BIOTIC AND ABIOTIC APPROACHES TO DEVELOP METHOD FOR ARBUSCULAR MYCORRHIZAL FUNGI SPORE PRODUCTION

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A Thesis Submitted in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy in Biotechnology
Suranaree University of Technology
Academic Year 2023

การใช้วิธีการทางชีวภาพ และกายภาพในการพัฒนาการผลิตสปอร์ของเชื้อราอาร์บัสคูลาร์ไมคอร์ไรซา

นายสุธี คิดดี

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

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คำสำคัญ : อาร์บัสคูลาร์ไมคอร์ไรซา/บรีวิบาซิลลัส สายพันธุ์ SUT47/บาซิลลัส เวเลเซนซิส สายพันธุ์ S141/แสงแอลอีดี/กรดไขมัน

ที่ผ่านมามีการศึกษาวิธีการทั้งทางชีวภาพ และทางกายภาพเพื่อเพิ่มประสิทธิภาพในการเพิ่มจำนวนสปอร์ของเชื้อราอาร์บัสคูลาร์ไมคอร์ไรซา (Arbuscular Mycorrhizal Fungi; AMF) เพื่อใช้ผลิตเป็นหัวเชื้อปุ๋ยชีวภาพในการเกษตร โดยในบรรดาวิธีการทางชีวภาพ พบว่าการใช้แบคทีเรียที่มีคุณสมบัติช่วยส่งเสริมการเจริญของเชื้อราอาร์บัสคูลาร์ไมคอร์ไรซา (Mycorrhization Helper Bacteria; MHB) มาปลูกร่วมกันกับพืชอาศัย เป็นวิธีการที่สามารถเพิ่มจำนวนสปอร์ของเชื้อราได้ โดยงานวิจัยนี้พบแบคทีเรียบางชนิดที่นอกจากจะมีความสามารถในการส่งเสริมการเจริญเติบโตของพืชแล้ว (Plant Growth Promoting Rhizobacteria; PGPR) ยังมีผลเชิงบวกต่อการเจริญและการเพิ่มจำนวนของเชื้อราภายใต้ระบบการผลิตแบบใช้วัสดุปลูกพืชทั่วไปด้วย ทั้งนี้การค้นพบประการแรก คือ สารที่ปลดปล่อยออกมา (secretion compounds) จากเชื้อแบคทีเรีย บรีวิบาซิลลัส สายพันธุ์ SUT47 (*Brevibacillus* sp. SUT47) เมื่อใช้ร่วมกันกับการปลูกเชื้อราอาร์บัสคูลาร์ไมคอร์ไรซาสายพันธุ์ *Acaulospora tuberculata* สามารถส่งเสริมการผลิตสปอร์ให้เพิ่มขึ้นในรากข้าวโพดได้ โดยการใช้สารที่ปลดปล่อยออกมาจากเชื้อแบคทีเรียเข้มข้นที่ระดับ 360 มิลลิกรัม ร่วมกับการปลูกเชื้อราให้ผลจำนวนการผลิตสปอร์มากที่สุดประมาณ 1,500 สปอร์ต่อต้น ในระยะเวลา 120 วัน หลังการปลูกเชื้อ อย่างไรก็ตามการประยุกต์ใช้แบคทีเรียในรูปแบบเซลล์ที่มีชีวิตยังคงส่งเสริมการผลิตจำนวนสปอร์ของเชื้อราอาร์บัสคูลาร์ไมคอร์ไรซาที่สูงกว่า โดยให้จำนวนสปอร์มากกว่า 2,000 สปอร์ต่อต้น ซึ่งแตกต่างอย่างมีนัยสำคัญเมื่อเทียบกับวิธีการใช้สารที่ปลดปล่อยออกมาจากแบคทีเรีย ประการที่สอง พบว่าเชื้อแบคทีเรีย *B. velezensis* S141 มีความสามารถในการส่งเสริมให้เชื้อราอาร์บัสคูลาร์ไมคอร์ไรซาสายพันธุ์ *Rhizophagus irregularis* เมื่ออยู่ร่วมกันแบบพึ่งพาอาศัย (symbiosis) กับต้นถั่วโลตัส (*Lotus japonicus*) โดยการปลูกเชื้อแบคทีเรีย สายพันธุ์ S141 ร่วมกันนี้ ส่งผลเชิงบวกต่อการเจริญเติบโตและการพัฒนาของเชื้อรา รวมถึงการเข้าครอบครองพื้นที่รากของเชื้อราอาร์บัสคูลาร์ไมคอร์ไรซา การเพิ่มจำนวนสปอร์ และการดูดซับธาตุอาหารของพืช ในลักษณะการมีปฏิสัมพันธ์แบบไตรภาคี อย่างไรก็ตามเมื่อตรวจสอบกลไกความสัมพันธ์ที่เกิดขึ้น พบว่าฮอร์โมนพืชในกลุ่มออกซิน (indole-3-acetic acid; IAA) ที่แบคทีเรียสายพันธุนี้สามารถผลิตได้ ไม่ใช่กลไกหลักที่ทำให้เกิดการส่งเสริมการอยู่ร่วมกันของพืช และเชื้อราอาร์บัสคูลาร์ไมคอร์ไรซา นอกจากนี้ยังเป็นที่น่าสนใจว่า

