiNO and PhiNPQ in all of rice cultivars. Therefore, it is assumed the growth

suppression by the AMF inoculation does not impair the efficiency of photosynthesis (Figure 11 A-C).

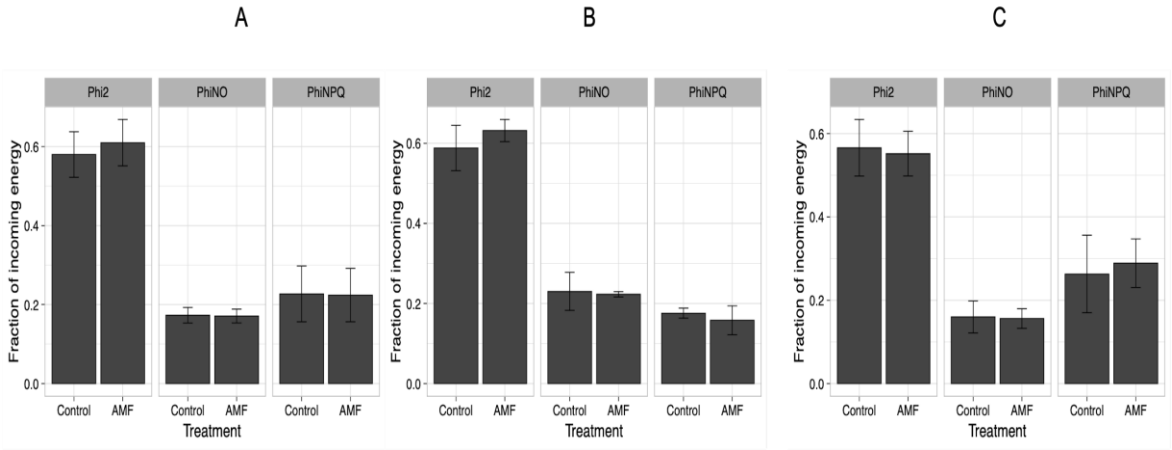

Figure 12 Effect of AMF inoculation on efficiency of photosynthesis. The fraction of incoming light energy for photosynthesis were measured in non-inoculated and inoculated rice seedlings of Nipponbare (A), SMP (B) and DK (C). The data corresponds to mean and SD of five biological replicates (n = 5). Statistical analysis was performed by Student's t-test. Asterisks indicate significant differences between the control and AMF-treated plants ($p < 0.05$).

3.5 The effect of cultured *Acaulospora morrowiae* inoculation on soil P availabilities.

To determine the effect of *A. morrowiae* inoculation on soil P status, we measured total soil P, available soil P and acid phosphatase activity in soils from each treatment at the end of experiment. The blank pots without rice seedling contained the sterile soil recolonized with native microbial solution. The results showed that the soil

P content in Nipponbare and SMP rice pots, seem to be slightly higher in AMF inoculation treatment than control but lower than the blank soil. In DK rice seedling, total P content in AMF inoculated soil was comparable to control but lower than the blank soil (Figure 12 A-C).

For soil P availabilities, the AMF inoculation seem to be slightly higher increased the from figure 13 available P content and acid phosphatase activity in the soils containing of the DK and Nipponbare rice see seedlings. In the soil used for the growth of SMP rice seedlings, the available P contents was not different between the non-inoculated and inoculated but lower than blank soil (Figure 13 A-C). However, in all of rice cultivars, the AMF inoculation significantly increases the activity of acid phosphatase in AMF-inoculated soil in all of rice cultivars (Figure 14 A-C). These results demonstrate that AMF inoculation improves soil P availability by increasing the acid phosphatase activity (Figure 14 A-C).

