ENHANCEMENT OF ARBUSCULAR MYCORRHIZAL INOCULANT PRODUCTION USING PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR)

Watcharin Yuttavanichakul

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biotechnology

Suranaree University of Technology

Academic Year 2015

การเพิ่มการผลิตหัวเชื้ออาร์บัสคูลาร์ไมคอร์ไรซาโดยการใช้แบคทีเรียที่ส่งเสริมการเจริญเติบโตของพืช

นายวัชรินทร์ ยุทธวานิชกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2558

ENHANCEMENT OF ARBUSCULAR MYCORRHIZAL INOCULANT PRODUCTION USING PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR)

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

Thesis Examining Committee

(Assoc. Prof. Dr. Montarop Yamabhai)

Chairperson

(Asst. Prof. Dr. Panlada Tittabutr)

Member (Thesis Advisor)

(Prof. Emeritus Dr. Nantakorn Boonkerd)

Member

(Prof. Dr. Neung Teaumroong)

Member

(Prof. Dr. Saisamorn Lumyong)

Member

(Prof. Dr. Sukit Limpijumnong)
Vice Rector for Academic Affairs and Innovation

(Prof. Dr. Neung Teaumroong)
Dean of Institute of Agricultural Technology

วัชรินทร์ ยุทธวานิชกุล : การเพิ่มการผลิตหัวเชื้ออาร์บัสคูลาร์ไมคอร์ไรซาโดยการใช้แบคทีเรียที่ส่งเสริมการเจริญเติบโตของพืช (ENHANCEMENT OF ARBUSCULAR MYCORRHIZAL INOCULANT PRODUCTION USING PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR)) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.พรรณลดา ติตตะบุตร, 82 หน้า.

การผลิตสปอร์อาร์บัสคูลาร์ไมคอร์ไรซาด้วยระบบการใช้วัสดุเพาะปลูกพืชทั่วไป (substrate-based production systems) ให้ผลผลิตจำนวนสปอร์ในระดับต่ำ ซึ่งเป็นอุปสรรคในการพัฒนาหัวเชื้ออาร์บัสคูลาร์ไมคอร์ไรซา ดังนั้นในการศึกษานี้จึงมีวัตถุประสงค์ในการวิจัย เพื่อใช้เชื้อแบคทีเรีย *Brevibacillus* sp. SUT47 ทำหน้าที่เป็นเชื้อแบคทีเรียผู้ช่วย ในการส่งเสริมการเจริญเติบโตของเชื้ออาร์บัสคูลาร์ไมคอร์ไรซา (Mycorrhization Helper Bacteria, MHB) ในการเพิ่มจำนวนสปอร์อาร์บัสคูลาร์ไมคอร์ไรซาจากเดิมเป็นสองเท่า โดยใช้ข้าวโพดเป็นพืชอาศัย และทำการศึกษาถึงกลไกในการเพิ่มขึ้นของจำนวนสปอร์ดังกล่าวโดยการศึกษาความสัมพันธ์ของโปรตีนระหว่างอาร์บัสคูลาร์ไมคอร์ไรซา พืชอาศัย และเชื้อ *Brevibacillus* sp. SUT47 ผลการศึกษาพบว่า ฮอร์โมนพืช Indole-3-Acetic Acid (IAA) ผลิตจาก SUT47 เป็นหนึ่งในปัจจัยสำคัญในการส่งเสริมการเพิ่มขึ้นของสปอร์อาร์บัสคูลาร์ไมคอร์ไรซา และจากผลการศึกษาโปรตีน พบว่าโปรตีนที่เกี่ยวข้องกับกระบวนการป้องกันตัวของพืช (plant defense response) และเอนไซม์ในการกำจัดอนุมูลอิสระของออกซิเจน (Reactive Oxygen Species (ROS)-scavenging enzymes) มีบทบาทสำคัญในการส่งเสริมการเพิ่มขึ้นของสปอร์อาร์บัสคูลาร์ไมคอร์ไรซา โดยเชื้อ *Brevibacillus* sp. SUT47 และการแสดงออกของยีนที่เกี่ยวข้องกับการต้านอนุมูลอิสระ รวมทั้งการตรวจสอบกิจกรรมของเอนไซม์ ที่เกี่ยวข้องกับการกำจัดอนุมูลอิสระแสดงให้เห็นว่า กลไกที่ *Brevibacillus* sp. SUT47 มีส่วนช่วยในการลดอนุมูลอิสระที่เกิดจากออกซิเจน รวมทั้งช่วยในการชะลอกระบวนการส่งสัญญาณของกรดซาลิซิลิก (salicylic acid signaling pathway) ของพืชในช่วงเริ่มต้นการเข้าอาศัยในพืชของเชื้ออาร์บัสคูลาร์ไมคอร์ไรซา อีกทั้ง elicitors โปรตีน ได้แก่ flagellin (*flg22*) และ elongation factor TU (*elf18*) ที่ปลดปล่อยออกมาจากเชื้อ *Brevibacillus* sp. SUT47 มีส่วนในการส่งเสริมกระบวนการก่อนการเข้าอาศัยในพืชของอาร์บัสคูลาร์ไมคอร์ไรซา (AM pre-penetration)

ดังนั้นจากการศึกษานี้สามารถนำมาประยุกต์ใช้ในการผลิตหัวเชื้ออาร์บัสคูลาร์ไมคอร์ไรซาต้นทุนต่ำได้เมื่อใช้ *Brevibacillus* sp. SUT47 ร่วมในการผลิต

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2558

ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

WATCHARIN YUTTAVANICHAKUL : ENHANCEMENT OF ARBUSCULAR MYCORRHIZAL INOCULANT PRODUCTION USING PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR). THESIS ADVISOR : ASST. PROF. PANLADA TITTABUTR, Ph.D. 82 PP.

AM/MAIZE/PGPR/SPORE PRODUCTION/PROTEOMICS/SCAVENGING ENZYME

The low number of AM spore producing under the substrate-based production systems is the main obstacle for development of AM inoculum. This study aimed at investigation the effect of *Brevibacillus* sp. SUT47 as Mycorrhization Helper Bacteria (MHB) on doubling the number of AM spore production in maize and elucidation its enhancing mechanisms through the protein interactions among AM, plant, and SUT47. Although indole-3-icetic icid (IAA) produced from SUT47 could be one of the factors that increase AM spore production, the proteomics analysis demonstrated that proteins involved in plant defense response and Reactive Oxygen Species (ROS)-scavenging enzymes were also the main players in this scenario. The qRT-PCR results and some antioxidant enzymes analyses represented the mechanisms in which SUT47 may govern a major role during the early infection and penetration stage of mycorrhiza by suppression of plant ROS through the activation of ROS-scavenging enzymes and retarding of SA signaling pathway. Moreover, the proteomics analysis of SUT47 secreted proteins represented some elicitors, such as flagellin (*flg22*) and elongation factor TU (*elf18*) could contribute in supporting AM pre-penetration in plant cell. These proposed mechanisms would facilitate the AM spore production

when co-inoculated with SUT47. The current data provide evidence that SUT47 can be used to enhance AM spore production and could be applied for supporting the production of AM inoculum at low cost.

School of Biotechnology

Academic Year 2015

Student's Signature_____

Advisor's Signature_____

Co-advisor's Signature_____

Co-advisor's Signature_____

ACKNOWLEDGEMENTS

It is a pleasure to thank many people who made this thesis possible through the support and inspiration of several individuals all of whom have my gratitude.

In this opportunity, I would like to thanks to the important person, my advisor Asst. Prof. Dr. Panlada Tittabutr, for her encouragement, valuable suggestion as well as giving me extraordinary experiences throughout the work. My deep appreciation is also expressed to my co-advisor, Prof. Emeritus Dr. Nantakorn Boonkerd and Prof. Dr. Neung Teaumroong, without them this thesis would not have been possible.

I gratefully thank all of my teachers for their constructive and helpful comments on this thesis.

I would acknowledge the Department of Agriculture (DOA) and the Suranaree University of Technology for providing bacterial strains.

I wish to thank Dr. Nantida Watanarojanaporn and Dr. Pongdet Piromyou for AM and PGPR strains.

I would also like to thank all of the people who have made me happy and provide several helps everything for my work, as well as all of my friends in the School of Biotechnology for their contributions.

Finally, I am deeply thankful to my parents that believed in me also give me for an inspiration and all of the possible supports during the good and difficult times.

Financial support was provided by Suranaree University of Technology (academic excellence scholarship for graduated student) and is most gratefully acknowledged.

Watcharin Yuttavanichakul

CONTENTS

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

CHAPTER I

INTRODUCTION

1.1 Significance of this study

Arbuscular mycorrhizal fungi (AM) are soil fungi forming symbiosis with widespread plant families. AM have a potential to improve plant productivity as well as increase plant resistance against abiotic and biotic stresses (Smith and Read, 2010). Currently, demand of AM is increasing in economic agriculture as biofertilizer, since people are conscious for safe and healthy food. Moreover, the long term sustainability agriculture and reducing on environmental pollution associated with agrochemical compounds are concerning (Smith and Smith, 2011; Abbasi et al., 2015) in agriculture or horticulture to increase crop yield and plant health as well as for environmental restoration (Johansson et al., 2004). Nevertheless, the AM production is time consuming and need many resources. IJdo and colleague (2011) classified the production system into three main categories (i) the substrate-based production system, (ii) the substrate-free cultivation system, and (iii) the *in vitro* cultivation system. Although numerous methods have been developed for decades for AM production, the obligate biotrophic nature of AM made their inoculum production complicated. Therefore, development of AM production in large-scale with cost-efficient is needed to obtain a high-quality AM inoculum to fit the demand of AM in agriculture.

Recently, the interactions between plant growth promoting rhizobacteria (PGPR) and AM are among the most interesting studies due to their contribution to the

productivity of agricultural systems and natural ecosystems (Requena et al., 1997; Xavier and Germida, 2003; Adesemoye and Kloepper, 2009). Many studies involving the combined application of PGPR and AM have shown the possibilities of using certain PGPR to stimulate the beneficial role of AM, and vice versa (Barea et al., 2002; Barea et al., 2005). Interestingly, *Azospirillum* sp. which is one of PGPR has been reported to enhance mycorrhizal colonization (Bhowmik and Singh, 2004). The beneficial PGPR that support mycorrhizae are called Mycorrhization Helper Bacteria (MHB) which are considered to perform one of these five main actions on mycorrhizae; (i) in the receptivity of root to the mycobiont, (ii) in root-fungus recognition, (iii) in fungal growth, (iv) in the modification of rhizospheric soil, and (v) in the germination of fungal propagules (Rigamonte et al., 2010). However, mechanism on how MHB stimulated mycorrhizal colonization and spore production is poorly understood. On the other hand, the information on using PGPR for arbuscular mycorrhizal mass inoculum production has never been reported.

Therefore, the aims of this study were to develop arbuscular mycorrhizal spore production method by using PGPR as MHB and to examine the mechanism of PGPR on supporting arbuscular mycorrhizal colonization and spore production. The effects of host plant, AM specie, and level of plant nutrients influence on enhancing AM spore production by PGPR were investigated, while the techniques of 2D-Gel electrophoresis, quantitative reverse transcriptase PCR (qRT-PCR), and some stress response enzyme activities were used to elucidate the interaction mechanisms between AM, host plant and PGPR. Hence, this research found the method of using PGPR to enhance AM spore production at low cost for mass production of AM inoculum, while the suppression of plant immune by PGPR during AM penetration and symbiosis could be one of the

possible mechanisms in which PGPR as MHB use to enhance AM spore production in host plant. The schematic of protein-protein interactions between AM, plant, and MHB on supporting of AM symbiosis was proposed.

1.2 Hypotheses

A. The AM colonization and spore production could be enhanced by using PGPR as MHB.

B. PGPR would have some mechanisms involved in symbiosis interaction that can enhance AM colonization and spore production.

1.3 Objectives

1.3.1 Main objective:

To develop the AM production by using PGPR and evaluate the mechanisms of PGPR upon AM production.

1.3.2 Specific objectives:

1. To determine the possible combinations of AM and PGPR strains that can enhance AM colonization and spore formation.

2. To determine the effect of host plant, AM specie, plant nutrient level phosphorus, and IAA on enhancing the AM colonization and spore formation by PGPR.

3. To investigate the interaction mechanisms of PGPR that can enhance mycorrhizal colonization and spore production in host plant.

CHAPTER II

REVIEW OF THE LITERATURES

2.1 Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal fungi belong to the phylum Glomeromycota, and order Glomales (Schüβler et al., 2001). They present exclusive characteristic of endomycorrhizal (i.e., fungi found within plant roots) dominated from other mycorrhizas, such as ectomycorrhizal and ericoid by special structures as arbuscules and vesicles (Smith et al., 2003). Primarily, nutrient and carbon source exchanges between AM and plant occur via the arbuscules, while the vesicles are a storage organ. AM has intraradical hyphae located inside the root, extraradical hyphae found outside the root and in the soil environment. Normally, the arbuscules, vesicles, and intraradical hyphae are regarded as the intraradical mycelium, and the group of extraradical hyphae is called extraradical mycelium (Fellbaum et al., 2012).

In AM-plant symbioses, AM translocate nutrients from the soil to host plant pass through the extraradical mycelium and host plant will be supply AM with a carbon source. Plant supplies carbon source from the photosynthesis for AM about 5-85% which depending on the plant species and its dependence on the association (Treseder and Allen, 2000). Apart from nutrient uptake, the extraradical mycelium also involved in spore formation and initiation of root colonization (Brundrett and Abbott, 1991). Spores, hyphae, and colonized root are the propagules of AM (Brundrett and Abbott, 1991). Arbuscular mycorrhizal fungi support plant

establishment through the supply of nutrients, especially phosphorus (P) (Koide and Kabir, 2000). They improved biotic and abiotic stresses in their plant host by inhibiting the growth of pathogenic organisms and increasing plant resistance to drought or other unfavorable conditions (Barea et al., 2005). AM play crucial roles in the soil system by increasing plant nutrient contented and influencing rhizodeposition by the plant, AM modify plant growth and alter rhizosphere processes (Richardson et al., 2009).

2.2 Importance of arbuscular mycorrhizal fungi to plant ecology

One of the most important contributions of AM to ecosystem function and productivity is the uptaking and transferring of plant nutrients. Phosphorus achievement is a major role of AM to promote plant growth and development. Phosphorus is an essential nutrient element. However, both the organic P and the insoluble inorganic P are unavailable for plant uptake (Koide and Kabir, 2000). Only some plants can produce organic acids and phosphatases to hydrolyze and release P from the inopportune environmental conditions (Kiers and Heijden, 2006). However, Tawaraya (2006) demonstrated that AM in associations with the plants could improve P availability by solubilizing the insoluble fraction of inorganic P, which significantly increased P uptake by onion (*Allium cepa* L.) and resulting in P concentration in the plant tissue.

Using a split-dish *in vitro* carrot mycorrhizal system developed by Koide and Kabir (2000) found that AM were capable of hydrolyzing organic P sources and able to translocate the P to host plant. Even through, the mycorrhizal plants access to organic P sources and can successfully compete symbiosis with soil microorganisms

for P. However, the authors pointed out that AM utilize organic P in a slower rate when compared with inorganic P. Because a large percentage of total P in organic P is less solubilization by AM (Koide and Kabir, 2000). Recently, Heijden (2010) showed that AM reduced P lost by leaching in microcosms established with a sandy surfaced soil that is vulnerable to nutrient loss. Therefore, AM have the ability to increase available soil P and reduce losses of the P. Moreover, AM also an influence on the plant nitrogen (N) source but was not high as in the case of P (Heijden, 2010), they provided their host access to different forms of N then the resulting in an increase of plant N uptake (Hodge et al., 2001). Hodge et al. (2001) verified the ability of AM to decompose organic matter and obtain N from the organic source. They presented that AM increased the diffusion rate of N to its host. Therefore, mycorrhizal plants have the ability to access to N sources compared with non-mycorrhizal plants. The most exciting part of their findings is that AM could be saprophytic, especially when decomposition is required for nutrient uptake. The intraradical hyphae decompose the amino acids to access the C and then transfer the remaining N as ammonium to the host plant. Other studies have shown that AM increase zinc (Zn) uptake via plants by increasing its bioavailability in the soil. Because only a small fraction of Zn is supplied by most crops plant, Zn absences in the human diet have been concerned; therefore, AM indirectly increase the nutritional value of human food through Zn achievement (Ryan and Graham, 2002). Other micronutrients assimilated by AM for plant use include copper (Cu) and iron (Fe) (Liu et al., 2000).

At low P concentrations, Cu, Fe and Zn contents in maize inoculated with *G. intraradices* were higher than those of a non-mycorrhizal plant, even without the additions of micronutrients. Hobbie et al. (2009) described the relationship between

water and nutrient uptake by plants since the transpiration drives water and nutrient transfer from soil to plant roots. Moreover, AM enhanced plant water and nutrient use efficiency by modifying plant stomata and transpiration rate. Other functions of the AM is the inducing of pathogenesis-related (PR) proteins of the host plant and increasing nutrient acquisition of the affected plant to compensate for the damage caused by the pathogen (Gosling et al., 2006). Despite there are numerous reports of AM on plants, several studies also showed beneficial effects of mycorrhizal symbioses on plant productivity and other functional roles are not yet to be determined (Govindarajulu et al., 2005).

2.3 Arbuscular mycorrhizal production

The number of small- to medium-sized companies producing arbuscular mycorrhizal fungal (AM) inocula around the world has been increasing, particularly listed 21 companies in North America, 8 in Europe, 2 in South America, and 2 in Asia, but there are certainly many more established companies pointing to produce and use AM inocula in various segments of plant production (Gianinazzi and Vosátka, 2004). The reasons for the development of this agricultural biotechnology industry producing AM inocula were multiple: (i) AM were progressively being considered as a natural plant health insurance and examples of their optimistic impact on plant development and health, land reclamation, and phytoremediation were continually increasing (Leyval et al., 2002); (ii) there was higher awareness of biodiversity issues, including those about soil microbial communities, and acceptance of these natural technologies as alternatives to agrochemicals (Barea, 2000); and (iii) society was

difficult more sustainable means of production, with a consequent feedback to farmers and land ecologists.

Producing microbial inocula was a complex procedure that involves not only the development of the necessary biotechnological expertise but also the ability to respond to the specifically related permitted, ethical, educational and commercial requests. This is particularly true in the case of obligate endosymbiotic microorganisms especially AM fungi because abundant the above-mentioned requirements is closely associated with the particular method of inoculum production.