เชื้อแบคทีเรีย สายพันธุ์ S141 สามารถเข้าสู่ภายในเซลล์รากของพืช และอาศัยอยู่ภายในเนื้อเยื่อพืชได้ (endophytic bacteria) และยังพบว่าแบคทีเรียชนิดนี้สามารถกระตุ้นการแสดงออกของกลุ่มยีนเครื่องหมาย (marker genes) ในพืชที่สัมพันธ์กับการเข้าครอบครองรากของเชื้อราอาร์บัสคูลาร์ไมคอร์ไรซาในระยะแรก การดูดซับธาตุอาหารในพืช รวมทั้งกระตุ้นการแสดงออกของยีนในเชื้อราอาร์บัสคูลาร์ไมคอร์ไรซาที่เกี่ยวข้องกับการควบคุมวงจรการแบ่งเซลล์ของเชื้อราชนิดนี้อีกด้วย

ในงานวิจัยนี้ยังได้ศึกษาวิธีการทางกายภาพ ด้วยเทคโนโลยีการปลูกพืชโดยใช้แสงจาก Light Emitting Diode (LED) ที่อัตราความเข้มแสงเฉพาะของแสงสีแดงและสีฟ้าในอัตราส่วน 60:40 ที่ความเข้มแสงรวม 300 ไมโครโมลต่อตารางเมตรต่อวินาที ส่งเสริมการเข้าครอบครองพื้นที่รากของเชื้อราอาร์บัสคูลาร์ไมคอร์ไรซาในรากต้นข้าวโพด (*Zea mays* L.) การใช้แสง LED สามารถเร่งกระบวนการผลิตสปอร์ของเชื้อราในระบบการผลิตแบบใช้วัสดุปลูก โดยให้จำนวนสปอร์ประมาณ 1,500 สปอร์ต่อต้น ในระยะเวลา 90 วัน หลังการปลูกเชื้อ นอกจากนี้ การประยุกต์ใช้กรดไมริสติกซึ่งเป็นกรดไขมันที่จำเป็นต่อการเจริญเติบโตของเชื้อรา ในระดับความเข้มข้นที่เหมาะสม (10-10,000 ไมโครกรัม) ร่วมกับการปลูกต้นข้าวโพด ส่งผลให้มีแนวโน้มจำนวนสปอร์ของเชื้อราที่ผลิตเพิ่มขึ้นเช่นกัน

ผลงานวิจัยที่ได้นี้สามารถใช้เป็นแนวทางวิธีการในการเพิ่มจำนวน และเร่งกระบวนการผลิตสปอร์ของเชื้อราอาร์บัสคูลาร์ไมคอร์ไรซาโดยใช้ระบบวัสดุปลูกที่สามารถเพิ่มกำลังการผลิตในระดับใหญ่ได้ต่อไป นอกจากนี้ยังแสดงให้เห็นถึงบทบาทของแบคทีเรียในกลุ่มพีจีพีอาร์ในฐานะแบคทีเรียผู้ช่วยในการสนับสนุนการอยู่ร่วมกันระหว่างพืชและเชื้อราอาร์บัสคูลาร์ไมคอร์ไรซา ที่สามารถนำไปประยุกต์ใช้ในวิถีการเกษตรแบบยั่งยืนได้อย่างมีศักยภาพต่อไป

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2566

ลายมือชื่อนักศึกษา _____
ลายมือชื่ออาจารย์ที่ปรึกษา _____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

SUTEE KIDDEE : BIOTIC AND ABIOTIC APPROACHES TO DEVELOP METHOD FOR ARBUSCULAR MYCORRHIZAL FUNGI SPORE PRODUCTION. THESIS ADVISOR : PANLADA TITTABUTR, Ph.D., 111 PP.