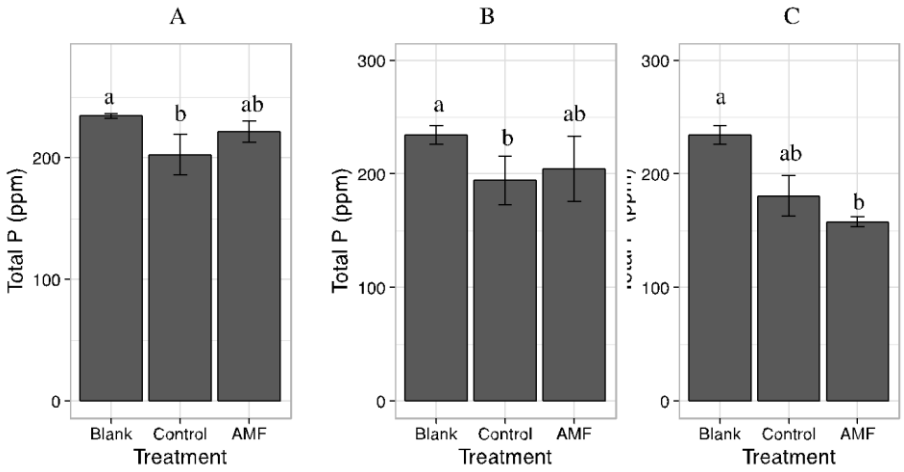

Figure 13 Effect of AMF inoculation on total soil P. Total soil P contents were measured in the non-inoculated soil and AMF-inoculated soil of Nipponbare (A), SMP (B) and DK (C) compared with the blank soil. Data correspond to the mean and SD of five biological replicates (n = 5). Statistical analysis was performed by one-way ANOVA followed by LSD. Different letters indicate significant differences among the treatments ($p < 0.05$).

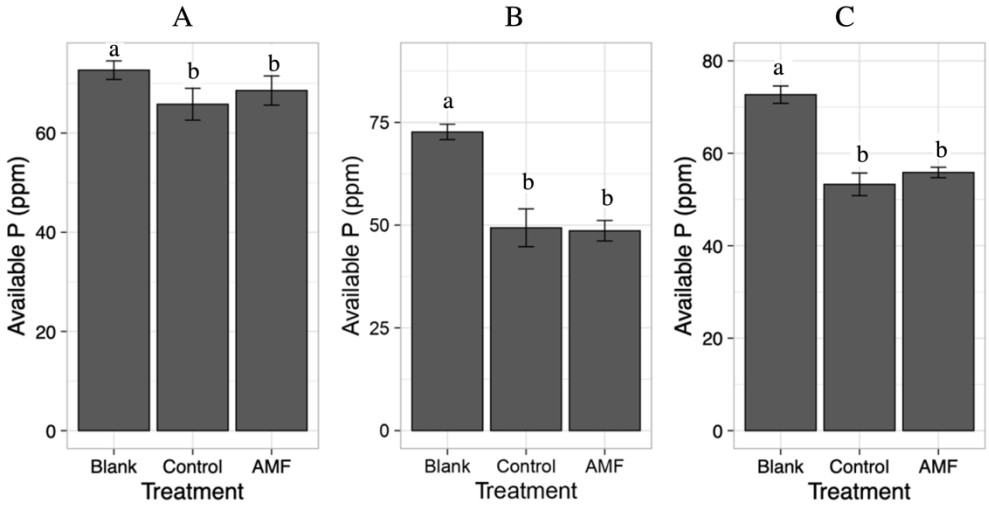

Figure 14 Effect of AMF inoculation on available soil P. Available P soil were measured in the non-inoculated soil and AMF-inoculated soil of Nipponbare (A), SMP (B) and DK (C) compared with the blank soil. Data corresponds to the mean and SD of five biological replicates (n = 5). Statistical analysis was performed by ne-way ANOVA followed LSD. Different letters indicate significant differences among the treatment ($p < 0.05$).