2.4 Inoculum production systems with commercial applications

Production systems of AM fungi were evolved considerably during recent years, from relatively simple technologies to more complex (Sylvia and Jarstfer, 1997). At present, the inoculum is produced for commercial purposes in the following ways: (i) nursery plots with soil in which inoculated plants are cultured in an open field or nursery beds. Advantages: simple, adapted for local use, low costs; disadvantages: limited in application, easily contaminated, and poorly adapted for the development of an industrial activity. (ii) containers (pots) with different substrates (Von Alten et al., 2002). Advantages: low technology input, undesirable contaminations fairly easily excluded, equitable costs; disadvantages: not pure, limited in its industrial development. (iii) aeroponic systems (Sylvia and Jarstfer, 1994), where preinoculated plant roots are incessantly misted with nutrient solution sprayed within cultivation boxes. Advantages: easier control of contaminants, carrier-free inoculum, adapted for microplants; disadvantages: relatively complicated technological setup. (iv) *in vitro* on roots transformed with *Agrobacterium rhizogenes*

(Declerck et al., 1998). Advantages: pure cultures, permits industrial development; disadvantages: high technological security, high costs, not all AM fungi successfully cultural in this system, and suitability of inoculum produced *in vitro*, in particular, its competitive ability toward other microbes in field soil, has yet to be tested.

2.5 Formulation of the inocula

Basically, the formulation procedure consisted of placing fungal propagules (root fragments colonized with AM fungi, fragments of fungal mycelium, and spores) in a given carrier (perlite, peat, inorganic clay, zeolite, vermiculite, sand, etc.) for a given application. Biological inoculants belong to diverse taxonomic groups varying considerably in physiology and, as a consequence, in their nutritional and environmental requirements. Therefore, the final formation of the formulation will result from a more or less technologically complex procedure, determined by the microbe involved, the way of producing inoculum, and the target inoculum application (bare-root plants, containerized plants, cuttings, seeds, potting mixes, soils, etc.). The fungi should be selected to be compatible with the target environment (Vosatka and Dodd, 2002). Following mass production, fungal propagules must be formulated in such a way that they can be stored and distributed under a wide range of temperatures and without losing viability. The formulation should be simple and economical, and the formulated inocula should be easy to transport and apply. Some companies producing AM fungal inocula have adopted the approach of one type of formulation (i.e., single fungal species) for all markets while others produce a range of products for their target buyers.

2.6 Quality control of mycorrhizal inocula

The industrial activity of inoculum producers has developed using different AM fungi, which are quite often not well characterized in terms of ecological requirements and stability. The lack of quality control for several marketed inocula is among the main reasons for the low reception of mycorrhizal technology in horticultural and agricultural practices. This situation has led to the need for this industry to develop, in its own interest, criteria that will satisfy minimum requirements of quality for the AM inoculum production. Whatever the mode of inoculum production chosen and the formulation procedure adopted by the companies, the marketed product has to meet the expected requirements of end-users (e.g., reduce phosphorus fertilizer inputs, increase plant tolerance to pollutants, improve flowering, favor ecological land restoration, and many others). Although these objectives may vary according to the companies, they should all aim at the use of AM fungi as a natural plant health coverage (Smith and Gianinazzi-Pearson, 1988).

In this context, the following criteria should be fulfilled by the companies: (i) plants to be inoculated must be able to form mycorrhizas; (ii) the AM fungal inoculum must be free of agents that could negatively affect normal plant growth and development; (iii) the shelf life of the inoculum should be sufficient to suit the end-user markets. The introduction of such criteria by the inocula producers could contribute to the definition of conditions for the recording of products at national or international levels (Von Alten et al., 2002). Furthermore, in the product description, the inclusion of the following recommendations for quality standards may be considered.

2.7 Classification of arbuscular mycorrhiza production systems

2.7.1 Substrate-based production systems

Classical production of AM fungi was generally performed by the cultivation of plants and associated symbionts in a soil- or sand-based substrate, even though a range of substrate substitutes and amendments were also commonly used. In the large-scale production, AM production may be achieved in single pots of various materials (e.g., earthenware or plastic) and sizes. The sizes may be varied from medium-size bags and containers to large raised or grounded beds (Douds et al., 2010). The production process was often conducted under controlled or semicontrolled conditions in greenhouses or performed in growth chambers for the easy handling and control of parameters such as humidity and temperature. However, depending on the host plant and climate conditions, large-scale production was sometimes conducted in open air, e.g., for on-farm production (Douds et al., 2010).

2.7.2 Substrate-free cultivation systems

A wide variety of substrate-free cultivation techniques also termed "solution culture techniques" exists. They mainly differ in the mode of aeration and application of the nutrient solution. In static systems, the nutrient solution needs to be aerated via an aeration pump to prevent roots from suffering from oxygen deprivation. However, strong movement of the nutrient solution and bursting of air bubbles might damage the development of the delicate extraradical hyphae. To prevent this problem, pumps may be switched on only periodically to minimize the impact on AM fungal growth and development (Hawkins and George, 1997). The use of a nutrient film that covers the roots increased the relative area for gas exchange and overcome problems due to insufficient aeration. This technique was used to culture AM fungi in the early

1980s and was patented by Mosse and Thompson in 1981. Aeroponics was a form of hydroponics in which the roots (and AM) were bathed in a nutrient solution mist (Zobel et al., 1976). Comparison to testing the suitability of different aeroponic techniques were performed by Jarstfer et al. (1998) and Mohammad et al. (2000). In the first study, Jarstfer et al. (1998) tested three types of aeroponic devices, atomizing disk, pressurized spray through a microirrigated nozzle, and an ultrasonically generated fog of nutrient solution with droplets of 3-10 μm diameter. They reported that pump and nozzle spray systems were the most adapted systems for AM fungi cultivation. In the study of Mohammad et al. (2000), atomizing disk was compared with the latest ultrasonic nebulizer technology (resulting into microdroplets of 1 μm in diameter). These authors reported the ultrasonic nebulizer as the most successful aeroponic method for the cultivation of *G. intraradices* associated to Sudan grass (*Sorghum sudanense* Staph.). In substrate-free production systems (i.e., hydroponics and aeroponics), precolonized plants are produced prior to their introduction into the systems.

2.7.3 *In vitro* production systems

The first attempt to culture AM fungi *in vitro* date back to late 1950s (Mosse, 1959). Mosse (1962) reported the first association of an *Endogone* species with a plant. Since then, several progresses paved the way to mass-production of AM. In the mid-1970s, Mosse and Hepper (1975) successfully established a culture of an AM associated with excised roots of tomato (*Lycopersicum esculentum* Mill.) and red clover (*T. pratense* L.) on a gelled medium. Ten years later, Mugnier and Mosse (1987) used Ri T-DNA transformed carrot roots as host in the so-called root organ culture (ROC) system. To facilitate the access to the AM and increase the production

of propagules. St-Arnaud et al. (1996) used a split-plate method, such as a bi-compartmental ROC, to separate a proximal compartment containing the root and AM from a distal compartment in which only the AM developed. Using this split-plate method, Douds (2002) demonstrated that the AM continued sporulation after medium from the distal compartment was partially replaced, and glucose was provided to the proximal compartment, which resulted in repeated harvests of the same Petri plate culture.

Different production systems were derived from the basic ROC in Petri plates. For example, Voets et al. (2005) and Boulois et al. (2006) developed two *in vitro* culture systems based on autotrophic plants. In the system of Voets et al. (2005), the shoot developed outside the Petri plate while the roots and AM were associated inside the Petri plates filled with a suitable agar medium. In the system of Boulois et al. (2006), the shoot developed in a sterile tube vertically connected to the top of a Petri plate in which the AM and roots developed. In the study of Boulois et al. (2006), ~1,600 spores were obtained in a period of 12 weeks in the root compartment while Voets et al. (2005) obtained on average 4,500 spores within the same period and more than 12,000 spores per Petri plate after 22 weeks of cultivation.

2.8 Plant growth-promoting rhizobacteria (PGPR)

Plant growth-promoting rhizobacteria (PGPR) are beneficial inhabitants of the rhizosphere and found in colonization with plant roots (Harrier and Watson, 2004). The beneficial effects of PGPR on several crops including cabbage (*Brassica oleracea* L.), canola (*Brassica rapa* L.), maize (*Zea mays* L.), potato (*Solanum tuberosum* L.), rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.) were reported (De Freitas

et al., 1997; Wright et al., 1998). As reviewed by Hayat et al. (2010), beneficial effects of PGPR on plant growth can be classified as direct or indirect. Direct effects of PGPR enhanced plant productivity via many different modes of action including increasing seedling emergence, shoot and root growth, nutrient content, seed yield and protein concentration and simulating ripening and senescence of plants after maturity (Dobbelaere et al., 2003). Consequently, the important factor for plant growth is the element and hormone such as nitrogen (N), phosphorus (P), potassium (K) iron (Fe), Calcium (Ca), magnesium (Mg), and Auxin.

Nitrogen is one of the most important elements for plant production; however, most organisms cannot use atmospheric N_2 directly. As a result, N frequently is a limiting 6 nutrients (Kloepper and Beauchamp, 1992). The role of bacteria in biological N_2 fixation has been reported as early as the 1800s (Silva et al., 2004), and there are reports that PGPR could promote growth because of their role in N_2 fixation (Vessey, 2003). For example, *Paenibacillus* found in the wheat rhizosphere was the PGPR due to its ability to fix N_2 (Bertrand et al., 2001). Most of the studies reported N_2 fixing rhizobia such as *Bradyrhizobium* and *Rhizobium* species as PGPR due to their atmospheric N_2 fixation (Adesemoye and Kloepper, 2009). Although some researchers have selected PGPR by observed nitrogenase activity which this activity may relate to growth promotion by PGPR (Vessey, 2003). However, some PGPR contained nitrogenase activity but did not promote soybean (*Glycine max* (L.) Merr.) growth. Thus, N_2 fixation may not be an important trait of PGPR (Touraine, 2004), and PGPR may not necessarily contribute considerably to plant only N nutrition (Richardson et al., 2009). Some PGPR increase the availability of other essential plant

nutrients, such as phosphorus (P), iron (Fe), Calcium (Ca), magnesium (Mg), and potassium (K) (Vessey, 2003; Adesemoye and Kloepper, 2009).

Phosphorus is a limiting soil nutrient because a considerable fraction of total soil P was organic P or insoluble inorganic P and cannot be absorbed by plants (Koide and Kabir, 2000). Phosphorus was mainly absorbed by plants in the orthophosphate forms; namely, monobasic ($H_2PO_4^{-1}$) and dibasic (HPO_4^{-2}) P (Vessey, 2003). Furthermore, soil iron and aluminum oxides form Fe and Al-hydroxylated surfaces that retain P, and at high pH, calcium carbonate reduced the bioavailability of soluble inorganic P in the soil solution. Similarly, when P was applied as fertilizer, it was easily immobilized, further increasing P deficiencies (Esitken et al., 2006). Therefore, mineralization of organic P and solubilization of inorganic P by PGPR is an important aspect of their association with plants. Some PGPR solubilizes insoluble inorganic and organic P compounds by secreting organic acids and enzyme phosphatases, respectively. De Freitas et al. (1997) demonstrated the rock phosphate solubilization by *Bacillus* and *Xanthomonas* species via their synthesis of organic acids. Organic acid acidified microbial substrates which induce proton exchange for calcium ions (Ca^{2+}), thereby releasing soluble P forms from mineral phosphate (Rodríguez and Fraga, 1999). The production of organic acids such as acetic, citric, lactic, oxalic, gluconic, and succinic acids were described (de Freitas et al., 1997; Rodríguez and Fraga, 1999; Adesemoye and Kloepper, 2009). Nevertheless, not all P solubilizing bacteria increase P uptake and P concentration in plant tissues. Freitas et al. (1997) showed that P solubilization by *Bacillus* and *Xanthomonas* species did not enhance P content of canola; rather the PGPR used other mechanisms such as hormone

production to stimulate plant growth. Calcium, Iron, magnesium, and potassium were among other essential nutrients supplied by PGPR to their host (Lucy et al., 2004).

Some PGPR enhanced nutrient availability via production of siderophore. Siderophores were yellow-green fluorescent pigments with high affinity for Iron (III) (Fe^{3+}) and were capable of reducing this Fe form to Iron (II) Fe^{2+} which can be absorbed by plant cells. This mechanism was especially important under Fe limiting conditions (Glick, 1995).

It is well recognized that PGPR produce phytohormones that stimulate plant growth (Glick, 1995; Vessey, 2003). Auxin [e.g., Indole-3-acetic acid (IAA)] was one of the important hormones produced by PGPR (Glick, 1995; Richardson et al., 2009). By increasing lateral roots and roots hair formation, IAA expanded the root surface area, and allowed greater exploration of soil regions for nutrients (de Freitas et al., 1997; Dobbelaere et al. 2003). Dobbelaere et al. (2003) related the yield increases of spring wheat to root development by IAA producing by *Azospirillum brasilense*. Idris et al. (2007) found the correlation of colonizing and growth promoting abilities of some strains of *Bacillus cereus* to IAA production. Furthermore, beneficial effects of IAA-producing PGPR on root growth increased colonization sites for other beneficial microorganisms, such as AM and symbiotic N_2 fixers (Vessey, 2003).

Indirect effect of PGPR to promote plant growth such as, the removing hazardous chemicals and inhibiting the growth of deleterious microorganisms (biocontrol) in the rhizosphere (Hayat et al., 2010). PGPR reduce the growth of pathogens through the production of antibiotics and siderophores (Whipps, 2001). Even though the mechanisms involved in growth promotion by PGPR are not completely understood (Nelson, 2004), atmospheric N_2 fixation, phytohormone

production, antagonism against pathogens, enhancement of plant nutrient uptake such as P solubilization, and stimulation of beneficial activities of other rhizosphere organisms were frequently reported.

2.9 Interactions between Plant growth-promoting rhizobacteria and arbuscular mycorrhizal fungi

There were documentaries evidence that the symbiotic associations of plants with AM were influenced by a number of rhizosphere organisms including PGPR (Walley and Germida, 1995; Barea et al., 2002; Vessey, 2003; Barea et al., 2005). In a similar way, the activities of PGPR were depend on their associations with other rhizosphere organisms, especially the commonly found AM (Walley and Germida, 1995; Roesti et al., 2006). A number of these studies revealed the beneficial associations between AM and PGPR, enhance plant growth and nutrient uptake (Roesti et al., 2006; Adesemoye and Kloepper, 2009). Mar Vázquez et al. (2000) observed the effect of *A. brasilense* on shoot and root weight of maize was only beneficial when co-inoculated with *G. deserticola*. Rodríguez-Romero and co-workers (2005) found that the co-inoculation of *G. manihotis* and *Bacillus* species increased banana growth and nutrient uptake, although the interaction effect was not significantly greater than the bacterial treatment. Interaction effects of these organisms also were observed on the uptake of micronutrients. Meyer and Linderman (1986) showed the increase in the concentrations of Cu, Fe, Ni, and Zn in a subterranean clover (*Trifolium subterraneum* L.) shoot when *Pseudomonas putida* was co-inoculated along with native AM species. This effect was higher than the individual effect of the PGPR or AM on plant.

The beneficial interactions observed between AM and PGPR on plant growth were based on a few known mechanisms (Barea et al., 2002, 2005; Richardson et al., 2009). Certain PGPR produce some phytohormones that enhance AM colonization by increasing root surface area and susceptibility of plant to AM hyphae penetration (Toro et al., 1997; Barea et al., 2002). In combination, AM and PGPR modified soil nutrient concentration ratios and nutrient mobility to facilitate nutrient retention in the plant tissue (Toro et al., 1997). Finally, they both influenced microbial community composition to benefit their host plant (Roesti et al., 2006). Although beneficial associations between AM and PGPR were frequently observed, neutral and detrimental ones also found (Walley and Germida, 1997; Vazquez et al., 2000; Wu et al., 2005; Adesemoye and Kloepper, 2009). Kim et al. (1997) reported that a phosphate solubilizing bacterium (PSB), *Enterobacter agglomerans*, had no influence on the percentage of tomato root colonized by *G. etunicatum*. In general, all these authors demonstrated that AM and PGPR interaction effects were unpredictable, and plant response to these effects was inconsistent. Microbial, soil, and other environmental factors were commonly used to explain these variabilities (Vazquez et al., 2000; Roesti et al., 2005).

The interaction effects between AM and PGPR were not limited to plants; a number of researchers implied these organisms in the removal of potentially toxic elements (Gonzalez-Chavez et al., 2004) in the soil (Barea et al., 2002). PGPR enhanced mycorrhizoremediation of contaminated soils and water (Khan, 2005). Mycorrhizoremediation may be defined as the involvement of AM in the reduction or removal of contaminants from affected soils and water by stimulating plant growth and nutrient uptake (Khan, 2005). PGPR facilitated mycorrhizoremediation by

increasing plant root surface area for AM colonization, modifying microbial community associations with the mycorrhizal root, and inducing the transfer of these contaminants to the rhizosphere where they can be easily accessed and taken up by plants (Barea et al., 2002, 2005). These studies verified the interactions and hence it was conceivable that the combined application of PGPR and AM could enhance mycorrhizal colonization. However, not much clear works have been done on this interaction, there is little knowledge on how its mycorrhizal colonization can be increased by using PGPR. There has also been little information used of PGPR for arbuscular mycorrhizal mass inoculum production. However, PGPR was reported by Bhowmik and Singh (2004) which the PGPR (*Azospirillum* sp.) considerably enhanced mycorrhizal colonization compared to yeast (*Saccharomyces cerevisiae*).

Since, many methods have been developed for increasing of arbuscular mycorrhizal such as the substrate-free cultivation systems and the *in vitro* cultivation systems but there still have high cost production. The classical system is commonly used in many countries and can be used in small – scale to large – scale of AM production but it can produce low spore production. Therefore, in this study, PGPR will be used to improve AM spore production and to determine the mechanisms of their interaction.

2.10 The regulation of the plant defense response in arbuscular mycorrhizal symbiosis

AM are considered to be biotrophic micro-organisms. Because of their obligate symbiotic nature, they cannot be cultured. The penetration into the root and intercellular growth of the AM fungi involves a complex sequence of biochemical and

cytological events and intracellular modifications (Balestrini and Bonfante, 2005), which imply that the fungus must clearly be recognized by the host plant. The mechanisms controlling AM development are largely unknown. However, the plant defense responses during the early stages of plant–AM interactions may be the induction or suppression effect. This was achieved through the recognition of specific signal molecules call elicitors. Elicitors can be secreted from the microbe (exogenous elicitors) or generated as a result of physical or chemical cleavage of the plant cell wall (endogenous elicitors). After rerception of an elicitor, a number of biochemical changes contribute to the early response in host cells. These processes include changes in the ion permeability of the plasma membrane, the activation of plasma membrane-bound enzymes, the activation of kinases, phosphatases, phospholipases, and the production of signal molecules, including active oxygen species. The result of these processes is the transcriptional activation of defense-related genes (Somssich and Hahlbrock, 1998). For example, the elicitor derived from an extract of extraradical mycelium of *Glomus intraradices* was able to induce phytoalexin synthesis in soybean cotyledons (Lambais et al., 2000).