Keyword: Arbuscular mycorrhizal fungi/*Brevibacillus* sp. SUT47/*Bacillus velezensis* S141/LED light/Fatty acid

Biotic and abiotic approaches have been explored to enhance the efficiency of arbuscular mycorrhizal fungi (AMF) spore propagation for the purpose of producing AMF inoculum to be used in agriculture. Among biotic approaches, the use of mycorrhization helper bacteria (MHB) is a technique that can boost AMF spore numbers when co-inoculated with AMF in host plants. In this study, some plant growth-promoting rhizobacteria (PGPR) had a positive effect on promoting fungal abundance under the substrate-based production system. Firstly, the secretion compounds from *Brevibacillus* sp. SUT47 were discovered to promote AMF spore production of *Acaulospora tuberculata* when co-inoculated on maize roots. The highest number of spores was produced when 360 mg of concentrated secretion compounds were applied which significantly increased the highest AMF spore number of 1,500 spores per plant at 120 days after inoculation. However, the application of whole cell bacteria still significantly promoted a higher number of AMF spores than that of co-inoculation with secretion compounds approximately at 2,000 spores per plant. Secondly, *Bacillus velezensis* S141 was found to promote *Rhizophagus irregularis* in symbiosis with *Lotus japonicus*. Co-inoculation with strain S141 positively influenced fungal growth and development, including AMF root colonization, spore number, and plant nutrient uptake during the tripartite interaction. Surprisingly, the ability of strain S141 to produce indole-3-acetic acid was not the key mechanism promoting this symbiosis. Nevertheless, S141 could penetrate into plant root cells and establish itself as an endophytic bacterium. Moreover, its presence induced the expression of marker genes related to the early phases of AMF colonization, nutrient uptake in host plants, and upregulation of AMF genes involved in cell cycle regulation.

Regarding abiotic approaches, Light Emitting Diode (LED) technology was employed specifically using a red and blue light ratio of 60:40, at a total light intensity of 300 µMol/m^2/s to promote *R. irregularis* colonization in maize (*Zea mays* L.) root. LED light demonstrated a significant acceleration in AMF spore production under the substrate-based condition. The AMF spore number of approximately 1,500 spores per plant was produced after 90 days after inoculation. Besides, the application of an appropriate concentration of myristic acid, a fatty acid required for AMF development, at the suitable concentration (10-10,000 µg) together with maize cultivation also tended to increase in the number of *R. irregularis* spore production.

These findings offered promising prospects to accelerate large-scale AMF spore production using substrate-based methods. Additionally, these findings highlight the potential role of PGPR as helper bacteria in supporting plant-AMF symbiosis and their potential application in sustainable agriculture practices.

School of Biotechnology
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ACKNOWLEDGEMENT

I would like to express my sincere gratitude to all the individuals who have contributed to the completion of this thesis. Their support and inspiration have been invaluable, and I am deeply thankful for their presence in my academic journey.

First and foremost, I extend my heartfelt appreciation to my advisor, Assoc. Prof. Dr. Panlada Tittabutr, whose encouragement, valuable suggestions, and extraordinary experiences have guided me throughout this work. Without her guidance and unwavering support, this thesis would not have been possible. I am truly grateful for her mentorship.

I would also like to thank my co-advisors, Emeritus Professor Dr. Nantakorn Boonkerd and Professor Dr. Neung Teaumroong, for their expertise and insights, which have significantly contributed to the development of this thesis. I am thankful for their invaluable contributions.

A special thank you goes to my co-advisor, Professor Dr. Katsuharu Saito, Faculty of Agriculture, Shinshu University, Japan. His encouragement, valuable suggestions, and expertise in mycorrhizal fungi have been instrumental in guiding me throughout this work. I am truly grateful for his dedicated mentorship.