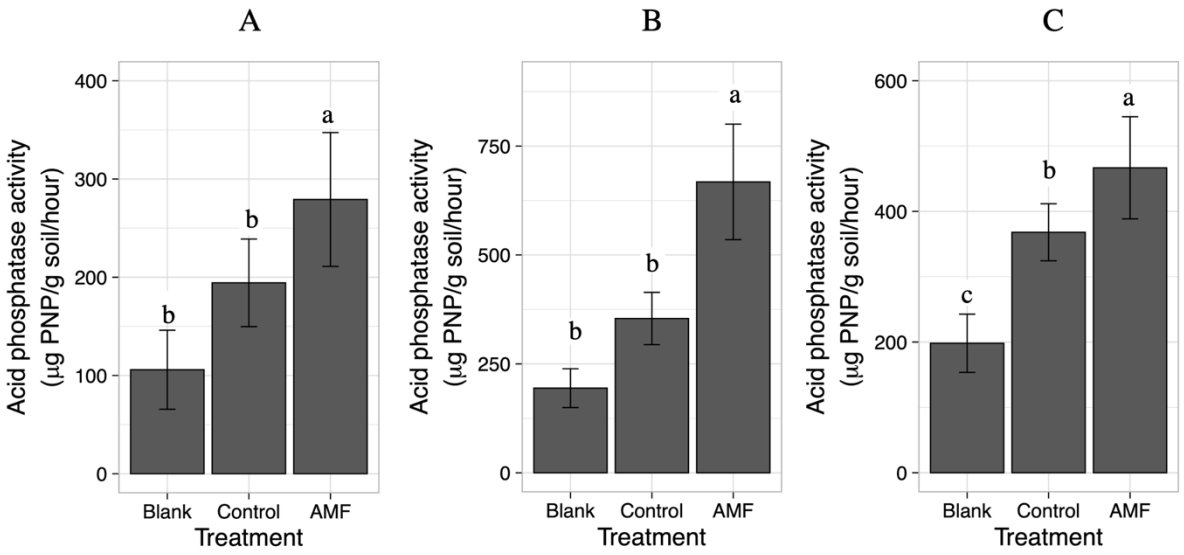

Figure 15 Effect of AMF inoculation on acid phosphatase activity. Acid phosphatase activities were measured in the non-inoculated soil and AMF-inoculated soil of Nipponbare (A), SMP (B) and DK (C) compared with the blank soil. Data corresponds to the mean and SD of five biological replicates (n = 5). Statistical analysis was performed by one-way ANOVA followed LSD. Different letters indicate significant differences among the treatments ($p < 0.05$).

CHAPTER4

Discussion

4.1 Flooding decreases AMF diversity in SMP rice roots.

There were 21 AMF OTUs in SMP roots grown in the organic rice paddy soil collected from Phatthalung rice research institute under non-flooded and flooded conditions. Flooding decreased the AMF diversity, richness, and evenness in rice roots. (Table 1). The reduction of AMF diversity was related to the absence of *Dentiscutata* fungi (Figure 4). *Dentiscutata* OUT 1-7 shared the highest sequences similarity with *Dentiscutata heterogama*. Their sequence abundance was depleted in the flooded condition while *Acaulospora* OUT 11-21 were enriched. In lowland ecosystem, flooding decreases AMF colonization because it accelerates the degradation of root cortical cells and the formation of aerenchyma in crown roots and large lateral roots, where the fungi form symbiosis. (Vallino et al., 2014). Furthermore, flooding reduces AMF diversity, abundance, and richness (Wang et al., 2016). From our previous study, we examined the effect of flooding on the fungal community by using the ITS2 primers to amplify fungal and AMF sequences. The results showed that flooding increases rice biomass and shapes the fungal and AMF community (Klinnawee et al., 2021). In this study, we investigate diversity of AMF in rice roots under the flooded condition by using Kruger primers to amplify the SSU-ITS-LSU region of rDNA. The Kruger primers were reported as the most efficient primer system covering the highest AMF sequences, compared to the other primer system such as ITS2, SSU, LSU, and Redecker

(Kohout et al., 2014b). In our study, flooding changed AMF community composition and AMF diversity.