An oxidative burst could be detected at sites where hyphal tips of *G. intraradices* attempted to penetrate a cortical root cell of *M. truncatula* (Salzer et al., 1999). The greatest evident effects were observed in the incompatible associations between AM and non-host plants or NOD^-mutant plants. The stronger defense response of PR-1 protein, and phenolics has been observed when pea NOD^- mutant plants were tested with an AM (Gollotte et al., 1993). Moreover, a hypersensitive response and some elements of signal transduction pathways activated after pathogen recognition by the plant have been observed during the early stages of

AM formation. The transient increase in catalase and peroxidase activity observed in tobacco mycorrhizal roots also matched with the accumulation of salicylic acid (SA) (Blilou et al., 2000).

SA is a signal molecule involved in the signal transduction pathway activated in plant–pathogen reactions (Gaudin et al., 1987). The accumulation of SA was also correlated to an increase in the expression of genes encoding function in plant defense response. The defense-related genes expressed during the early stages of AM fungal penetration are also activated by pathogen infection, treatment with elicitors, or by SA. Among these components, salicylic acid and reactive oxygen species (ROS) have been involved as second messengers in AM associations. Thus, levels of H_2O_2 and other ROS have been measured in AM roots and suggested that the levels of H_2O_2 in mycorrhizal associations are increased (Salzer et al., 1999).

Alterations in the pattern of anti-oxidative enzymes, such as peroxidase and catalase in AM roots may indicate that oxidative compounds are produced during the colonization process (Lambais, 2000). The increase in catalase and peroxidase activity could be due to their function as antioxidants for any active oxygen molecules generated during the initial stages of fungal penetration. Since H_2O_2 and other reactive oxygen species are involved in signal transduction cascades in plant–pathogen interactions, it is possible that degradation of H_2O_2 by catalase in AM could be a possible mechanism for avoiding the activation of defense response genes. Another example of regulation of catalase and peroxidase has been shown in bean and wheat colonized by *G. intraradices* (Blee and Anderson, 2000). The accumulation of these enzymes in cells containing arbuscules may be due to a localized regulation of the defense mechanism. Nevertheless, the findings that transient increases of SA are

detected in tobacco and rice AM roots and that the exogenous application of SA to rice roots did not affect hyphopodium formation, but did cause a delay of mycorrhization of root suggest that the regulation of defense response in plants against AM fungi may be through the SA pathway.

However, the role and significance of the defense mechanism activated during the AM interaction remains unclear. Whether the alteration of defense gene expression has a particular or functional role in the establishment of the AM symbiosis. An investigation of the signaling mechanisms underlying the activation and cross-talk processes that participate in the regulation of the AM symbiosis (Fig 2.1) may contribute to understanding of the formation and functioning of this symbiosis, including the repercussions that these mechanisms could have on the induction of the plant resistance against pathogens observed in mycorrhizal plants. Figure 2.1 presents the binding of fungal elicitors to plant receptors triggers the activation of the signal transduction pathway leading to defense gene activation. Numerous events participate in the signal transduction pathway, including generation of reactive oxygen species and increases in SA.

The action of fungal hydrolases on plant cell wall components could also generate endogenous elicitors that contribute to the generation of the signal transduction chain after elicitor perception. Constitutive inducible plant hydrolases are the enzymes responsible for elicitor degradation and, consequently, plant defense attenuation. In parallel, the recognition of a putative fungal symbiotic signal by the plant cell leads to the activation of a specific symbiotic pathway (García-Garrido and Ocampo, 2002).

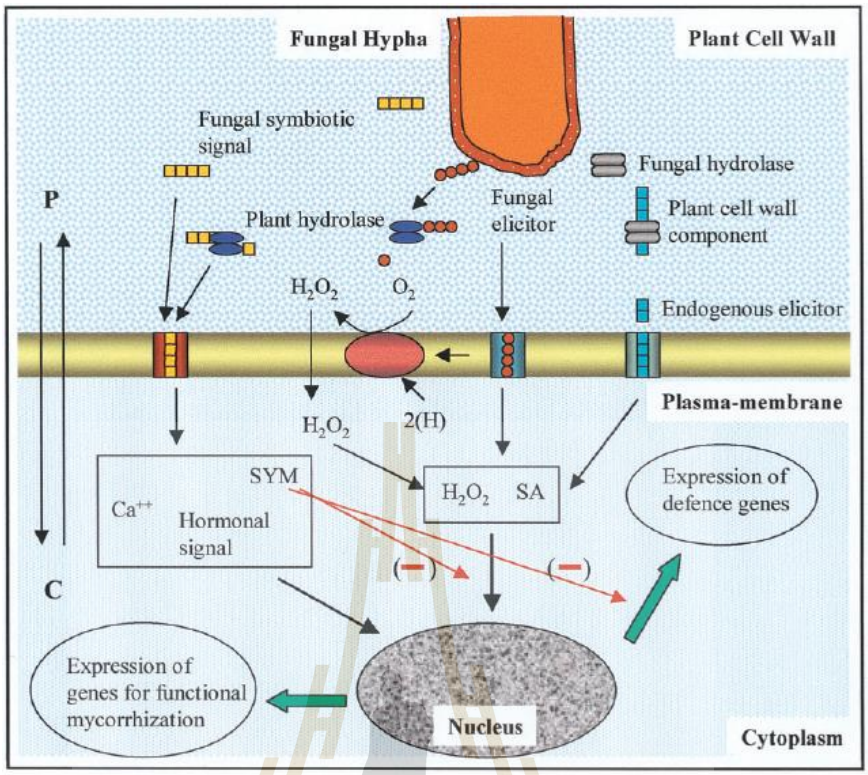

Figure 2.1 Hypothetical model representing the regulatory mechanisms involved in the plant defense response during the establishment of the AM symbiosis.

2.11 Proteomics and application on study plant-mycorrhizal symbiosis interaction

The term proteome refers to the complete set of proteins studies (genomics) reveal or suggest what could theoretically happen or provides insights into the information involved in mediating specific cellular processes. Two-dimensional electrophoresis (2-DE) is a common technique used to analysis protein in proteomic research. This technique involves on the separation of proteins based on their isoelectric points by isoeletric focusing (IEF), and then based on their molecular masses, under denaturing condition, by sodium dodecyl sulfate polyacrylamide gel

electrophoresis (SDS-PAGE). Many of the techniques used in proteomics, in particular two-dimensional electrophoresis (2-DE) were developed two decades before the term proteomics was created (O'Farrell, 1975).

However, technical improvements in the quality and reproducibility of 2-DE gels, analytical Mass Spectrometry (MS) and the development of software packages to process digitized images of gels have been contributed by scientist plant biology (Kav et al., 2007). The identification of proteins involved in plant-microbe symbioses and elucidating their functions in mycorrhizal symbioses is the important information to understand in their relationship. The completion of the genome sequence and proteomics techniques may now be utilized to study the expression of the protein in plant-mycorrhizal symbioses interactions.

The levels of protein expression using 2-DE during the early stages of mycorrhiza development showed significant changes in protein synthesis at a few hours of *Eucalyptus globulus* inoculated with *Pisolithus tinctorius* association (Hilbert et al., 1991). 2-DE performed the comparison of protein profiles of un-inoculated roots with roots synchronized for *Glomus intraradices* in *Medicago truncatula*-arbuscular mycorrhizal symbiosis (Amiour et al., 2006). The changes in the root proteome of *M. truncatula* were reported, leading to differences in their colonization intensity upon an infection with an arbuscular mycorrhizal fungus. The differential accumulation of proteins involved in plant defense reactions, cytoskeleton rearrangements, and auxin signaling upon symbiont contact was observed. These putative pathways by which symbiosis-related gene(s) may regulate very early AM formation.

The investigation of *A. fruticosa* mycorrhizal proteins at the maturation stage found that a total of 3,473 proteins were identified, of which 77 showed dramatic changes in their root expression levels; 33 increased, and 44 decreased. Plant proteins were assigned into 11 categories: metabolism-related (32%), protein folding and degradation-related (22%), energyrelated (10%), protein synthesis-related (8%), stress and defense-related (24%), transcription-related (6%), membrane and transport-related (4%), cellular structure-related (2.5%), signaling transductionrelated (11%) and unknown proteins (5%). These metabolic characteristics and molecular mechanisms are involved in AM symbioses (Song et al., 2015). However, the function of these proteins on symbiosis pathway has not been elucidated.

CHAPTER III

MATERIALS AND METHODS

3.1 Arbuscular mycorrhizal fungi spore propagation

Pure cultures spore of *Claroideoglomus etunicatum* and *Acaulospora tuberculata* were obtained from School of Biotechnology, Suranaree University of Technology, Thailand and propagated as described by Watanarojanaporn et al. (2013) using maize (*Zea mays* Linn.) as the host. The spores were extracted from pot samples using the wet-sieving method at maturity stage 90 dai (days after inoculation), described by Dandan and Zhiwei, (2007). Spores were rinsed into a small Petri dish, and 100 spores were collected in each tube and stored at 4ºC.

3.2 Plant growth promoting rhizobacteria (PGPR) preparation

PGPR including *Brevibacillus* sp. strain SUT47, *Pseudomonas* sp. strain SUT19 and *Bacillus* sp. strain SUT1 were obtained from School of Biotechnology, Suranaree University of Technology, Thailand, which all of them could promote maize growth, and their plant growth promoting characters were described by Piromyou et al. (2011). Each PGPR strain was grown in liquid LG culture medium (Thuler et al., 2003) for 2 days at 28ºC to obtain bacterial cell between 10^7-10^8 cells/ml to use in the experiment.

3.3 Assay of AM root colonization and spore production promoted by PGPR

One hundred spores of each AM strain were co-inoculated with 1 ml of each PGPR strain onto a sterilized maize seedling in 15-cm diameter plastic pot contained 1:1 (v/v) autoclaved vermiculite and sand mixture. The pot cultures were treated weekly with 50 ml Hoagland solution (Hoagland and Arnon, 1950) for 90 dai, then AM root colonization and spore production were determined. The AM root colonization was estimated by using trypan blue histochemical staining procedure (1970). After staining, the intensity of AM colonized root cortex was observed by using a light microscope and measured with MYCOCALC software (http://www.dijon.inra.fr/MychintecMycocalcprg/download.html) (Duc et al., 1989). Then, the AM spores were isolated from plant and the number of spore was counted above with the method of International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM) (http://invam.wvu.edu/methods/spores).

3.4 Determination of spore production in different host plants and AM species

The sorghum (*Sorghum bciolor* Linn.), onion (*Alium cepa* Linn.), and maize (*Zea mays* Linn.) seeds were sterilized and germinated for a day. The seeds were inoculated with 1 ml (10^8 cells/ml) of *Brevibacillus* sp. strain SUT47 together with 50 spores of each AM specie per seed. The Hoagland solution was used in the experiment. The number of AM spore was determined after planting 90 dai as described above compared with AM plant without SUT47 co-inoculation.

3.5 Assay of AM root colonization and spore production influenced by concentration of plant nutrients

The concentration of nutrients and phosphorous was prepared into four treatments with 5 replicates by using the Hoagland solutions modified by Millner and Kitt (1992) including full strengths (F), full strengths with low phosphate (0.02 ppm) (FL), half strengths (H), and half strengths with low phosphate (HL). Each treatment was inoculated with 1 ml (10^8 cells/ml) of strain SUT47 and 50 spores of *A. tuberculata* to each seedling. At 90 dai, the AM root colonization and spore production were determined by comparison between AM plant with and without strain SUT47 co-inoculation.

3.6 Analysis of maize root architecture affected by co-inoculation of *A. tuberculata* with *Brevibacillus* sp. strain SUT47

The germinated maize seeds were co-inoculated with 1 ml (10^8 cells/ml) of strain SUT47 or none PGPR strain *Escherichia coli* DH5alpha (negative control) with 50 spores of *A. tuberculata* in pouch as a describted by Hoagland and Broyer, (1936). The maize root architecture was analyzed at 7 dai by using Smart Root Program (Lobet et al., 2011) available on (http://www.uclouvain.be/en-smartroot).

3.7 Determination of *A. tuberculata* spore production in maize treated with different concentrations of indole-3-acetic acid (IAA)

The germinated sterilized maize seeds were inoculated with 50 spores of *A. tuberculata* and treated weekly with 50 ml Hoagland solutions containing different

IAA concentrations (25, 50, 75, 100, and 150 ppm) for 90 dai and then the AM spore production was determined as described above.

3.8 Protein extraction from maize root co-inoculated with *A. tuberculata* and *Brevibacillus* sp. strain SUT47

Experiment was designed into four treatments with 3 replicates of non-inoculated maize (control), maize inoculated with *A. tuberculata* (AM), maize inoculated with SUT47 (SUT47), and maize co-inoculated with *A. tuberculata* and SUT47 (AM+SUT47). One ml of SUT47 and 50 spores of *A. tuberculata* were used in each treatment as previously described. Full strength Hoagland solutions was supplied to each treatment to obtain 40-50% relative humidity. At 30 dai, total proteins were extracted from each 500 mg roots using 10% sucrose in SDS buffer modified from protocol according to Wu et al. (2014).

3.9 Extraction of proteins secreted from *Brevibacillus* sp. strain SUT47 in response to maize root exudates

Brevibacillus sp. strain SUT47 was cultured in liquid LG culture medium for 24 hours at 28^{o}C (Thuler et al., 2003). To extract secreted proteins of SUT47 in response to 7-day maize root exudates, the incubation method was followed the technique of Rigamonte et al. (2010). The SUT47 culture was treated with and without the 7-day maize root exudates and incubation for 12 hours. The SUT47 culture without maize root exudates was used as control condition. After incubation, the supernatant of cell culture was collected by centrifuged at 12,000 rpm for 10 min. The SUT47 secreted proteins were extracted from the supernatant and lyophilized,

then secreted proteins were rehydrated in 25 ml deionized water. Phenol extraction, precipitation of proteins and two-dimensional (2D) gel electrophoresis were carried out as described by Süß et al. (2006). The protein spots were analyzed by comparison with the supernatant of SUT47 without root exudate treated condition.

3.10 Protein analysis by 2D-Gel electrophoresis

Precast 13 cm nonlinear pH 3-10 IPG strips (Immobiline DryStrip gels, GE Healthcare Life Sciences) were rehydrated for overnight with 250 ug/ml of each total protein sample in 250 µl of 8 M Urea, 20 mMDTT, 2% (v/v) CHAPS, 2% (v/v) IPG buffer pH 3-10 and Bromophenol blue for the first-dimension isoelectric focusing (IEF). IEF was performed automatically using Ettan IPGphor 3 Isoelectric Focusing System (GE Healthcare) and then IPG strips were immediately equilibrated (Görg et al., 1987). Then, proteins were separated in the second dimension on 12% pH 8.8 SDS-polyacrylamide gels electrophoresis (PAGE). Gels were run at 25ºC for 10 min at 35 V, and then at 80 V until the dye front reached the bottom of the gels using an Ettan DALTsix electrophoresis (GE Healthcare). The stained gels were scanned with ImageQuant 5.2 software (GE Healthcare) on a Typhoon 9200 scanner (GE Healthcare) with a resolution of 300. The spot intensity were performed with ImageMaster 2D Platinum 7.0 analysis software (GE Healthcare). Protein staining spots were quantified by calculation of spot volume using the total spot volume normalization method multiplied by the total area of all spots. The data of spot volume and density were carried out with Excel 2013. Each experiment was performed in triplicate.

Proteins of interest were manually excised from coomassie blue staining gel. The interested spots were destained with 20 mM ammonium bicarbonate (AmB) for 10 min, and solution was discarded. Then the gels were destained with 20 mM AmB in 50% acetonitrile (ACN) for 10 min, and then the solution was discarded again. These two steps were repeated three times to eliminate coomassie blue staining followed by drying the gels in a SpeedVac concentrator. The dried gels were rehydrated in 10 mM dithiothreitol in 20 mM AmB at 56ºC for 45 min to reduce the proteins then the solutions were discarded. After that 55 mM iodoacetamide in 20 mM AmB was added to the gels and incubated in the dark at room temperature for 45 min to alkylate the proteins. Upon removal of the liquid phase, the gels were washed with 25 mM AmB in 50% ACN and 100% ACN, consecutively. The proteins were digested with 40 µl of sequencing grade trypsin (20 ng trypsin; Promega, Madison, WI) in 25 mM AmB for overnight at 37ºC. The resulting peptides were extracted from gel pieces with 5% formic acid in 50% ACN by ultrasonic bath from Elma (Singen, Germany), model Transsonic TI-H-5 at 100% power 25ºC for 25 min.

3.11 Protein identification

Digested proteins were analyzed by nano liquid chromatography-electrospray ionization quadrupole-time of flight MS (nano-LC-ESI-MS/MS) using an EASY-nLCII spectrometer coupled with a MicroTOF QII (Bruker, Germany). The tandem mass spectra of the tryptic peptides were searched from Mascot database (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=MIS). The precursor and MSMS tolerances were set to ±1.2 Da and ±0.6 Da, respectively (Niyomploy et al., 2014).

3.12 Protein categorization and network construction

The expressed proteins were classified and functional searched on the PIR (Protein Information Resource) (http://pir.georgetown.edu) and PANTHER (ProteinANalysis THrough Evolutionary Relationships) systems (http://www.pantherdb.org). The differentially expressed protein interaction network was built automatically by the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) system. The gene symbol list of these proteins was input to search against the database, which contain known and predicted protein-protein interactions.

3.13 Quantitative reverse transcriptase PCR (qRT-PCR) of genes expressed in maize co-inoculated with AM and *Brevibacillus* sp. strain SUT47

The experiment was designed into four treatments with 3 replicates as described in protein extraction from maize root. At 7, 14, 21 and 30 dai after inoculation, 250 mg of root from each treatment were ground with liquid nitrogen and total RNA was isolated using RNeasy Plant Mini Kit (QIAGEN, USA). The cDNA was synthesized using QuantiTect Reverse Transcription Kit (QIAGEN, USA) according to the manufacturer's instructions. The primers for reference gene *MEP* (membrane protein PB1A10.07c) and primers design for interested genes were shown in Table 3.1 based on the information of maize transcription (Manoli et al., 2012). qRT-PCR (Roche LightCycler® 480 (LC480)) system experiments were conducted (Bustamante et al., 2014). Normalized value (R) of the expression level relative to the calibrator is determined by the formula:

$$R = 2^{-\Delta\Delta Ct}$$

The proposed mathematical model, which includes the normalization with the reference gene is expressed as follows:

$$R = \frac{(E_{\text{studied gene}})^{\Delta Ct \text{ studied gene (control-sample)}}}{(E_{\text{control gene}})^{\Delta Ct \text{ studied gene (control-sample)}}}$$

where: E = amplification efficiency; ΔCt = subtract of threshold cycle designated respectively for studied or control gene in the calibration and in the samples (Pfaffl, 2001).