Furthermore, I am sincerely thankful for the support and constructive feedback provided by all my teachers, which greatly enhanced the quality of this thesis.

Additionally, I would like to express my appreciation to the member of my thesis committee, Associate Professor Dr. Sophon Boonlue. Their valuable insights, thoughtful suggestions, and rigorous evaluations contributed significantly to this thesis. Their expertise, dedication, and willingness to share their insights have been invaluable in shaping the direction and methodology of this research.

I must acknowledge the support and assistance of Ms. Prangkhaw Prukhett, whose guidance was invaluable throughout my academic journey.

My heartfelt appreciation goes out to the members of my research lab (NPN lab) for their unwavering support and assistance during challenging times. Their

continuous help and encouragement have been invaluable in overcoming difficulties throughout this thesis journey.

I am deeply indebted to my family for their unwavering belief in me, their constant inspiration, and their unwavering support during both good and difficult times.

Finally, I acknowledge the financial support provided by The Royal Golden Jubilee (RGJ) Ph.D. Program scholarship, The Thailand Research Fund (TRF), and Suranaree University of Technology (SUT). Their support has enabled my doctoral studies and played a crucial role in facilitating this study.

Once again, I would like to express my deepest gratitude to everyone who has contributed to the realization of this thesis.

Sutee Kiddee

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LIST OF ABBREVIATIONS

AMF	=	Arbuscular mycorrhizal fungi
AMF (-)	=	no Arbuscular mycorrhizal fungi inoculation
AMF (+)	=	inoculation with Arbuscular mycorrhizal fungi
Apr	=	April
ATP	=	Adenosine triphosphate
cDNA	=	Complementary deoxyribonucleic acid
CFU	=	Colony forming unit
cm	=	Centimeter
COS	=	Chitooligosaccharide
Ct	=	Cycle threshold
DNA	=	Deoxyribonucleic acid
DAE	=	Day after exposure
DAI	=	Day after inoculation
GH	=	Greenhouse
h	=	hour
HCl	=	Hydrochloric acid
H^+	=	Proton
Jan	=	January
Kg	=	Kilogram
KOH	=	Potassium hydroxide
L	=	Litter
LB	=	Luria-Bertani
LCO	=	Lipochitooligosaccharide
LED	=	Light emitting diode
MHB	=	Mycorrhization helper bacteria
ml	=	Mililiter
mm	=	Milimeter
miRNA	=	micro-Ribonucleic acid

LIST OF ABBREVIATIONS (Continued)

n	=	Number
N	=	Nitrogen
NI	=	Non inoculation
nm	=	Nanometer
nmol	=	Nanomole
n.s.	=	Non statistically significant
OD_{600}	=	Optical density measurements at a wavelength of 600 nanometer
P	=	Phosphorus
p-value	=	Probability value
PBS	=	Phosphate buffered saline
PCR	=	Polymerase chain reaction
PGPR	=	Plant growth promoting rhizobacteria
Pi	=	Inorganic phosphate
polyP	=	Polyphosphate
qRT-PCR	=	Quantitative reverse transcription polymerase chain reaction
RNA	=	Ribonucleic acid
SD	=	Standard deviation
SPAD	=	The Soil Plant Analysis Development
s.e.m.	=	Standard error of mean
SYTO 9	=	Green-fluorescent nucleic acid staining
v/v	=	Volume per volume
w/v	=	Weight per volume
µl	=	Microliter
µg	=	Microgram
µM	=	Micromolar
µm	=	Micrometer
%	=	Percentage sign

LIST OF ABBREVIATIONS (Continued)

F (%)	=	the frequency of mycorrhiza in the root system
M (%)	=	the intensity of the mycorrhizal colonization in the root system
m (%)	=	the intensity of the mycorrhizal colonization in the root fragments
a (%)	=	the arbuscule abundance in the mycorrhizal parts of the root fragment
A (%)	=	The arbuscule abundance in the root system.
^{o}C	=	Degree Celsius