In a lowland ecosystem, *Acaulospora* and *Dentiscutata* are found in different hydrologic gradients. *Dentiscutata* is dominant in a predominantly dry zone while *Acaulospora* is more often detected in an intermittent wet zone. Moreover, both rarely appear in a predominant wet zone. However, some AMF genera were predominantly detected under flooded condition. In lowland rice paddies, *Acaulospora* and *Dentiscutata* is reported as a dominant genus under lowland ecosystem (Sarkodee-Addo et al., 2020; Watanarojanaporn et al., 2013)

In this study, we detected that under flooded conditions, the dominant AMF genus in lowland rice was *Acaulospora*. The *Acaulospora* genera also revealed high relative abundance in comparison to other indigenous endophytic AMF in rice roots (Figure 4). Similar results were also reported in some studies that up to 80 % of AMF spores found in lowland rice paddy soil were Acaulospora (Sarkodee-Addo et al., 2020; Watanarojanaporn et al., 2013; Xavier Martins & Rodrigues, 2020). Even in mangrove ecosystem, *Acaulospora* was detected as the most common AMF genus associated with most of the mangrove plant species (Gaonkar & Rodrigues, 2020). Therefore, *Acaulospora* might be considered an anaerobic tolerant fungus, which is sufficient for plant growth under flooded conditions.

4.2 *Acaulospora* are propagated by trap culture

We propagated the AMF by trap culture method by using maize as a host plant grown under a mix of the organic paddy soil and sterile sand. The results showed that

only *Acaulospora* spores were detected in soil at the end of propagation. The result indicate that *Acaulospora* might be more aggressive to colonized compared with other indigenous AMF.

(Graham & Abbott, 2000) also found that *Acaulospora* genera can aggressively colonize roots under P-sufficient soil and P-deficient soil.

Almost all cultured *Acaulospora* spore sequences shared percentage of sequence similarity at 98.4-99.4 % with the dominant AMF OTU13-14 in the SMP rice roots, and the other two spore sequences showed the highest similarity to the OTU14 at 95%. The cultured *Acaulospora* spore could be classified into two species based on the standard 97% sequence similarity for clustering of microbial rDNA sequence (Stackebrandt & Goebel, 1994). However, classification of AMF species using the rDNA gene sequence similarity threshold of 97% may overestimate the actual number of AMF species, especially when the sequence contains the ITS regions (House et al., 2016; Maeda et al., 2018). For example, the intra-genomic similarity in the ITS2 region from the ten-rDNA paralogous of the *Rhizophagus irrigularis*, a model AMF species, is 90.28% (Maeda et al., 2018).

To identify the cultured AMF spore at the species level by molecular approach, we constructed phylogenetic tree from our cultured *Acaulospora* sequences and sequences of all the *Acaulospora* species obtained from Kruger et al (2012). The results showed that our cultured *Acaulospora* sequences were deposited in the same clade with *Acaulospora morrowie*. Therefore, we considered that the cultured *Acaulospora* fungi are the *A. morrowiae* species (Figure 7).

4.3 Inoculation of *Acaulospora morrowiae* suppresses the growth of rice seedlings

Our study revealed that about 70% of roots in all three rice cultivars were colonized with *A. morrowiae* and mature arbuscules were found in the inoculated roots. (Figure 8A). However, the proportion of arbuscules in SMP roots was relatively lower than Nipponbare and DK. The low arbuscule formation in SMP could be positively correlated to the presence of *Pup1*-K46 region, which confers tolerance to P-deficiency in rice (Chin et al., 2010a). SMP, an *indica* lowland rice, may have more abundance of *Pup1* region (Chankaew et al., 2019) compared to Nipponbare, a *japonica* lowland rice, which reportedly lacks *Pup1*-K46 region (Chin et al., 2010). However, DK shows high efficiency of AMF symbiosis compared with Nipponbare and SMP. This is explained by the fact that when AMF was inoculated in upland rice (DK), we observed plant growth promotion, whereas AMF inoculation in lowland rice showed plant growth suppression (Diedhiou et al., 2016).