3.14 Antioxidant enzymes activity assays

The measurement of enzyme activities of superoxide dismutase (SOD; EC 1.15.1.1), ascorbate peroxidase (APX; EC 1.11.1.11), and peroxidase (POD, EC1.11.1.7) of maize root co-inoculated with *Brevibacillus* sp. strain SUT47 and AM were performed (Krantev et al., 2008; Muñoz et al., 2015). The 500 mg of roots from each treatment were ground to a fine powder in liquid nitrogen, with a pestle and mortar. Then, 300 µl of 50 mM potassium phosphate buffer were prepared in different pH and EDTA concentrations for each enzyme (pH 7.4 for SOD, pH 7 for APX and POD enzymatic activity, and EDTA concentration at 1, 0.5, and 0.1 mM for SOD, APX, and POD enzymatic activity, respectively), and 2% (w/v) polyvinylpolypyrrolidone (PVP) (MW. 360,000) were added. The homogenate was centrifuged at 10,000×g for 30 min at 4ºC, and the supernatant was transferred into a sterile tube and used for subsequent determination of enzymatic activities.

Table 3.1 The primers used for qRT-PCR.

Protein name (Gene)	**Primers 5'-3'**	**TM ºC**	**Size (bp)**
Pathogenesis-related protein 1 (*PR-1*)	Fw = CCTGGGTGTCCGAGAAGCA	60	133
	Rw = ACAGCCGATGGCGGTGGAGT	60	
Superoxide dismutase (*SOD_Mn*)	Fw = TTTGGAAGAACCTCAAGCCT	58	125
	Rw = GCCTTCTGCATTCATCCTCT	59	
APx1 - Cytosolic Ascorbate Peroxidase (*APX*)	Fw = TAGGGAGGACAAGCCTCAAC	59	72
	Rw = CTCAGGTGGTCAGAACCCTT	59	
Ascorbate peroxidase (*POD1*)	Fw = CAGGGAGGACAAGCCTCAG	58	63
	Rw = CAGAACCCTTAGTGGCATCA	58	
Glutathione S-transferase 1 (*GST-I*)	Fw = AGAGCTGTTGAGGGAAGGAA	56	82
	Rw = AGCGGTGTACTGGTTAGCCT	59	

3.15 The catalase production and sensitivity of *Brevibacillus* sp. strain SUT47 to H_2O_2

Brevibacillus sp. strain SUT47 was cultured in liquid LG culture medium for 24 hours at 28ºC (Thuler et al., 2003). Each 100 µl of the bacteria culture and liquid LG culture medium without bacteria cell were dropped onto a microscope slide, then 20 µl of 3% (v/v) hydrogen peroxide were dropped onto both samples. The O_2- forming foam were detected as the presence of catalase activity (Iwase et al., 2013). The sensitivity of SUT47 to H_2O_2 (Sigma-Aldrich, USA) was assessed in agar diffusion test in which a filter soaked with 5 µl of different H_2O_2 concentrations including 3%, 10%, 20%, and 30% (v/v) was placed on 24 hours-SUT47 cultured agar plate and incubated for overnight at 37ºC (Alquéres et al., 2013).

3.16 Salicylic acid (SA) concentration assay

The SA concentration in maize was measured by addition 1:1 volume of 0.1% aqueous solution of $FeCl_3$ to a suspension of maize root extracted (as described above) using a pestle and mortar in liquid nitrogen. The series of standard complex solutions (100-1000 µg/ml) were prepared using > 99% purity of SA (Sigma-Aldrich) (St. Louis, Mo., U.S.A.), and the absorbance was measured at 540 nm by spectrophotometer (Thermo Spectronic, USA) (Warrier et al., 2013).

3.17 Statistical analysis

The results presented were the means of three independent experiments. Sample variability was given as the standard deviation of the mean. The significance of differences between control and treatment mean values was analyzed by analysis of

variance (ANOVA). When confirming a statistically significant value in the F-test ($P \leq 0.05$), a post hoc test (Duncan's multiple-range test at ($P \leq 0.05$) was used as a multiple comparison procedure (Duncan, 1955) by SPSS software for WINDOWSTM,Version14.0; SPSS, Chicago, IL) (Colman and Pulford, 2011).

CHAPTER IV

RESULTS

4.1 AM spore production and root colonization could be promoted by PGPR

Each of PGPR including *Brevibacillus* sp. strain SUT47, *Pseudomonas* sp. strain SUT19 and *Bacillus* sp. strain SUT1 was separately co-inoculated with *A. tuberculata* or *C. etunicatum* to determine the effect of PGPR on AM root colonization and spore production. Co-inoculation with SUT47 significantly enhanced spore production of both *A. tuberculata* and *C. etunicatum* (approximately 3 and 2 folds, respectively) when compared with control (non-PGPR inoculation) (Figure 4.1A). SUT47 and SUT19 were significantly increase root colonization of *A. tuberculata* (2 folds) but did not showed significantly increase root colonization of *C. etunicatum* when compared with control (Figure 4.1B). SUT1 showed neither differences in root colonization nor spore production from control.

4.2 Host plant and AM species influence the spore production of AM promoted by PGPR

The different common temperate host plants using in AM propagation including sorghum, onion, and maize were tested with different AM species and co-inoculated with SUT47. It was found that *A. tuberculata* co-inoculated with

SUT47 was significantly increase spore production in all host plants when compared with non-SUT47 co-inoculation, while there was no significant difference of spore production among plant hosts (Figure 4.2A). On the other hand, *C. etunicatum* co-inoculated with SUT47 significantly increase spore production in sorghum and maize but not in onion as a host. However, using maize as a host produced higher spore number of *C. etunicatum* than that of sorghum (Figure 4.2B). Therefore, both host plant species and AM species affected the AM spore production promoted by SUT47. However, maize was selected as a host plant for further experiments.

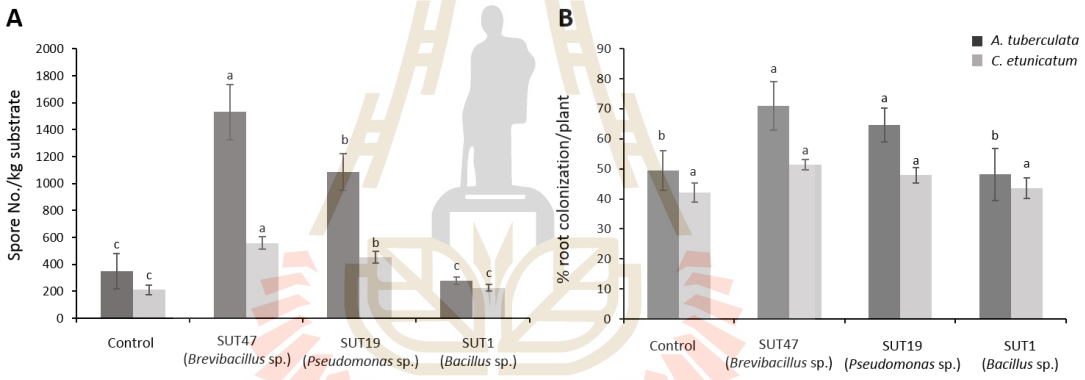

Figure 4.1 Effect of different PGPR co-inoculated with *Acaulospora tuberculata* or *Claroideoglomus etunicatum* on AM spore production in maize (A), and percentage of root colonization (B), after propagation for 90 days in maize. Values indicated with different letters in the same species of mycorrhiza were significantly different according to Duncan's multiple range test $P \leq 0.05$.

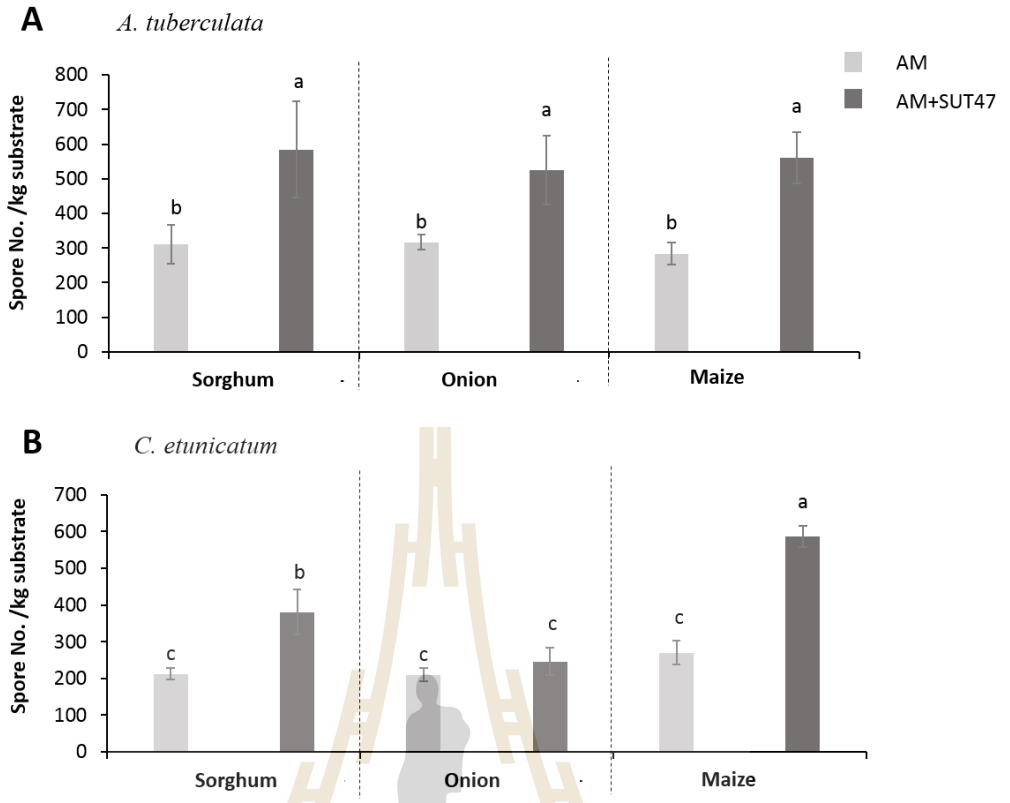

Figure 4.2 Effect of host plants (sorghum, onion, and maize) on AM spore production when co-inoculation *A. atuberculata* (A) and *C. etunicatum* (B) with and without *Brevibacillus* sp. SUT47. Values indicated with different letters were significantly different according to Duncan's multiple range test $P \leq 0.05$.

4.3 Lower concentration of plant nutrients stimulated AM root colonization but not spore production in plant co-inoculated with *Brevibacillus* sp. strain SUT47

Co-inoculation of *Brevibacillus* sp. strain SUT47 with *A. tuberculata* showed the highest spore production when treated with full strength nutrient and low phosphate Hoagland solution (FL), but the spore number was not significantly

different from thaose of maize treated with full strength (F), half strength (H) and half strength of Hoagland solution with low phosphate (HL). Nevertheless, plants treated with low phosphate significantly increased spore production when co-inoculated with SUT47 than that of control (without SUT47 co-inoculation) (Figure 4.3A). For the root colonization, co-inoculation of SUT47 with *A. tuberculata* clearly increased roots colonization of AM when treated with HL solution (Figure 4.3B). Therefore, reducing plant nutrients together with co-inoculation of SUT47 could promote the AM colonization and probably further increase the spore production in maize as a host plant.

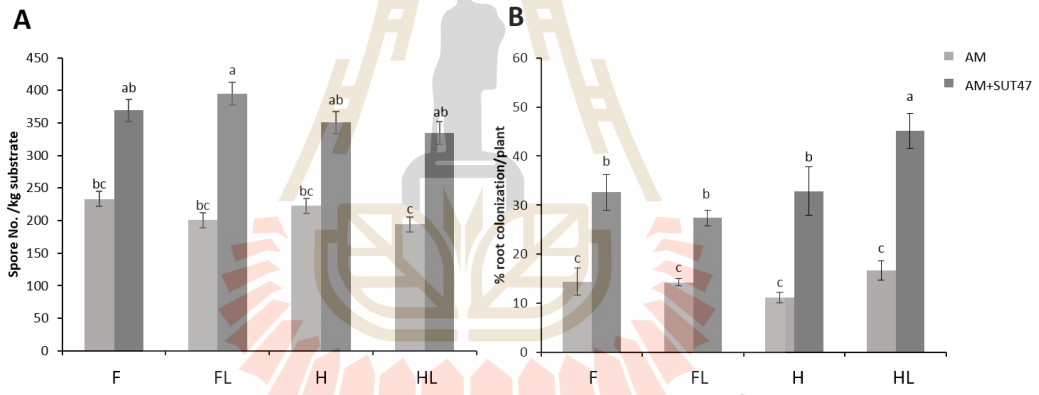

Figure 4.3 The spore production in maize (A) and percentage of root colonization (B) of *Acaulospora tuberculata* co-inoculated with and without *Brevibacillus* sp. SUT47 under different concentrations of Hoagland solutions of full strengths (F), full strengths with low-p (0.04 ppm) (FL), half strengths (H), and half strengths with low-p (0.02 ppm) (HL) after propagated for 90 days. Values followed by different letters were significantly different according to Duncan's multiple range test $P \leq 0.05$.

4.4 Maize root architecture affected by co-inoculation of *A. tuberculata* with *Brevibacillus* sp. strain SUT47

The morphology of maize root co-inoculated *A. tuberculata* with SUT47 was observed in comparison with non-SUT47 co-inoculation. The Smart Root Program was applied to determine maize root architecture. The result demonstrated that roots inoculated with SUT47 and co-inoculation of *A. tuberculata* with SUT47 performed significantly decrease in distance from the tip to first root hair but increase the root hair density when compared with plant inoculated with *E. coli* DH5alpha (none PGPR strain) and control (Table 4.1 and Figure 4.4). This result reveals the role of SUT47 on changing plant roots morphology, especially increase root hair density which may subsequently promote the chance of AM infection. According to Piromyou et al. (2011), it is possible that IAA highly produced from SUT47 would be the key role that support root architecture changes and finally promote AM root colonization and spore production.

However, although it was found that the IAA concentration at 100 ppm significantly increased AM spore production in maize, the number of spore production treated with IAA still lower than that of maize co-inoculated *A. tuberculata* with SUT47, and once the concentration of IAA increased up to 150 ppm/ml, the AM spore production did not increase (Figure 4.5). Thus, it could be implied that not only IAA, but also some other mechanisms from SUT47 facilitated AM spore production in maize root.

Table 4.1 Root architecture measurement of plant inoculated with AM and SUT47.

Root Data	Values					
	Control	**AM**	***E. coli***	**SUT47**	**AM+ *E. coli***	**AM+ SUT47**
Primary root						
Growth rate (cm d^{-1})	2.54±0.35	3.35±0.08	3.03±0.15	2.83±0.03	2.83±0.19	3.00±0.38
Diameter (cm)	0.076±0.013	0.085±0.004	0.073±0.007	0.077±0.003	0.082±0.010	0.084±0.004
Branching density (LR cm^{-1})	3.29±0.25	2.75±0.53	2.67±0.66	3.73±0.81	2.94±1.11	3.12±0.44
LAUZ (cm)	10.19±1.92	12.37±0.63	11.09±1.27	8.79±1.79	10.75±0.70	10.05±2.62
Seminal root						
Growth rate (cm d^{-1})	$2.11±0.25^{ab}$	$3.02±0.14^{a}$	$2.32±0.28^{ab}$	$1.91±0.37^{b}$	$2.27±0.25^{ab}$	$2.86±0.29^{a}$
Diameter (cm)	$0.046±0.006^{b}$	$0.069±0.007^{ab}$	$0.070±0.002^{ab}$	$0.069±0.004^{ab}$	$0.049±0.021^{b}$	$0.090±0.018^{a}$
Lateral root						
Growth rate (cm d^{-1})	0.143±0.035	0.172±0.050	0.100±0.053	0.340±0.235	0.223±0.063	0.184±0.047
Diameter (mm)	$0.008±0.005^{cd}$	$0.26±.005^{c}$	$0.053±.003^{ab}$	$0.004±.001^{d}$	$0.047±.007^{b}$	$0.068±0.012^{a}$
Insertion angle (˚)	$65.48±0.107^{ab}$	$75.50±1.25^{a}$	$60.87±3.38^{b}$	$70.19±4.49^{ab}$	$60.76±4.85^{b}$	$73.79±6.75^{ab}$
Root hair						
Distance (µm)	$532.00±67.78^{a}$	$518.00±52.48^{a}$	$438.00±60.94^{ab}$	$286.00±44.68^{b}$	$432.00±83.63^{ab}$	$276.00±39.06^{b}$
Length (µm)	$252.00±43.17^{ab}$	$224.20±31.36^{b}$	$194.00±31.08^{b}$	$333.00±29.82^{a}$	$264.60±41.34^{ab}$	$337.60±19.41^{a}$
Density (no. μm^{-1})	$0.014±0.00075^{b}$	$0.017±0.00135^{ab}$	$0.014±0.00158^{b}$	$0.018±0.00175^{a}$	$0.013±0.00120^{b}$	$0.021±0.00148^{a}$

Mean and standard deviation of two independent experiments with five replicates per experiment are presented. Value followed by different letters in the same column indicate significant differences among treatments ($P \leq 0.05$). LR = Lateral Root, LAUZ = Leangth of the Apical Unbranching Zone, Distance = Tip to the first root hair distance.

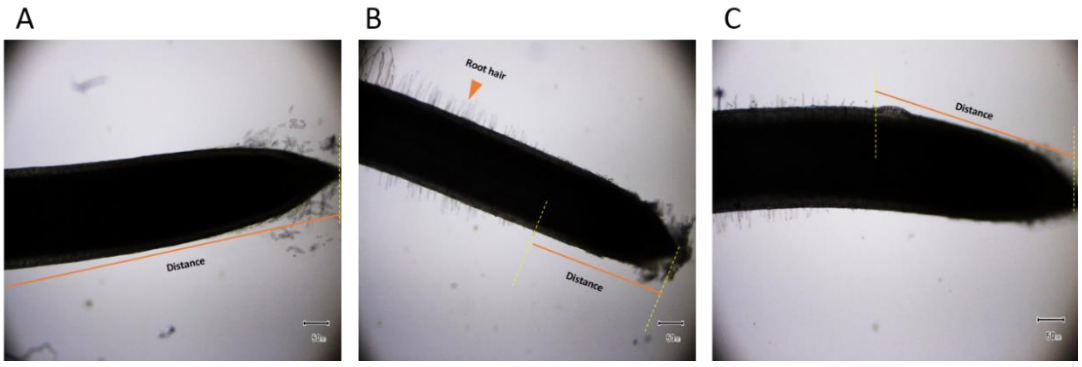

Figure 4.4 The effect of *Brevibacillus* sp. SUT47 on maize root architecture. (A) non-inoculation, (B) plant inoculated with *Brevibacillus* sp. SUT47, and (C) plant inoculated AM with *Brevibacillus* sp. SUT47. Distance = Tip to the fist root hair distance.

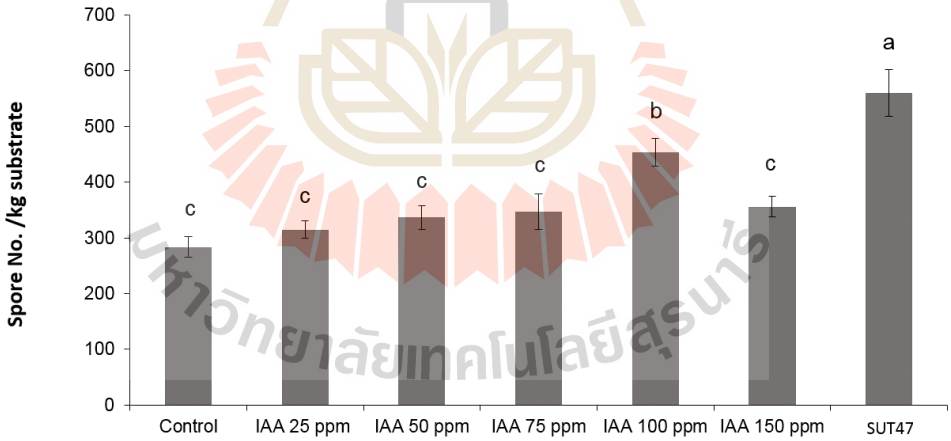

Figure 4.5 The effect of different concentrations of IAA on *Acaulospora tuberculata* spore production and compared with co-inoculated with *Brevibacillus* sp. SUT47. Values indicated with different letters were significantly different according to Duncan's multiple range test $P \leq 0.05$.

4.5 Maize protein profile altered by *A. tuberculata* co-inoculated with *Brevibacillus* sp. strain SUT47

The 2DE images of proteins extracted from control maize, maize with AM, and maize with AM+SUT47 were presented in Figure 4.6. The protein maps from control maize, maize with AM, and maize with AM+SUT47 were compared using ImageMaster 2D platinum software to identify the protein spot variations. The significant differentially expressed protein spots ($P < 0.05$) with 2-fold or more decreased or increased intensity when compared with control maize were selected. Totally, 23 up-regulated protein spots and 9 down-regulated protein spots were found in maize inoculated with AM alone treatment, while 16 up-regulated protein spots and 16 down-regulated protein spots were found in maize co-inoculated with AM+SUT47. However, only 15 spots were selected for further protein identification based on criteria: (i) spots must be appeared or disappeared in all 3 independent experiments when compared with control; (ii) spots showed high or low density more than control maize; (iii) show high or low density less then control maize. The 2D Image of identified proteins, the biological process, and fold changes ratio of each protein were presented in Figure 4.6A-C and Table 2. The PANTHER classification system classifying proteins according to their functional properties was used to understand the biological relevance of the differentially expressed maize proteins in response to AM and AM+SUT47 inoculation. According to their ontology and biological process, these proteins could be classified into 3 groups (i) plant defense response; (ii) ROS-scavenging enzyme, and (iii) Energy metabolism (Table 4.2). Protein spots involved in plant defense response and ROS-scavenging enzyme including (i) up regulated protein in AM maize root: spot number 0 (Sb07g006390); (ii) up regulated protein in

AM+SUT47 maize root: spot number 1 (PR1); (iii) up regulated protein in both AM and AM+SUT47 maize root: spot number 2 (SOD_Mn) and 5 (APX); (iv) down regulated protein in both AM and AM+SUT47 maize root: spot number 8 (POD1), 20 (GST-I), and 28 (APx-I) were interesting proteins and the expanded regions differentially expressed were displayed in Figure 4.6D.

Thirteen identified proteins were analyzed the protein-protein interaction network using STRING system (Figure 4.7). These protein networks showed the different protein-protein interactions among interested proteins involved in plant defense response and reactive oxygen species correlated with protein in the energy metabolism group.

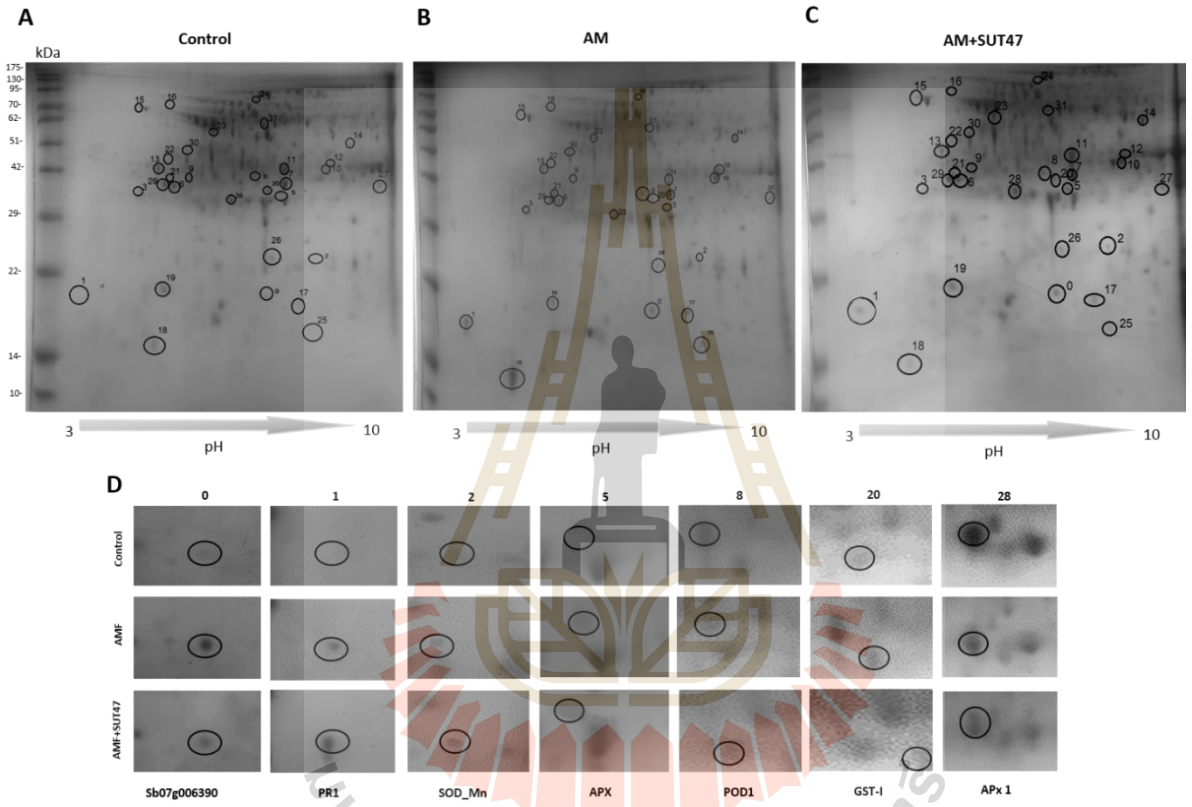

Figure 4.6 The representative 2DE images of maize root (control) (A); maize root inoculated with *Acaulospora tuberculata* (B); maize root co-inoculated with *Acaulospora tuberculata* and *Brevibacillus* sp. strain SUT47 (C); and the expanded regions of selective differentially expressed protein spots (D). Spot number referred to spot identified in Table 4.2.

Table 4.2 Identified proteins of maize root extracts differentially expressed after the inoculation with AM and SUT47.

Spot No.	Top hit protein	Gene symbol	Protein Ontology/ Biological Process	Organisms	No. of peptides matched	Mascot score	Mass (kDa)	PI	Fold change ratio [a]	
									AM	AM + SUT47
	Plant defense response									
0	Putative uncharacterized protein	Sb07g006390	Hydro-lyase activity / defense response to fungus	Sorghum	6	223	24.5	9.27	44.66±0.67	3.00±0.13
1	Pathogenesis-related protein 1	PR1	Cellular component	*Zea mays*	1	55	15.3	4.38	5.66±0.12	36.00±0.15
15	DIMBOA UDP-glucosyltransferase BX9	BX9	Transferase activity	*Zea mays*	4	255	50.3	5.15	5.33±0.33	21.66±0.74
	ROS-scavenging enzyme									
2	Superoxide dismutase	SOD_Mn	Superoxide dismutase activity	*Zea mays*	10	466	25.6	6.71	24.33±0.33	31.60±0.67
5	Ascorbate peroxidase	APX	Peroxidase activity	*Zea mays*	15	524	27.5	5.67	28.00±0.21	31.33±0.33
8	Ascorbate peroxidase	POD1	Peroxidase activity	*Zea mays*	15	571	27.5	5.64	-8.66±0.30	-8.33±0.23
20	Glutathione S-transferase 1	GST-I	Glutathione transferase activity	*Zea mays*	9	339	24.0	5.44	-10.00±0.46	-13.70±0.08
28	APx1 - Cytosolic Ascorbate Peroxidase	APx1	Peroxidase activity/response to oxidative stress	*Zea mays*	12	455	27.5	1.03	-2.00±0.08	-
	Energy metabolism									
7	Lactoylglutathione lyase	GlxI	Lactoylglutathione lyase activity	*Zea mays*	17	684	35.3	6.62	18.00±0.17	19.66±0.20
12	Malate dehydrogenase	MDH	Malate dehydrogenase activity	*Zea mays*	4	268	35.7	7.63	5.66±0.77	12.66±0.17
30	Glutamine synthetase	Gln4	Catalytic activity	*Zea mays*	8	342	39.6	5.24	-8.33±0.18	-11.00±0.08
21	Putative actin family protein isoform 1	Actin	ATP binding	*Zea mays*	13	477	42.0	5.24	-8.99±0.31	-
19	Chaperonin	Cpn	ATP binding	*Zea mays*	7	364	25.8	7.72	15.66±0.28	-
6	Triosephosphate isomerase	TPI	Isomerase activity	*Zea mays*	5	223	26.9	5.12	5.66±0.16	15.33±0.13
18	Uncharacterized protein	-	Transferase activity	*Zea mays*	5	284	19.5	5.14	32.66±0.63	-

[a] The fold change ratio of one protein was obtained from the protein abundance ratios between the un-inoculated and inoculated treatments.

± The standard deviation of protein ratios of the spot. - : vanished protein.

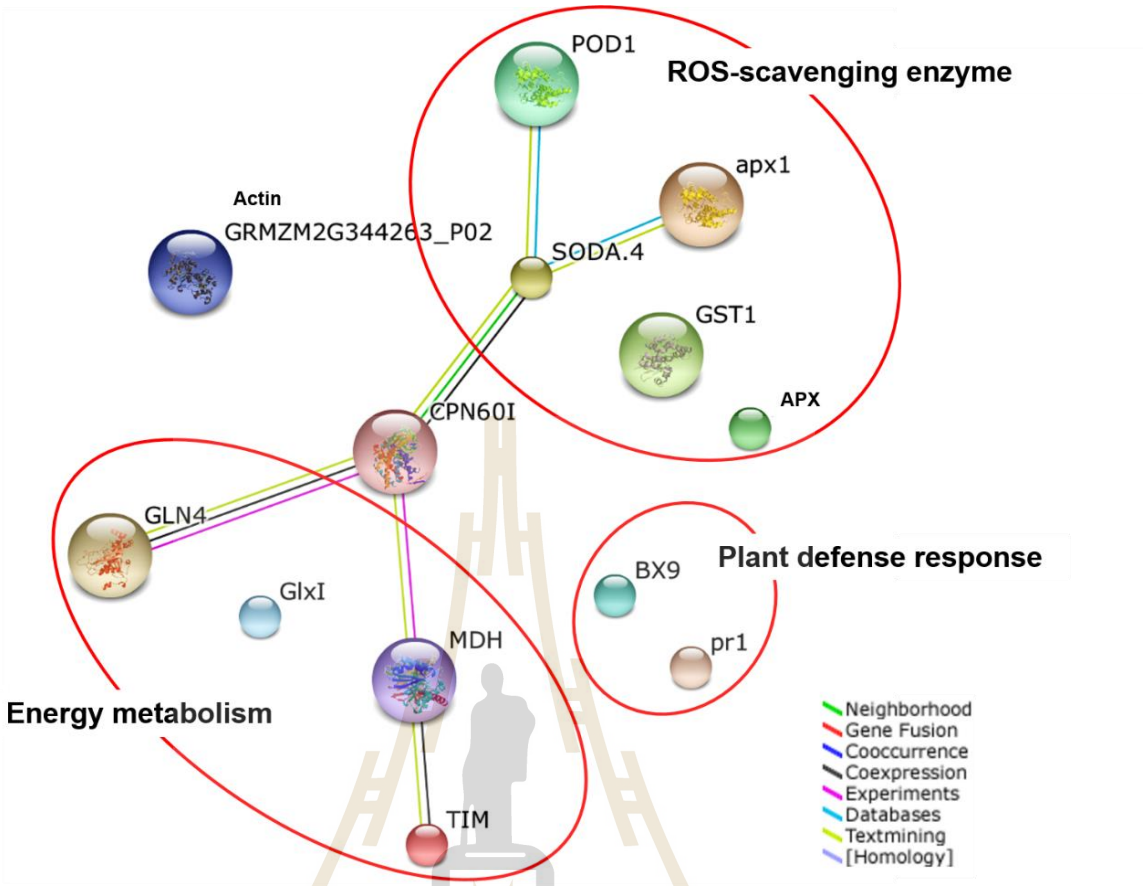

Figure 4.7 The protein-protein interaction network of the identified differentially expressed proteins. The network containing 13 identified differentially expressed proteins was mapped using the STRING system based on evidence with different types. The links between proteins represent possible interaction. Different line colors represent the types of evidence for the associations, which are shown in the legend.

4.6 *Brevibacillus* sp. strain SUT47 proteins secretion profile altered in response to maize root exudates

The identified secretion proteins from SUT47 were presented in Table 4.3. The significant differentially expressed protein spots ($P < 0.05$) with decreased or increased intensity when compared with control were selected. Totally, 11 up-regulated protein spots and 9 down-regulated protein spots were found in the SUT47 proteins secretion when responded to 7-day root exudates. Some interesting proteins secretion from SUT47, such as the effector protein elongation factor TU and the flagellin protein were presented in response to 7-day maize root exudates.

Table 4.3 Identified differentially expressed proteins of SUT47 protein secretion at 7 dai.

Spot No.	Top hit protein	Gene ontology/ Biological Process	Organisms	Mascot score	Mass	Peptide matched	PI	FD[a]
1	enamine deaminase RidA	deaminase activity	*Brevibacillus* sp.	74	13554	3	5.03	-0.21±0.08
7	molecular chaperone GroES	ATP binding	*Brevibacillus* sp.	137	10355	5	4.92	-0.59±0.03
23	elongation factor Tu	translational elongatio	*Brevibacillus* sp.	307	43054	15	5.18	0.82±0.07
25	50S ribosomal protein L25	RNA binding	*Brevibacillus* sp.	117	21245	5	5.97	1.18±0.02
49	acetylglutamate kinase	kinase activity	*Brevibacillus* sp.	58	31754	3	5.10	-0.19±0.07
54	C4dicarboxylate ABC transporter	transport	*Brevibacillus* sp.	150	42316	5	9.11	-0.05±0.006
55	4hydroxytetrahydrodipicolinate synthase	catalytic activity	*Brevibacillus* sp.	76	32210	8	5.24	-0.20±0.03
58	chromosome partitioning protein ParB	DNA binding	*Brevibacillus* sp.	150	33817	3	6.36	0.45±0.05
64	cytochrome-c peroxidase	oxidation-reduction	*Brevibacillus* sp.	174	36216	3	5.41	-0.58±0.07
83	flagellin	structural molecule activity	*Brevibacillus* sp.	240	37712	18	4.70	-3.22±0.04
86	acetyl CoA acetyltransferase	catalytic activity	*Brevibacillus* sp.	144	40825	9	5.84	-0.31±0.01
102	membrane protein	integral component of membrane	*Brevibacillus* sp.	360	53056	23	5.75	0.20±0.02
106	serine protease	peptidase activity	*Brevibacillus* sp.	698	52504	13	5.46	0.05±0.003
108	methylmalonatesemialdehyde dehydrogenase	oxidation-reduction process	*Brevibacillus* sp.	84	54178	3	5.80	0.05±0.007
117	ABC transporter substratebinding protein	polyamine binding	*Brevibacillus* sp.	79	58520	7	6.06	0.29±0.02
120	glutamine synthetase	nitrogen fixation	*Brevibacillus* sp.	420	52367	10	5.22	0.03±0.003
124	acyl CoA dehydrogenase	acyl-CoA dehydrogenase activity	*Brevibacillus* sp.	171	64879	21	5.34	0.19±0.01
127	30S ribosomal protein S1	translation	*Brevibacillus* sp.	166	62562	21	4.99	-0.07±0.004
133	biotin carboxylase subunit of acetyl CoA carboxylase	catalytic activity	*Brevibacillus* sp.	95	72720	8	5.30	0.19±0.03
134	hypothetical protein	-	*Brevibacillus* sp.	58	74083	1	5.93	0.18±0.02

[a] The fold change ratio of one protein was obtained from the protein abundance ratios between the un-inoculated and inoculated treatments. - : vanished protein.

4.7 Validation of protein expression level by qRT-PCR

The qRT-PCR experiments were performed to verify the expression of selected candidated proteins from proteomic studies, including SOD, POD, APX, GST, and PR1 which were the proteins involving in scavenging reactive oxygen species and plant defense response. Figure 4.8 displayed the results of qRT-PCR analyses of genes in maize at different times after AM or AM+SUT47 inoculation. At 7 dai, the gene expression level of defense-related proteins (*PR1*) and scavenging reactive oxygen species (*SOD*, *POD*, *APX*, and *GST*) were up-regulated in maize inoculated with AM alone, while these genes were down-regulated in maize co-inoculated AM with SUT47 (Figure 4.8A). At 14 dai, the expression level of *POD*, *APX*, and *GST* were decreased in both treatments whereas *SOD* and *PR1* were increased. *PR1* was the highest expressed in maize inoculated with AM alone and the expression was higher than that of maize co-inoculated AM with SUT47, while SOD was also expressed in lower level in both maize inoculated with AM and co-inoculated AM with SUT47 (Figure 4.8B). However, all tested genes were down regulated at 21 dai (Figure 4.8C). Interestingly, all tested genes especially *PR1* in maize co-inoculated AM with SUT47 were up regulated at 30 dai, while these genes were down regulated in maize inoculated with AM alone (Figure 4.8D).