The AMF inoculation suppressed the growth of rice seedlings by significantly reducing the plant shoot and root dry weight in Nipponbare and DK. From the result, we assumed that both Nipponbare and DK is a non-locally adapted to the *A. morrowiae*. The growth suppression in Nipponbare and DK might be the less experience in nutrient exchange between rice and fungus. For SMP rice seedling, the AMF inoculation did not suppress shoot and root growth. It is assumed that the native *A. morrowiae* is locally adapted to SMP since they live in the same habitat for a long time. Their biological interaction has been evolutionarily adapted for many generations. Therefore, the growth suppression in SMP was not detected. In addition, it was reported that a native grass species more compatible to the native AMF species than commercial AMF species was

grown in a field by increasing plant height, leaves number and tiller of host plants. The benefits from these conditions were spread to neighboring plants surrounding the inoculated plant up to two meters from the inoculation point. The native AMF inoculum is consisted of *Acaulospora spinosa, Entrophospora infrequens, Claroideoglomus claroideum, Claroideoglomus lamellosum, Funneliformis mosseae, Racocetra fulgida and Cerataspora pellucida w*hile the commercial inoculum contained with the mixture of *Acaulospora columbiana, Rhizophagus clarus, Claroideoglomus etunicatum, and Rhizophagus intraradices* (Middleton et al., 2015). Moreover, native AMF promote number of branches, root biomass and shoot length in local plant under a field condition (Shah et al., 2008). Furthermore, the growth response from AMF inoculation is depended on the combination of plants and AMF species under a pot contained sterile soil in a greenhouse condition. A mutualistic relationship between plant and AMF was occurred when local plant colonized by native AMF. In contrast, a parasitic relationship was occurred when non- local plant inoculated with native AMF (Klironomos, 2003). It is agreed with the growth suppression in DK and Nipponbare since they are non-local rice cultivars.

Unsurprisingly, the growth suppression can be founded in another plant inoculated with a non-native AMF species such as a sorghum grown under high P soil concentrations (Kim et al., 2018). Moreover, *A. morrowiae* suppress the growth of wheat plants under high P soil concentrations since these fungi reduced the concentration of sucrose in roots (Graham & Abbott, 2000). Therefore, it is highly likely that AMF inoculation suppresses plants when the plant is grown under P-sufficient soils.

In addition, the benefit of AMF symbiosis is dependent on the plant cultivar. For example, the percentage of AMF colonization was different in thirteen woody fruit tree cultivars even if inoculated with the similar AMF species grown under sterile soil without a native microbial community. Surprisingly, the AMF inoculation suppresses plant biomass. The growth suppression positively correlated with the higher of percent AMF colonization. For example, when the percent AMF colonization was higher resulting to more growth suppresses in plants (Bâ et al., 2000). Furthermore, AMF inoculation had a differential effect on twenty-three maize cultivars grown under metal-polluted soil (Yin et al., 2021).

In this study, the growth suppression due to AMF inoculation might be a result of the soil P condition. The available P content of the soil was 37.28 ppm, which is considered a high P level for rice seedlings (Pinit et al., 2020). This level of available P concentration is generally detected in lowland rice paddies (Uwasawa et al., 1988). The symbiosis between rice and AMF was inhibited under a high P concentration. However, the cultured *Acaulospora* colonizing rice roots may be due to a tolerance of high P soil. Some AMF species, including *A. laevis*, can aggressively colonize roots in P-sufficient soils. This fungus reduced the growth of the host plant and do not enhance plant P uptake. In conventional fertilizer dose, AMF inoculation does not promote plant fresh weight but in reduced fertilizer dose, AMF promotes plant fresh weight. Moreover, under reduced fertilizer AMF increases fruit number and enhances host plant resistance against root pathogens (Martínez-Medina et al., 2011).

We hypothesize that the growth suppression due to AMF inoculation found in this study might be resulting from high P concentration in the soil. The P-sufficient

soils induce the photosynthetic activities in plants, which can increase the photosynthesis product such as sugar.

(Veronica et al., 2017). Unfortunately, under this situation, plants may provide a large quality of nutrients to the AMF, leading to reducing in plant growth. Purin & Rillig, (2008) reported that AMF can change from mutualistic to parasitic symbiosis under the P-sufficient soils. Therefore, it is highly likely that AMF inoculation suppresses plant growth under P-sufficient soils. However, the AMF inoculation did not alter the efficiency of photosynthesis in the leaves. These results strongly indicate that the interaction between AMF and rice was not a parasitic relationship. The behavior is unlike behaviors of other plants infected by fungal root parasites such as *Fusarium oxysporum* and *Setophoma terrestris*, which decrease the plant growth and efficiency of photosynthesis of partner plants. (Sayago et al., 2020; Ye et al., 2004). For abiotic stress such as heat stress was reduced the efficiency of photosynthesis (Salvucci & Crafts-Brandner, 2004). Under drought stress, it reduced the efficiency of photosynthesis in weeping lovegrass plants (Poaceae) (Colom & Vazzana, 2003).

4.4 Inoculation of *A. morrowie* enhances root Pi content.

AMF inoculation did not promote the accumulation of Pi in the shoot in all rice cultivars but significantly increased the accumulation of P in the mycorrhizal root of Nipponbare and DK rice seedlings (Figure 10). For SMP, AMF inoculation did not alter the accumulation of P in six weeks old rice root. AMF inoculation has been reported to induce the level of gene expression, which involve in Pi transporter gene in the plant

(Liu et al., 2018). Moreover, AMF inoculation induced the mycorrhizal P uptake pathway in rice through the mycorrhiza-specific phosphate transporter *OSPT11* (Chen et al., 2013b). The level of *OSPT11* is positively correlated with the arbuscule abundance in rice roots (Vallino et al., 2014). Therefore, the lower P content in mycorrhizal SMP roots might have resulted from the lower activity of *OSPT11* associated with the density of mature arbuscules in the roots.

The increase in Pi content in roots is due to the AMF take up of mineral nutrients, especially P in the inoculated soil, leading to higher P in roots of AMF inoculated soil (Figure 10). We anticipate that AMF inoculation might cut down the P loss by delivering the P into rice roots and stored in the roots before they are lost. In the lowland rice ecosystem, AMF was reported to prevent P loss via runoff and leaching, which increases the shoot and root biomass of rice (S. Zhang et al., 2020). In the pot experiment, inoculation of AMF did not promote shoot and root growth but reduced yield loss of rice under drought conditions (Chareesri et al., 2020). Moreover, AMF inoculation did not promote P uptake even if plants are grown under P deficiency but increased efficiency of photosynthesis and grain yield in salt stress conditions (Tisarum et al., 2020). Therefore, it is highly likely that AMF promotes plant performance under extreme conditions.

4.5 AMF inoculation improves soil P availability by increasing acid phosphatase activity.

Our study revealed that AMF inoculation significantly increased the amount of available P and the acid phosphatase activity in AMF-inoculated soil (Figure 13 and 14). Acid phosphatase is an important enzyme involved in P mineralization and hydrolysis. It converts organic phosphorus such as phosphate esters, quinquevalent phosphorus acids, phosphine oxide, phosphonium salt and phosphoranes. into inorganic Pi, which is available for plant uptake (Smitth and Read., 2008). To improve soil P availability, AMF inocula has been cultured in both greenhouse and natural conditions. Inoculation of AMF, such as *Funneliformis mosseae* in maize increased soil P mineralization, consistent with higher activities of acid phosphatase in the rhizospheric soil compartment (Wang et al., 2020). Furthermore, inoculation of *Acaulospora laevis* in plants grown under sterile soil in a greenhouse improved P acquisition because of the fungus activity of acid phosphatase in the soil (Prasad et al., 2012). The activity of soil alkaline phosphatase is exclusively increase in high P soil whereas acid phosphatase is increased independently of soil P plants. Indicating that plant could utilize the higher amount of P when they form AMF symbiosis.

AMF provide the symbiotic plants with direct and indirect strategies to increase acid phosphatase activities in soils. There is evidence that AMF *Rhizophagus clarus* directly release acid phosphatase from their external hyphae into the soil, which is positively correlated to available P levels in soils (Sato et al., 2015). Moreover, inoculation of G*lomus versiforme* strongly activates the expression of secreted purple acid phosphatase *PtPAP1* gene in root of *Poncirus trifoliata* L. Raf (Shu et al., 2014). Therefore, AMF elevate the activity of acid phosphatase through the direct exudation

via their external hypha and the activation of root acid phosphatase via transcriptional manipulation in host roots.