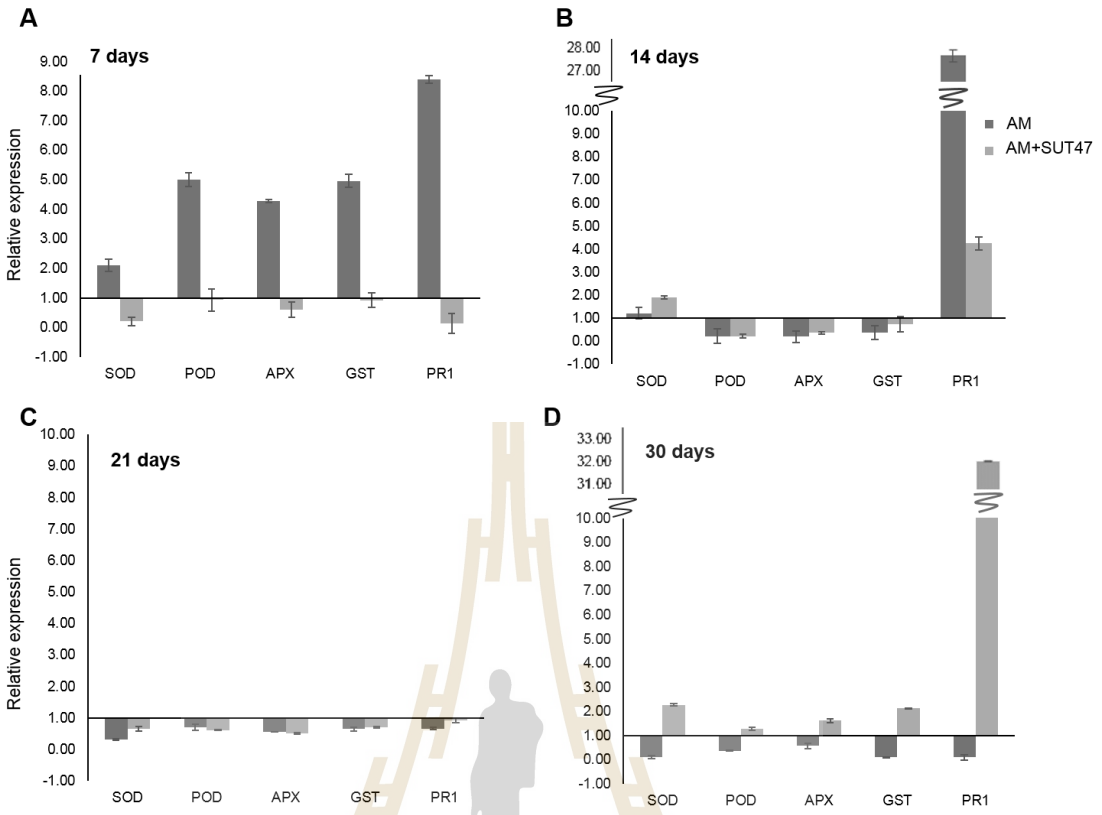

Figure 4.8 Expression analysis of *SOD*, *POD*, *APX*, *GST*, and *PR1* using qRT-PCR in response to AM and AM+SUT47 at 7 (A), 14 (B), 21 (C), and 30 (D) dai. The results were shown in relative expression of the target genes normalized with reference gene *MEP* in each day after inoculation.

4.8 Antioxidant enzymes activities and content of salicylic acid of maize were altered after co-inoculation with *Brevibacillus* sp. strain SUT47

The results of gene expression were confirmed by antioxidant enzyme activity measurement. SOD activity was produced in high level at 7 dai in the roots of maize from all treatments. Maize inoculated with AM alone showed the highest SOD

activity, while this enzyme activity was significant lower when SUT47 was co-inoculated with AM into maize root. Then, the SOD activity decreased more than 10 folds at 14, 21, and 30 dai (Figure 4.9A). On the other hand, the APX activity started to produce in root maize at 14, 21, and 30 dai. The un-inoculated plant tended to reduce the level of APX activity at 14, 21, and 30 dai, while the APX activity tended to increase in maize inoculated with AM alone or AM+SUT47. However, the APX activity of maize co-inoculated AM+SUT47 was significant lower than APX activity in maize inoculated with AM alone at 14 and 21 dai. Interestingly, the APX activity of maize co-inoculated AM+SUT47 was significantly increased than other treatments at 30 dai (Figure 4.9B). Changing of POD activity among the treatments was found in maize root at 7, 21, and 30 dai. At 7 and 30 dai, the POD activity was significantly increased in maize co-inoculated AM+SUT47, while at 21 dai POD activity was significantly increased in maize inoculated with AM alone (Figure 4.9C).

Salicylic acid (SA) production was tested to determine the plant immune response in correlation with PR1 function. At 7 and 21 dai, the un-inoculated maize root significantly showed higher SA concentration than maize inoculated with AM or AM+SUT47. However, the SA concentration of maize inoculated with AM+SUT47 was significant lower than SA from maize treated with AM alone. Then, AM inoculated maize tended to accumulate SA in high level at 30 dai, while the SA concentration in maize co-inoculated AM+SUT47 was significant lower than other treatments (Figure 4.9D). Moreover, the detection of catalase activity was shown in SUT47 culture (Figure 4.10A) and SUT47 was not sensitive to H_2O_2 (Figure 4.10B). These results revealed some clues that co-inoculation SUT47 with AM altered the

plant immune response in plant. The interaction between maize, AM, and SUT47 were proposed (Figure 4.11) and explained in discussion.

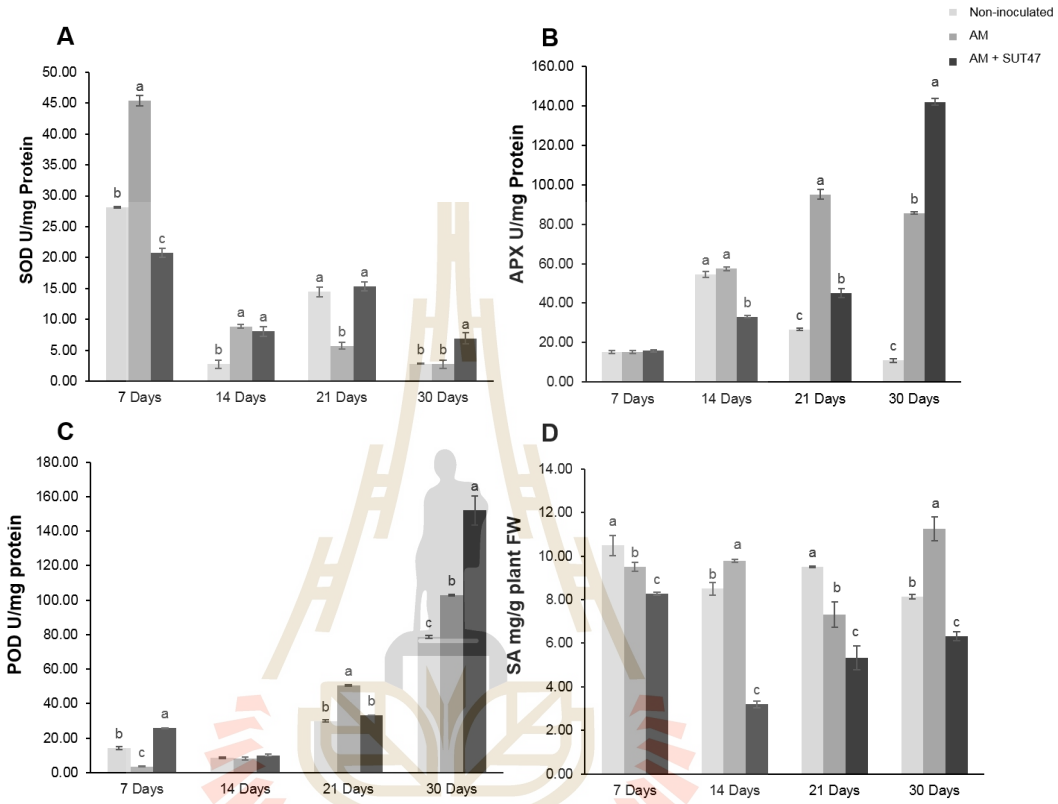

Figure 4.9 Enzymatic activity of *SOD* (A), *APX* (B), *POD* (C), and salicylic acid (SA) analysis (D) of non-inoculated plant, plant inoculated with AM, and plant co-inoculated AM+SUT47 at 7, 14, 21, and 30 dai. Values indicated with different letters in the same day were significantly different according to Duncan's multiple range test $P \leq 0.05$.

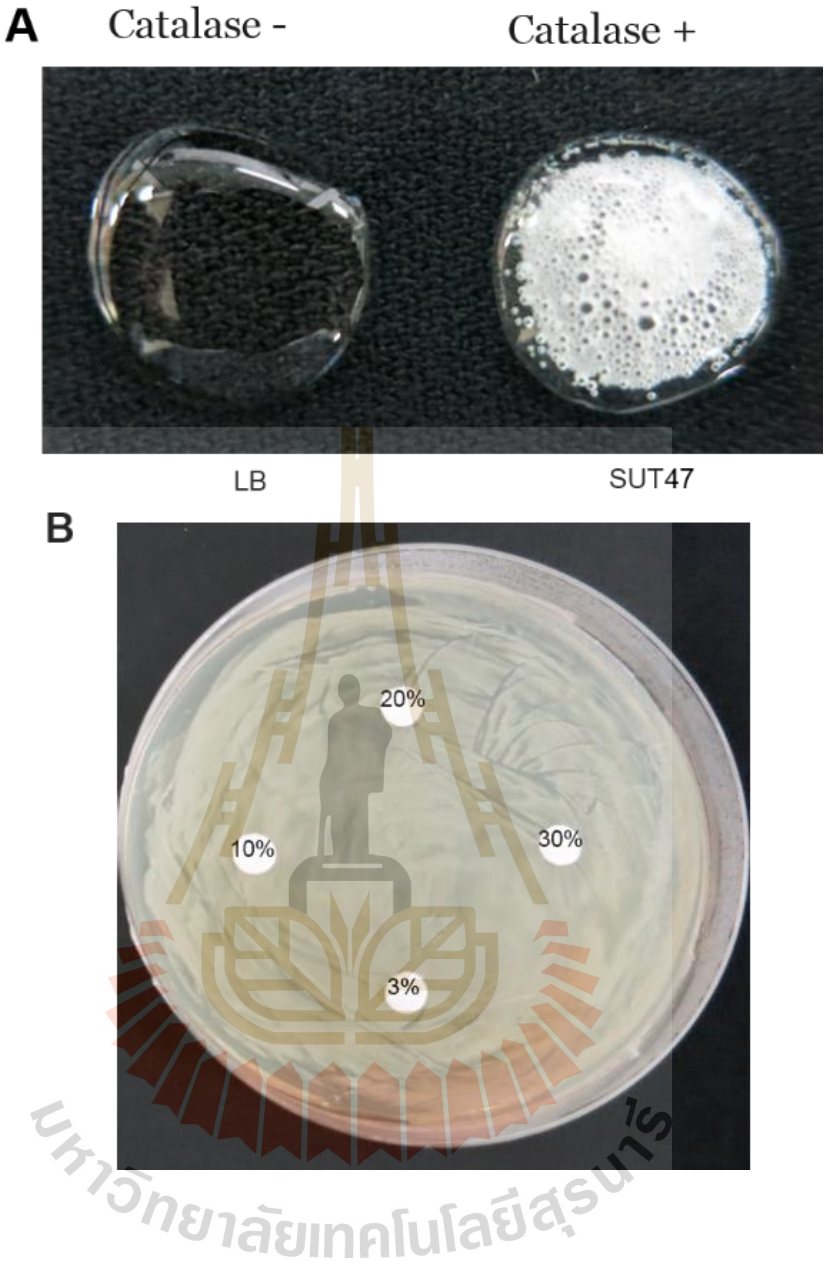

Figure 4.10 The detection of catalase (A) and sensitivity of SUT47 to H_2O_2 was assessed in agar diffusion test in which a filter soaked with 10 µl of H_2O_2 in (3, 10, 20, and 30% [vol/vol]) were placed on SUT47 LB agar incubated overnight at 28ºC (B).

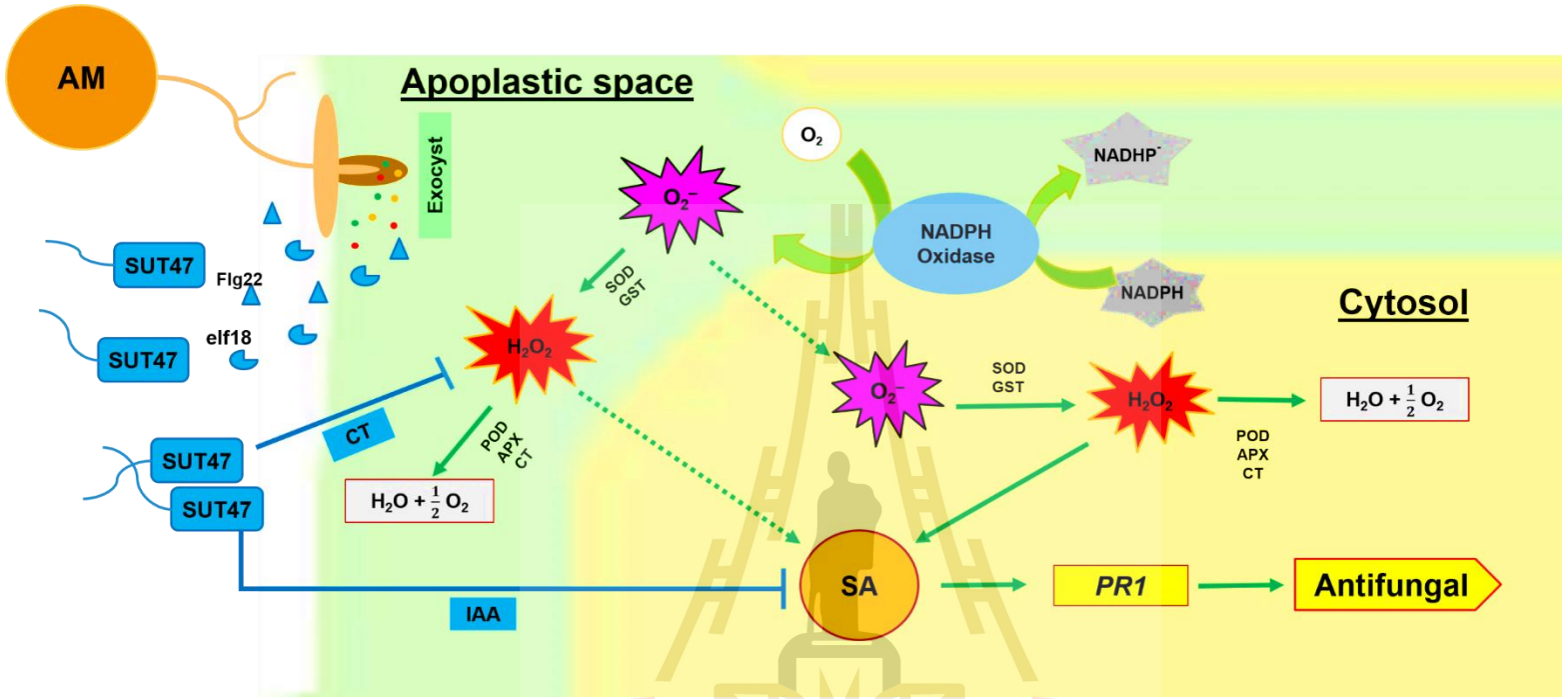

Figure 4.11 The proposed mechanism of *Brevibacillus* sp. SUT47 to promote AM during infection and penetration stage by produce ROS-scavenging enzyme to suppress innate plant immunity. IAA and elicitors produced from SUT47 may also inhibit of SA mediated PR1signaling pathway and activate pre-penetration of AM, respectively. SOD = Superoxide dismutase, POD = Peroxidase, GST = Glutathione transferase, APX = Peroxidase activity, CT = Catalase, NADPH = Nicotinamide adenine dinucleotide phosphate, Flg22 = flagellin, elf18 = elicitors elongation factor TU, SA = Salicylic acid, and PR1 = Pathogenesis-related protein 1.

CHAPTER V

DISCUSSION

Even though many studies have provided evidences of plant growth promotion supported by either PGPR or AM via their different properties in last decades, ability of PGPR as Mycorrhization Helper Bacteria (MHB) has still ambiguous. This study focused on elucidation the role of PGPR as MHB on enhancing AM spore production. It was found that three main factors, including PGPR strains, AM species and host plant species influence on AM spore production. PGPR containing N-fixation, P- and K-solubilization abilities have been reported to promote sporulation and AM root infection rate (Singh, 1992; Wu et al., 2005). Nevertheless, not all PGPR strains promote AM spore production in our study, although the species of *Pseudomonas* and *Bacillus* were found to promote mycorrhiza as MHB (Wu et al., 2012; Labbé et al., 2014). On the other hand, IAA has been reported to affect strigolactones involved in *Pisum sativum*-mycorrhizal symbiosis (Foo, 2013). Since *Brevibacillus* sp. strain SUT47 could produce IAA higher than other tested PGPR strains in this study (Piromyou et al., 2011), the level of IAA production by SUT47 might be another factor to promote AM root colonization and spore production. Moreover, limitation of nutrient could also promote AM root colonization and spore production (Millner and Kitt, 1992). Our result showed that low level of phosphate stimulated AM root colonization. It was proposed that plant may access to phosphorus by allowing more AM colonization. As reported by Sylvia et al. (2003), the spore production and

colonization ability also depends on the genetic control of the host and the AM fungus. However, our study indicates a complex interaction of all symbiotic partners including AM, PGPR, and host plants have influence on AM spore production.

As mentioned above, SUT47 can produce high concentration of IAA. Ludwig-Müller and Güther, (2007) suggested that an increase of auxins in mycorrhizal plant could be a signals that enable AM colonization process. Change in root architecture resulting from IAA also contributed to increase AM infection areas (Gamalero et al., 2004; Wang et al., 2011). Therefore, IAA produced from SUT47 may promote early root hair production that increase root hair density and subsequently enhance AM spore production. In our experiment, optimal dose of purified IAA is required to promote AM spore production. Gryndler et al. (1998) also reported that too high concentration of IAA suppressed *Glomus fistulosum* hyphal growth. Interestingly, even though the level of IAA produced by SUT47 was in the optimal range of 75-100 ppm, AM spore production in maize inoculated with SUT47 was still higher than that of maize treated with purified IAA. This result implies that IAA is not the main mechanism for supporting AM spore production.

The proteomics analysis of plant interacted with AM and AM+SUT47 preliminary demonstrated that some proteins involved in plant defense response, ROS-scavenging enzymes and energy metabolism which played important roles in symbiosis. During the infection and penetration stage, plant inoculated with AM+SUT47 produced ROS-scavenging enzymes (SOD, POD, and APX) and plant defense response (PR1) lower than that of plant inoculated with AM alone. It has been reported that ROS, such as superoxide anion (O_2^-), hydroxyl radical (OH^-), and hydrogen peroxide (H_2O_2) were generated in pathogenic fungi infecting plant

resulting in interference of plant cellular homeostasis due to oxidative stress (Salzer et al., 1999). It was indicated that plant response to the stress occurred from invaders by ROS accumulation. On the other hand, ROS-scavenging enzymes, such as POXs, APXs, and GSTs were accumulated higher in mycorrhizal roots than nonmycorrhizal roots (Campos-Soriano et al., 2010). In the same way, ROS-scavenging enzyme accumulation was found in AM plant more than that of plant inoculated with AM+SUT47, it is implied that the level of stress during AM infection and penetration stage was reduced when co-inoculation with SUT47. This result was confirmed by the strongest activity of SOD in AM plant which response to oxidative stress by catalyzing O_2^- to H_2O_2 during infection stage, while SOD activity of plant inoculated with AM+SUT47 was in lower level. At later stage during mycorrhizal symbiosis, although the low expression of genes encoded for ROS-scavenging enzymes was observed, the fluctuation of these enzyme activities in all plants was still detected. These enzyme activities may be responsible for controlling ROS generally produced from various metabolic pathways as byproduct during plant development (Heyno et al., 2011). ROS are also generated at high level in plant cell containing arbuscule to initiate the fungal program for senescence or lead to arbuscular degradation (Fester and Hause, 2005) and since the life-span of arbuscule is only 4-15 days (Goltapeh et al., 2008), the fluctuation of ROS scavenging enzyme activities in both AM and AM+SUT47 plant may be detected in higher level than non-inoculated plant.