In addition to acid phosphatase, plants also exude phytase to mineralize phytic acids which are the most abundance phosphorus form in soils. AMF inoculation is improved soil P availability by exudation of the phytase from external hyphae. The increases in phytase activity are related to enhancement of P uptake in maize (Wang et al., 2017). Furthermore, AMF inoculation enhance the phytate-P uptake through improve soil bacterial diversity, which increase plant biomass and alkaline phosphatase activity (Cao et al., 2015). Moreover, in a field experiment, AMF stimulate organic P mobilization by changing bacterial community, which can improve host plant release of phytase (Zhang et al., 2018). AMF inoculations also promoted utilization of phytate-P by increasing of phytase activity in root grown under P deficiency. In addition, the gene involved in the release of phytase in plants are upregulated by AMF inoculation (Shu et al., 2014).

Under P deficiency, plants exude organic acids and phenolic acids to enhance P solubilities in soils. Root exudates such as malic acid, citric acid, lactic acid, fumaric acid and ascorbic acid enhance P solubility under low P condition (Bononi et al. 2020; Mukai et al., 2021). Moreover, plants form symbiosis with AMF to improve soil P mobilization since the fungi accelerate the release of organic acids mainly citric acid into the soil and they dissolve the insoluble inorganic P. The increase in citric acid can contribute to enhanced P acquisition in mycorrhizal roots (Tawaraya et al., 2006). AMF inoculations change root exudation composition since the AMF enhance the secretion of phenolic acids, which are related to the higher P mobilization (Lǔ et al., 2019; Scagel

& Lee, 2012). In addition, AMF inoculation increase soil P mobilities by solubilization of soil inorganic precipitated P (della Mónica et al., 2020). It is suggested that the organic acids, which are involved in P mineralization must be investigated in further study.

CHAPTER5

Conclusion

There were 21 Glomaromycota found in the roots of SMP lowland *indica* rice grown in the organic lowland rice paddies. *D. heterogama* and *A. morrowiae* were defined as the dominant AMF species. Because of the abundance of SSU-ITS-LSU sequences in the SMP rice roots, *Acaulospora morrowiae* was more tolerant to flooding. Inoculation of *A. morrowiae* in rice improves rice cultivation since it could be considered as a soil improver and plant growth promoter. *A. morrowiae* increased soil P availability via the release of acid phosphatase. In this study, we found that inoculation of *A. morrowiae* suppressed the growth of rice seedlings grown in the sterile compost and sand at a ratio of 1:1, considering the high nutrient in the soil. We anticipate that mycorrhizal growth suppression might have resulted from the rice-AMF interaction under the high nutrient soil in which the mycorrhizal nutrient uptake pathway is not necessary whereas some carbon sources from the host plants are shared in the AMF symbiosis. In our further study, different soil compositions and sources will be elucidated to investigate the soil conditions delivering mycorrhizal growth promotion to rice. In addition, the *A. morrowiae* -rice compatibility will be tested in other landrace rice varieties (Figure16)

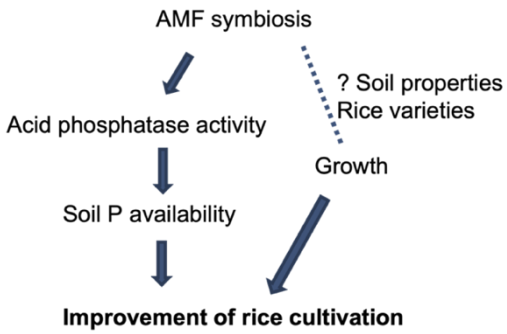

Figure 16 Graphical summary of the role of native AMF in rice. AMF symbiosis provides the two vital strategies for the improvement of rice cultivation. First, they act like soil P improvers since increase activity of acid phosphatase. Second, AMF symbiosis act like as a plant growth promoter, which is dependent on soil properties and rice varieties.

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