Furthermore, *PR1* was another gene, which expressed in AM plant higher than plant co-inoculated with SUT47 during infection and penetration stage. Torres et al. (2006) proposed that ROS are one of messengers triggering salicylic acid (SA) and resulting in mediating plant defensive responses. It has been reported that SA induces

the expression of systemic acquired resistance (SAR) and *PR* gene (Cao et al., 1997). Lower level of SA concentration was correlated with the lower expression of *PR1* in AM plant co-inoculated with SUT47. Since PR1 protein has been reported as an indicator of the plant defense response which have antifungal property (Mitsuhara et al., 2008; Sels et al., 2008), therefore, SUT47 would somehow involves in retard plant defense system during AM infection and penetration. Although enhancing of SA level in plants could delay AM root colonization, this activity does not affect the symbiotic potential (Cao et al., 1997). Thus, AM still successfully colonize plant in both treatments (AM and AM+SUT47 plants) at later stage of symbiosis. However, increase of ROS-scavenging enzymes and *PR1* expression in AM+SUT47 at 30 dai was observed. It has been reported that the colonization of AM once reach its peak, large number of vesicles and arbuscules were accumulated. Besides, extension of hyphe from the plant root surface to infect neighboring roots within the roots was detected (Song et al., 2015). Therefore, it is possible that AM+SUT47 plant may also contain high quantity of mycelium earlier than AM plant, then the new infection of neighboring roots may subsequently activate plant defense responses.

The role of IAA production by SUT47 may be one of factors that play a role in interference the pathway of SA signaling. Since, it has been reported that IAA suppresses the expression of *PR1* through the SA-mediated defenses pathway (Wang et al., 2007; Mutka et al., 2013). Moreover, the catalase (CT) activity was also found in SUT47 and this strain was not sensitive to H_2O_2 (Figure 4.10). PGPR could produce catalase to alleviate the oxidative damage from plant stress (Sen and Chandrasekhar, 2015). Similarly, SOD and glutathione reductase (GR) produced from *G. diazotrophicus* strain PAL5 play an important role in suppression of ROS and

increased endophytic colonization of rice plants (Alquéres et al., 2013). These results suggested that SUT47 may facilitate the AM infection through the reduction of ROS produced in plant apoplastic space during early stage of interaction.

The proteomics analysis of SUT47 secretion protein (Table 4.3) when induced with root exudates showed two important proteins including flagellin (*flg22*) and elicitors elongation factor TU (*elf18*) that may be participated at the AM pre-penetration site. It has been proposed that the host defense proteins do not contact the fungal cell due to AM morphophysiological modifications in the host plant occur during AM symbiosis (Balestrini and Bonfante, 2005), and the trafficking plasma membrane protein, exocytosis, and secretory pathway are also required in the AM penetration site (Genre et al., 2012; Leborgne-Castel and Bouhidel, 2014). In AM-*Arabidopsis* interaction, the perifungal membrane proliferation process of AM also required exocyst complex protein takes place ahead of penetrating and intracellularly growing hyphal tips (Genre et al., 2012). Exocyst subunits, including EXO70B2, H1, H2, and H7 were found to be induced by bacterial elicitor, such as flg22 and elf18 (Pečenková et al., 2011). This result might be correlated with the presence of these elicitors from SUT47 secreted protein that assist the perifungal membrane biogenesis of AM in plant cell. The roles of SUT47 on promoting AM early infection and penetration were proposed in Figure 4.11. However, the proteins proposed in this model may not cover all of proteins involved in the tripartite interaction during symbiosis due to the limitation of this proteomic approach, which the loss of some low abundance proteins could be happened. Besides, the mycorrguzal protein profile directly involved in this tripartite interaction should also be further investigated.

In conclusion, this study demonstrated the using of *Brevibacillus* sp. SUT47 as MHB to doubling the AM spore production in the substrate-based production systems. The mechanisms by which SUT47 may use for promoting spore production are (i) the suppression of ROS by increasing the ROS-scavenging enzyme, (ii) retarding of SA signaling pathway through IAA production, and (iii) supporting AM pre-penetration via elicitors secreted from SUT47. Therefore, the suppression of plant immunity by the function of SUT47 would be the main mechanism of increasing AM spore production in plant.

REFERENCES

REFERENCES

Abbasi, Hisamuddin, Akhtar, A., and Sharf, R. (2015). Vesicular arbuscular mycorrhizal (VAM) fungi: A tool for sustainable agriculture. **American Journal of Plant Nutrition and Fertilization Technology.** 5: 40-49.

Adesemoye, A. O., and Kloepper, J. W. (2009). Plant-microbes interactions in enhanced fertilizer-use efficiency. **Applied Microbiology and Biotechnology.** 85(1): 1-12.

Alquéres, S., Meneses, C., Rouws, L., Rothballer, M., Baldani, I., Schmid, M., and Hartmann, A. (2013). The bacterial superoxide dismutase and glutathione reductase are crucial for endophytic colonization of rice roots by *Gluconacetobacter diazotrophicus* PAL5. **Molecular Plant-Microbe Interactions.** 26(8): 937-945.

Amiour, N., Recorbet, G., Robert, F., Gianinazzi, S., and Dumas-Gaudot, E. (2006). Mutations in DMI3 and SUNN modify the appressorium-responsive root proteome in arbuscular mycorrhiza. **Molecular Plant-Microbe Interactions.** 19(9): 988-997.

Balestrini, R., and Bonfante, P. (2005). The interface compartment in arbuscular mycorrhizae: A special type of plant cell wall. **Plant Biosystems-An International Journal Dealing with All Aspects of Plant Biology.** 139(1): 8-15.

Barea, J. M., Azcón, R., and Azcón-Aguilar, C. (2002). Mycorrhizosphere interactions to improve plant fitness and soil quality. **Antonie van Leeuwenhoek.** 81(1): 343-351.

Barea, J. M., Pozo, M. J., Azcón, R., and Azcón-Aguilar, C. (2005). Microbial co-operation in the rhizosphere. **Journal of Experimental Botany.** 56(417): 1761-1778.

Bertrand, H., Nalin, R., Bally, R., and Cleyet-Marel, J. C. (2001). Isolation and identification of the most efficient plant growth-promoting bacteria associated with canola (*Brassica napus*). **Biology and Fertility of Soils.** 33(2): 152-156.

Bhowmik, S. N., and Singh, C. S. (2004). Mass multiplication of AM inoculum: Effect of plant growth-promoting rhizobacteria and yeast in rapid culturing of *Glomus mosseae*. **Current Science.** 86(5): 705-709.

Blee, K., and Anderson, A. (2000). Defense responses in plants to arbuscular mycorrhizal fungi. In G. K. Podila and D. D. Douds (eds.). **Current advances in mycorrhizae research** (pp. 27-44). APS Press, St. Paul: Minn.

Blilou, I., Bueno, P., Ocampo, J. A., and García-Garrido, J. M. (2000). Induction of catalase and ascorbate peroxidase activities in tobacco roots inoculated with the arbuscular mycorrhizal *Glomus mosseae*. **Mycological Research.** 104(06): 722-725.

Boulois, D., Dupré, H., Voets, L., Delvaux, B., Jakobsen, I., and Declerck, S. (2006). Transport of radiocaesium by arbuscular mycorrhizal fungi to *Medicago truncatula* under in vitro conditions. **Environmental Microbiology.** 8(11): 1926-1934.

Brundrett, M. C., and Abbott, L. K. (1991). Roots of jarrah forest plants. I. Mycorrhizal associations of shrubs and herbaceous plants. **Australian Journal of Botany.** 39(5): 445-457.

Bustamante, M., Jin, J., Casagran, O., Nolan, T., and Riechmann, J. L. (2014). Gene expression analysis by quantitative real-time PCR for floral tissues. **Flower Development.** 1110: 363-382.

Campos-Soriano, L., García-Garrido, J. M., and Segundo, B. S. (2010). Activation of basal defense mechanisms of rice plants by *Glomus intraradices* does not affect the arbuscular mycorrhizal symbiosis. **New phytologist.** 188(2): 597-614.

Cao, H., Glazebrook, J., Clarke, J. D., Volko, S., and Dong, X. (1997). The *Arabidopsis NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. **Cell.** 88(1): 57-63.

Colman, A. M., and Pulford, B. D. (2011). **A crash course in SPSS for Windows: updated for versions 14, 15, and 16.** John Wiley & Sons.

Dandan, Z., and Zhiwei, Z. (2007). Biodiversity of arbuscular mycorrhizal fungi in the hot-dry valley of the Jinsha River, southwest China. **Applied Soil Ecology.** 37(1): 118-128.

De Freitas, J. R., Banerjee, M. R., and Germida, J. J. (1997). Phosphate-solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of canola (*Brassica napus* L.). **Biology and Fertility of Soils.** 24(4): 358-364.

Declerck, J., Feldmar, J., and Ayache, N. (1998). Definition of a four-dimensional continuous planispheric transformation for the tracking and the analysis of left-ventricle motion. **Medical Image Analysis.** 2(2): 197-213.

Dobbelaere, S., Vanderleyden, J., and Okon, Y. (2003). Plant growth-promoting effects of diazotrophs in the rhizosphere. **Critical Reviews in Plant Sciences.** 22(2): 107-149.

Douds, D. D. (2002). Increased spore production by *Glomus intraradices* in the split-plate monoxenic culture system by repeated harvest, gel replacement, and resupply of glucose to the mycorrhiza. **Mycorrhiza.** 12(4): 163-167.

Douds, D. D., Nagahashi, G., and Hepperly, P. R. (2010). On-farm production of inoculum of indigenous arbuscular mycorrhizal fungi and assessment of diluents of compost for inoculum production. **Bioresource Technology.** 101(7): 2326-2330.

Duc, G., Trouvelot, A., Gianinazzi-Pearson, V., and Gianinazzi, S. (1989). First report of non-mycorrhizal plant mutants (Myc-) obtained in pea (*Pisum sativum* L.) and fababean (*Vicia faba* L.). **Plant Science.** 60(2): 215-222.

Duncan, D. B. (1955). Multiple range and multiple F tests. **Biometrics.** 11(1): 1-42.

Esitken, A., Pirlak, L., Turan, M., and Sahin, F. (2006). Effects of floral and foliar application of plant growth promoting rhizobacteria (PGPR) on yield, growth and nutrition of sweet cherry. **Scientia Horticulturae.** 110(4): 324-327.

Fellbaum, C. R., Gachomo, E. W., Beesetty, Y., Choudhari, S., Strahan, G. D., Pfeffer, P. E., Kiers, E. T., and Bücking, H. (2012). Carbon availability triggers fungal nitrogen uptake and transport in arbuscular mycorrhizal symbiosis. **Proceedings of The National Academy of Sciences.** 109(7): 2666-2671.

Fester, T., and Hause, G. (2005). Accumulation of reactive oxygen species in arbuscular mycorrhizal roots. **Mycorrhiza.** 15(5): 373-379.

Foo, E. (2013). Auxin influences strigolactones in pea mycorrhizal symbiosis. **Journal of Plant Physiology.** 170(5): 523-528.

Gamalero, E., Trotta, A., Massa, N., Copetta, A., Martinotti, M. G., and Berta, G. (2004). Impact of two fluorescent pseudomonads and an arbuscular mycorrhizal fungus on tomato plant growth, root architecture and P acquisition. **Mycorrhiza.** 14(3): 185-192.

García-Garrido, J. M., and Ocampo, J. A. (2002). Regulation of the plant defence response in arbuscular mycorrhizal symbiosis. **Journal of Experimental Botany.** 53(373): 1377-1386.

Gaudin, I., Raschdorf, K., Schmid, E., Blum, W., and Inverardi, B. (1987). Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. **EMBO.** 6: 3209.

Genre, A., Ivanov, S., Fendrych, M., Faccio, A., Žárský, V., Bisseling, T., and Bonfante, P. (2012). Multiple exocytotic markers accumulate at the sites of perifungal membrane biogenesis in arbuscular mycorrhizas. **Plant and Cell Physiology.** 53(1): 244-255.

Gianinazzi, S., and Vosátka, M. (2004). Inoculum of arbuscular mycorrhizal fungi for production systems: Science meets business. **Canadian Journal of Botany.** 82(8): 1264-1271.

Glick, B. R. (1995). The enhancement of plant growth by free-living bacteria. **Canadian Journal of Microbiology.** 41(2): 109-117.

Gollotte, A., Gianinazzi-Pearson, V., Giovannetti, M., Sbrana, C., Avio, L., and Gianinazzi, S. (1993). Cellular localization and cytochemical probing of resistance reactions to arbuscular mycorrhizal fungi in a ‘locus a’myc− mutant of *Pisum sativum* L. **Planta.** 191(1): 112-122.

Goltapeh, E. M., Danesh, Y. R., Prasad, R., and Varma, A. (2008). Mycorrhizal fungi: What we know and what should we know? **Mycorrhiza.** 3-27.

Gonzalez-Chavez, M. C., Carrillo-Gonzalez, R., Wright, S. F., and Nichols, K. A. (2004). The role of glomalin, a protein produced by arbuscular mycorrhizal fungi, in sequestering potentially toxic elements. **Environmental Pollution.** 130(3): 317-323.

Görg, A., Postel, W., Weser, J., Günther, S., Strahler, J. R., Hanash, S. M., and Somerlot, L. (1987). Elimination of point streaking on silver stained two-dimensional gels by addition of iodoacetamide to the equilibration buffer. **Electrophoresis.** 8(2): 122-124.

Gosling, P., Hodge, A., Goodlass, G., and Bending, G. D. (2006). Arbuscular mycorrhizal fungi and organic farming. **Agriculture, Ecosystems & Environment.** 113(1): 17-35.

Govindarajulu, M., Pfeffer, P. E., Jin, H., Abubaker, J., Douds, D. D., Allen, J. W., Bücking, H., Lammers, P. J., and Shachar-Hill, Y. (2005). Nitrogen transfer in the arbuscular mycorrhizal symbiosis. **Nature.** 435(7043): 819-823.

Gryndler, M., Hršelová, H., Chvátalová, I., and Vosátka, M. (1998). *In vitro* proliferation of *Glomus fistulosum* intraradical hyphae from mycorrhizal root segments of maize. **Mycological Research.** 102(9): 1067-1073.

Harrier, L. A., and Watson, C. A. (2004). The potential role of arbuscular mycorrhizal (AM) fungi in the bioprotection of plants against soil-borne pathogens in organic and/or other sustainable farming systems. **Pest Management Science.** 60(2): 149-157.

Hawkins, H. J., and George, E. (1997). Hydroponic culture of the mycorrhizal fungus *Glomus mosseae* with *Linum usitatissimum* L., *Sorghum bicolor* L. and *Triticum aestivum* L. **Plant and Soil.** 196(1): 143-149.

Hayat, R., Ali, S., Amara, U., Khalid, R., and Ahmed, I. (2010). Soil beneficial bacteria and their role in plant growth promotion: a review. **Annals of Microbiology.** 60(4): 579-598.

Heijden, M. G. A. (2010). Mycorrhizal fungi reduce nutrient loss from model grassland ecosystems. **Ecology.** 91(4): 1163-1171.

Heyno, E., Mary, V., Schopfer, P., and Krieger-Liszkay, A. (2011). Oxygen activation at the plasma membrane: Relation between superoxide and hydroxyl radical production by isolated membranes. **Planta.** 234(1): 35-45.

Hilbert, J. L., Costa, G., and Martin, F. (1991). Ectomycorrhizin synthesis and polypeptide changes during the early stage of eucalypt mycorrhiza development. **Plant Physiology.** 97(3): 977-984.

Hoagland, D., and Broyer, T. (1936). General nature of the process of salt accumulation by roots with description of experimental methods. **Plant Physiology.** 11(3): 471.

Hoagland, D. R., and Arnon, D. I. (1950). **The water-culture method for growing plants without soil.** (2nd ed). Berkeley, Calif: College of Agriculture, University of California.

Hobbie, E. A., Hoff, C. J., Bryce, J. G., Colpaert, J. V., and Hallett, R. A. (2009). Nutrient supply rate and mycorrhizal colonization control patterns of element distribution in ectomycorrhizal pine. **Communications in Soil Science and Plant Analysis.** 40(21-22): 3503-3523.

Hodge, A., Campbell, C. D., and Fitter, A. H. (2001). An arbuscular mycorrhizal fungus accelerates decomposition and acquires nitrogen directly from organic material. **Nature.** 413(6853): 297-299.

IJdo, M., Cranenbrouck, S., and Declerck, S. (2011). Methods for large-scale production of AM fungi: past, present, and future. **Mycorrhiza.** 21(1): 1-16.

Iwase, T., Tajima, A., Sugimoto, S., Okuda, K.-i., Hironaka, I., Kamata, Y., Takada, K., and Mizunoe, Y. (2013). A simple assay for measuring catalase activity: A visual approach. **Scientific Reports.** 3: 3081.

Jarstfer, A. G., Farmer-Koppenol, P., and Sylvia, D. M. (1998). Tissue magnesium and calcium affect arbuscular mycorrhiza development and fungal reproduction. **Mycorrhiza.** 7(5): 237-242.

Johansson, J. F., Paul, L. R., and Finlay, R. D. (2004). Microbial interactions in the mycorrhizosphere and their significance for sustainable agriculture. **FEMS Microbiology Ecology.** 48(1): 1-13.

Kav, N. N., Srivastava, S., Yajima, W., and Sharma, N. (2007). Application of proteomics to investigate plant-microbe interactions. **Current Proteomics** 4(1): 28-43.

Khan, A. G. (2005). Role of soil microbes in the rhizospheres of plants growing on trace metal contaminated soils in phytoremediation. **Journal of Trace Elements in Medicine and Biology.** 18(4): 355-364.

Kiers, E. T., and Heijden, M. G. A. (2006). Mutualistic stability in the arbuscular mycorrhizal symbiosis: exploring hypotheses of evolutionary cooperation. **Ecology.** 87(7): 1627-1636.

Kloepper, J. W., and Beauchamp, C. J. (1992). A review of issues related to measuring colonization of plant roots by bacteria. **Canadian Journal of Microbiology.** 38(12): 1219-1232.

Koide, R. T., and Kabir, Z. (2000). Extraradical hyphae of the mycorrhizal fungus *Glomus intraradices* can hydrolyse organic phosphate. **New Phytologist.** 148(3): 511-517.

Krantev, A., Yordanova, R., Janda, T., Szalai, G., and Popova, L. (2008). Treatment with salicylic acid decreases the effect of cadmium on photosynthesis in maize plants. **Journal of Plant Physiology.** 165(9): 920-931.

Labbé, J. L., Weston, D. J., Dunkirk, N., Pelletier, D. A., and Tuskan, G. A. (2014). Newly identified helper bacteria stimulate ectomycorrhizal formation in Populus. **Frontiers in Plant Science.** 5: 579.

Lambais, M. (2000). Regulation of plant defense-related genes in arbuscular mycorrhizae. **Journal of Experimental Botany.** 53(377): 1377-1386.

Lambais, M. R., Goldman, M. H., Camargo, L. E., and Goldman, G. H. (2000). A genomic approach to the understanding of *Xylella fastidiosa* pathogenicity. **Current Opinion in Microbiology.** 3(5): 459-462.

Leborgne-Castel, N., and Bouhidel, K. (2014). Plasma membrane protein trafficking in plant–microbe interactions: A plant cell point of view. **Frontiers in plant Science.** 5 : 735.

Liu, A., Hamel, C., Hamilton, R., Ma, B., and Smith, D. (2000). Acquisition of Cu, Zn, Mn and Fe by mycorrhizal maize (*Zea mays* L.) grown in soil at different P and micronutrient levels. **Mycorrhiza.** 9(6): 331-336.

Lobet, G., Pagès, L., and Draye, X. (2011). A novel image-analysis toolbox enabling quantitative analysis of root system architecture. **Plant Physiology.** 157(1): 29-39.

Ludwig-Müller, J., and Güther, M. (2007). Auxins as signals in arbuscular mycorrhiza formation. **Plant Signal Behavior.** 2: 194-196.

Manoli, A., Sturaro, A., Trevisan, S., Quaggiotti, S., and Nonis, A. (2012). Evaluation of candidate reference genes for qPCR in maize. **Journal of Plant Physiology.** 169(8): 807-815.

Mar Vázquez, M., César, S., Azcón, R., and Barea, J. M. (2000). Interactions between arbuscular mycorrhizal fungi and other microbial inoculants (*Azospirillum, Pseudomonas, Trichoderma*) and their effects on microbial population and enzyme activities in the rhizosphere of maize plants. **Applied Soil Ecology.** 15(3): 261-272.

Meyer, J. R., and Linderman, R. G. (1986). Selective influence on populations of rhizosphere or rhizoplane bacteria and actinomycetes by mycorrhizas formed by *Glomus fasciculatum*. **Soil Biology and Biochemistry.** 18(2): 191-196.

Millner, P. D., and Kitt, D. G. (1992). The Beltsville method for soilless production of vesicular-arbuscular mycorrhizal fungi. **Mycorrhiza.** 2(1): 9-15.

Mitsuhara, I., Iwai, T., Seo, S., Yanagawa, Y., Kawahigasi, H., Hirose, S., Ohkawa, Y., and Ohashi, Y. (2008). Characteristic expression of twelve rice PR1 family genes in response to pathogen infection, wounding, and defense-related signal compounds (121/180). **Molecular Genetics and Genomics.** 279(4): 415-427.

Mohammad, A., Khan, A. G., and Kuek, C. (2000). Improved aeroponic culture of inocula of arbuscular mycorrhizal fungi. **Mycorrhiza.** 9(6): 337-339.

Mosse, B. (1959). The regular germination of resting spores and some observations on the growth requirements of an *Endogone* sp. causing vesicular-arbuscular mycorrhiza. **Transactions of the British Mycological Society.** 42(3): 273-286.

Mosse, B. (1962). The establishment of vesicular-arbuscular mycorrhiza under aseptic conditions. **Journal of General Microbiology.** 27(3): 509.

Mosse, B., and Hepper, C. (1975). Vesicular-arbuscular mycorrhizal infections in root organ cultures. **Physiological Plant Pathology.** 5(3): 215-218.

Mugnier, J., and Mosse, B. (1987). Vesicular-arbuscular mycorrhizal infection in transformed root-inducing T-DNA roots grown axenically. **Phytopathology** 77(7): 1045-1050.

Muñoz, V., Ibáñez, F., Tordable, M., Megías, M., and Fabra, A. (2015). Role of reactive oxygen species generation and Nod factors during the early symbiotic interaction between bradyrhizobia and peanut, a legume infected by crack entry. **Journal of Applied Microbiology.** 118(1): 182-192.

Mutka, A. M., Fawley, S., Tsao, T., and Kunkel, B. N. (2013). Auxin promotes susceptibility to *Pseudomonas syringae* via a mechanism independent of suppression of salicylic acid-mediated defenses. **The Plant Journal.** 74(5): 746-754.

Nelson, L. M. (2004). Plant growth promoting rhizobacteria (PGPR): Prospects for new inoculants. **Crop Management.** 10: 1094.

Niyomploy, P., Srisomsap, C., Chokchaichamnankit, D., Vinayavekhin, N., Karnchanatat, A., and Sangvanich, P. (2014). Superoxide dismutase isozyme detection using two-dimensional gel electrophoresis zymograms. **Journal of Pharmaceutical and Biomedical Analysis.** 90: 72-77.

O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. **Journal of Biological Chemistry.** 250(10): 4007-4021.

Pečenková, T., Hála, M., Kulich, I., Kocourková, D., Drdová, E., Fendrych, M., Hana, T., and Žárský, V. (2011). The role for the exocyst complex subunits Exo70B2 and Exo70H1 in the plant–pathogen interaction. **Journal of Experimental Botany.** 402.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. **Nucleic Acids Research.** 29(9): 45.

Phillips, J., and Hayman, D. (1970). Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. **Transactions of The British Mycological Society.** 55(1): 158-161.

Piromyou, P., Buranabanyat, B., Tantasawat, P., Tittabutr, P., Boonkerd, N., and Teaumroong, N. (2011). Effect of plant growth promoting rhizobacteria (PGPR) inoculation on microbial community structure in rhizosphere of forage corn cultivated in Thailand. **European Journal of Soil Biology.** 47(1): 44-54.

Requena, N., Jimenez, I., Toro, M., and Barea, J. (1997). Interactions between plant-growth-promoting rhizobacteria (PGPR), arbuscular mycorrhizal fungi and *Rhizobium* spp. in the rhizosphere of *Anthyllis cytisoides*, a model legume for revegetation in mediterranean semi-arid ecosystems. **New Phytologist.** 667-677.

Richardson, A. E., Barea, J. M., McNeill, A. M., and Prigent-Combaret, C. (2009). Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. **Plant and Soil.** 321(1): 305-339.

Rigamonte, T. A., Pylro, V. S., and Duarte, G. F. (2010). The role of mycorrhization helper bacteria in the establishment and action of ectomycorrhizae associations. **Brazilian Journal of Microbiology.** 41(4): 832-840.

Rodríguez, H., and Fraga, R. (1999). Phosphate solubilizing bacteria and their role in plant growth promotion. **Biotechnology Advances.** 17(4-5): 319-339.

Rodríguez-Romero, A. S., Guerrra, M. S. P., and Jaizmen-Vega, M. D. (2005). The effect of arbuscular mycorrhizal fungi and rhizobacteria on banana growth and nutrition. **Agronomy for Sustainable Development Journal.** 25: 395-39.

Roesti, D., Gaur, R., Johri, B. N., Imfeld, G., Sharma, S., Kawaljeet, K., and Aragno, M. (2006). Plant growth stage, fertiliser management and bio-inoculation of arbuscular mycorrhizal fungi and plant growth promoting rhizobacteria affect the rhizobacterial community structure in rain-fed wheat fields. **Soil Biology and Biochemistry.** 38(5): 1111-1120.

Ryan, M. H., and Graham, J. H. (2002). Is there a role for arbuscular mycorrhizal fungi in production agriculture? **Plant and Soil.** 244(1): 263-271.

Salzer, P., Corbière, H., and Boller, T. (1999). Hydrogen peroxide accumulation in *Medicago truncatula* roots colonized by the arbuscular mycorrhiza-forming fungus *Glomus intraradices*. **Planta.** 208(3): 319-325.

Schüβler, A., Schwarzott, D., and Walker, C. (2001). A new fungal phylum, the *Glomeromycota* : phylogeny and evolution. **Mycological Research.** 105(12): 1413-1421.

Sels, J., Mathys, J., De Coninck, B. M., Cammue, B. P., and De Bolle, M. F. (2008). Plant pathogenesis-related (PR) proteins: A focus on PR peptides. **Plant Physiology and Biochemistry.** 46(11): 941-950.

Sen, S., and Chandrasekhar, C. (2015). Effect of PGPR on enzymatic activities of rice (*Oryza sativa* L.) under salt stress. **Asian Journal of Plant Science and Research.** 5(6): 44-48.

Silva, H. S. A., Romeiro, R. S., Macagnan, D., Halfeld-Vieira, B. A., Pereira, M. C. B., and Mounteer, A. (2004). Rhizobacterial induction of systemic resistance in tomato plants: Aon-specific protection and increase in enzyme activities. **Biological Control.** 29(2): 288-295.

Singh, C. (1992). Mass inoculum production of vesicular-arbuscular (VA) mycorrhizae: II. Impact of N 2-fixing and P-solubilizing bacterial inoculation on VA-mycorrhiza. **Zentralblatt Für Mikrobiologie.** 147(8): 503-508.

Smith, F. A., and Smith, S. E. (2011). What is the significance of the arbuscular mycorrhizal colonisation of many economically important crop plants? **Plant and Soil.** 348(1-2): 63-79.

Smith, S. E., and Gianinazzi-Pearson, V. (1988). Physiological interactions between symbionts in vesicular-arbuscular mycorrhizal plants. **Annual Review of Plant Physiology and Plant Molecular Biology.** 39(1): 221-244.

Smith, S. E., and Read, D. J. (2010). **Mycorrhizal Symbiosis**. London: Academic press.

Smith, S. E., Smith, F. A., and Jakobsen, I. (2003). Mycorrhizal fungi can dominate phosphate supply to plants irrespective of growth responses. **Plant Physiology.** 133(1): 16-20.

Somssich, I. E., and Hahlbrock, K. (1998). Pathogen defence in plants a paradigm of biological complexity. **Trends in Plant Science.** 3(3): 86-90.

Song, F., Qi, D., Liu, X., Kong, X., Gao, Y., Zhou, Z., and Wu, Q. (2015). Proteomic analysis of symbiotic proteins of *Glomus mosseae* and *Amorpha fruticosa*. **Scientific Reports.** 5:18031.

St-Arnaud, M., Hamel, C., Vimard, B., Caron, M., and Fortin, J. A. (1996). Enhanced hyphal growth and spore production of the arbuscular mycorrhizal fungus *Glomus intraradices* in an in vitro system in the absence of host roots. **Mycological Research.** 100(3): 328-332.

Süß, C., Hempel, J., Zehner, S., Krause, A., Patschkowski, T., and Göttfert, M. (2006). Identification of genistein-inducible and type III-secreted proteins of *Bradyrhizobium japonicum*. **Journal of Biotechnology.** 126(1): 69-77.

Sylvia, D. M., Alagely, A. K., Kane, M. E., and Philman, N. L. (2003). Compatible host/mycorrhizal fungus combinations for micropropagated sea oats. **Mycorrhiza.** 13(4): 177-183.

Sylvia, D. M., and Jarstfer, A. G. (1994). Production of inoculum and inoculation with arbuscular mycorrhizal fungi. **Development in Plant and Soil Sciences.** 56: 231-231.

Sylvia, D. M., and Jarstfer, A. G. (1997). Distribution of mycorrhiza on competing pines and weeds in a southern pine plantation. **Soil Science Society of America Journal.** 61(1): 139-144.

Tawaraya, K., Naito, M., and Wagatsuma, T. (2006). Solubilization of insoluble inorganic phosphate by hyphal exudates of arbuscular mycorrhizal fungi. **Journal of Plant Nutrition.** 29(4): 657-665.

Thuler, D. S., Floh, E. I. S., Handro, W., and Barbosa, H. R. (2003). *Beijerinckia derxii* releases plant growth regulators and amino acids in synthetic media

independent of nitrogenase activity. **Journal of Applied Microbiology.** 95(4): 799-806.

Toro, M., Azcon, R., and Barea, J. (1997). Improvement of arbuscular mycorrhiza development by inoculation of soil with phosphate-solubilizing rhizobacteria to improve rock phosphate bioavailability ((sup32) P) and nutrient cycling. **Applied and Environmental Microbiology.** 63(11): 4408-4412.

Torres, M. A., Jones, J. D., and Dangl, J. L. (2006). Reactive oxygen species signaling in response to pathogens. **Plant physiology.** 141(2): 373-378.

Touraine, B. (2004). Nitrate uptake by roots-transporters and root development. Nitrogen acquisition and assimilation in higher plants. **Springer.** 1-34.

Treseder, K. K., and Allen, M. F. (2000). Mycorrhizal fungi have a potential role in soil carbon storage under elevated CO_2 and nitrogen deposition. **New Phytologist.** 147(1): 189-200.

Vandenkoornhuyse, P., Baldauf, S. L., Leyval, C., Straczek, J., and Young, J. P. W. (2002). Extensive fungal diversity in plant roots. **Science.** 295(5562): 2051-2051.

Vessey, J. K. (2003). Plant growth promoting rhizobacteria as biofertilizers. **Plant and Soil.** 255(2): 571-586.

Voets, L., Dupré de Boulois, H., Renard, L., Strullu, D. G., and Declerck, S. (2005). Development of an autotrophic culture system for the in vitro mycorrhization of potato plantlets. **Federation of European Microbiological Societies Microbiology Letters.** 248(1): 111-118.

Von Alten, H., Blal, B., Dodd, J., Feldmann, F., and Vosatka, M. (2002). Quality control of arbuscular mycorrhizal fungi inoculum in Europe. In S. Gianinazzi, H. Schüepp, J. M. Barea, ans K. Haselwandter (pp. 281-296). **Mycorrhizal**

technology in agriculture: From genes to bioproducts. Birkhäuser Verlag AG, Basel: Switzerland.

Vosatka, M., and Dodd, J. (2002). Ecological considerations for successful application of arbuscular mycorrhizal fungi inoculum. In S. Gianinazzi, H. Schüepp, J. M. Barea, ans K. Haselwandter (pp. 235-247). **Mycorrhizal technology in agriculture: From genes to bioproducts.** Birkhäuser Verlag AG, Basel: Switzerland.

Walley, F. L., and Germida, J. J. (1995). Failure to decontaminate *Glomus clarum* NT4 spores is due to spore wall-associated bacteria. **Mycorrhiza.** 6(1): 43-49.

Wang, D., Pajerowska-Mukhtar, K., Culler, A. H., and Dong, X. (2007). Salicylic acid inhibits pathogen growth in plants through repression of the auxin signaling pathway. **Current Biology.** 17(20): 1784-1790.

Wang, X., Pan, Q., Chen, F., Yan, X., and Liao, H. (2011). Effects of co-inoculation with arbuscular mycorrhizal fungi and rhizobia on soybean growth as related to root architecture and availability of N and P. **Mycorrhiza.** 21(3): 173-181.

Warrier, R., Paul, M., and Vineetha, M. (2013). Estimation of salicylic acid in Eucalyptus leaves using spectrophotometric methods. **Genetics and Plant Physiology.** 3(1-2): 90-97.

Watanarojanaporn, N., Boonkerd, N., Tittabutr, P., Longtonglang, A., Young, J. P. W., and Teaumroong, N. (2013). Effect of rice cultivation systems on indigenous arbuscular mycorrhizal fungal community structure. **Microbes and Environments.** 28(3): 316-324.

Whipps, J. M. (2001). Microbial interactions and biocontrol in the rhizosphere. **Journal of Experimental Botany.** 52(1): 487-511.

Wright, D. P., Scholes, J. D., and Read, D. J. (1998). Effects of VA mycorrhizal colonization on photosynthesis and biomass production of *Trifolium repens* L. **Plant, Cell & Environment.** 21(2): 209-216.

Wu, S., Cao, Z., Li, Z., Cheung, K., and Wong, M. (2005). Effects of biofertilizer containing N-fixer, P and K solubilizers and AM fungi on maize growth: A greenhouse trial. **Geoderma.** 125(1): 155-166.

Wu, X. Q., Hou, L. L., Sheng, J. M., Ren, J. H., Zheng, L., Chen, D., and Ye, J. R. (2012). Effects of ectomycorrhizal fungus *Boletus edulis* and mycorrhiza helper *Bacillus cereus* on the growth and nutrient uptake by *Pinus thunbergii*. **Biology and Fertility of Soils.** 48(4): 385-391.

Wu, X., Xiong, E., Wang, W., Scali, M., and Cresti, M. (2014). Universal sample preparation method integrating trichloroacetic acid/acetone precipitation with phenol extraction for crop proteomic analysis. **Nature Protocols.** 9(2): 362-374.

Xavier, L. J. C., and Germida, J. J. (2003). Bacteria associated with *Glomus clarum* spores influence mycorrhizal activity. **Soil Biology and Biochemistry.** 35(3): 471-478.

Zobel, R. W., Del Tredici, P., and Torrey, J. G. (1976). Method for growing plants aeroponically. **Plant Physiology.** 57(3): 344.

BIOGRAPHY

Mr. Watcharin Yuttavanichakul was born on May 28, 1984 at Nong Khai, Thailand. He got his Bachelor degree of Science in Crop Production Technology from Suranaree University of Technology, Thailand in 2006. After graduation, he used to work at Chia Tai Company in 2007 as a seed quality controller. Then, he started for his Master degree in 2008 and graduated in 2010 at School of Biotechnology, Suranaree University of Technology, Nakhon Ratchasima. During his Master degree study, he had experiences from his thesis work in "Improvement of peanut rhizobial inoculant by incorporation of Plant Growth Promoting Rhizobacteria (PGPR) as biocontrol against seed borne pathogenic fungi". Some parts of his work was also presented in 8th International Symposium on Biocontrol and Biotechnology in October, 4-6, 2010 at Pattaya, Thailand in the topic of "Effects of Biocontrol of Seed-borne Pathogenic Fungi of Peanut by Rhizobial Inoculants". His work was also published in the Biological Control Volume 63, Issue 2, Pages 69-230 (November 2012) in the title of "Improvement of peanut rhizobial inoculant by incorporation of plant growth promoting rhizobacteria (PGPR) as biocontrol against the seed borne fungus, *Aspergillus niger*". He received a scholarship from Tsukuba University and Tokyo University of Agriculture and Technology to do some part of his research in Japan from April 5 to July 4, 2010 and July 7 to September 7, 2014.