CHAPTER I
INTRODUCTION

1.1 Significanct of study

Arbuscular mycorrhizal fungi (AMF) are widely distributed in terrestrial plants. This symbiosis occurs between plant roots and fungi, with AMF having the potential to enhance plant nutrient uptake (Marins and Carrenho, 2017). This is particularly valuable in agriculture because most plants, including major food crops, form symbiotic relationships with AMF, leading to increased plant yields (Berruti, Lumini, Balestrini, and Bianciotto, 2015). The demand for mycorrhizal inoculants has been increasing due to their versatile applications with various plants and their ability to support plant growth by enhancing nutrient and moisture acquisition, which is particularly valuable in the face of climate change. This confirms by the global market data from 2012 revealed that rhizobia inoculants were the most widely used, constituting 79% of the world's demand, followed by phosphate-mobilizing bio-inoculants (15%), and others like mycorrhizal inoculants (7%) (Mukhongo et al., 2016). Although the demand for AMF inoculum has risen in several countries, a significant bottleneck is the limited availability of AMF inoculants on the market, primarily due to the laborious and time-consuming nature of AMF inoculant production. Therefore, research efforts should focus on improving the efficiency of AMF spore production in this context.

In this study, both biotic and abiotic approaches were explored to enhance the efficiency of AMF spore production. Regarding biotic approaches, the use of mycorrhization helper bacteria (MHB) is one technique that can boost AMF spore numbers when co-inoculated with AMF in the host plant. For instance, *Brevibacillus* sp. SUT47 has been shown to increase the spore production of *Acaulospora tuberculata* in maize (*Zea may* L. Suwan 5) by approximately two-fold compared to using AMF fungi alone (Yuttavanichakul, 2015). However, this method may not be suitable when high purity of AMF fungal spores is required, as it may introduce

contamination from SUT47 cells. Therefore, this study considered novel methodologies that could enhance AM fungal spore production using the secretion compounds of SUT47. Moreover, the application of *Bacillus velezensis* S141 as a plant growth-promoting rhizobacterium (Prakamhang et al. 2015; Sibponkrung et al. 2017) on promoting AMF, *Rhizophagus irregularis* on symbiosis with *Lotus japonicus* was investigated in this study. The effect of *B. velezensis* S141 inoculation that influence on fungal growth and development, plant nutrients uptake, and the expression of some marker genes in the host plant and AMF under the tripartite interaction were investigated in this study.

In terms of abiotic approach, the application of light-emitting diodes (LEDs) in AMF inoculant production has gained attention. Research has shown that radiation plays a significant role in the biochemical and morphological responses of some fungi, such as *Phycomyces blankeslleanus*, affecting their growth and differentiation (Schroeter-Zakrzewsaka, Borowiak, and Wolna-Maruwka, 2016). Recent findings suggest that red-blue LED treatments stimulate AMF spore production on glass beads, while blue LED light inhibits it. In contrast, red LED light promotes fungal growth and spore germination. The combination of red and blue LED light is the most efficient for AMF colonization and sporulation (Freire Cruz, 2016). However, the effects of LED light, including intensity and exposure time, on AMF spore production depend on the AM species and the type of host plant. This study focused on the optimization of LED light condition for enhance the production of *R. irregularis* in maize roots.

Fatty acids play a crucial role in the growth and development of fungi, hence serving as an additional biotic approach (Wewer et al., 2014). According to Jiang et al. (2017), the AMF *R. irregularis* is classified as a fatty acid auxotroph, as it depends on the fatty acids produced by host plants in order to maintain mycorrhizal colonization. Moreover, it has been observed that AMF belonging to the order Glomerales have the ability to generate vesicles, which serve as reservoirs for lipids, hence serving as a crucial energy and carbon source (Brands et al., 2018). Recent reports have demonstrated that various types of fatty acids supplied in asymbiotic cultures can promote fungal growth and spore production (Sugiura et al., 2020). Consequently, this study explored the potential of incorporating fatty acids or organic compounds

containing fatty acids, such as Myristic acid, palmitoleic acid and palmitic acid, into a substrate-based production system together with the host plant and investigated the influence on AMF colonization and spore production.

1.2 Research objectives

1. To evaluate the potential of using the secretion compounds from *Brevibacillus* sp. SUT47 for enhancing AMF colonization and spore production.

2. To investigate the application of mycorrhization helper bacteria (MHB) on enhancing the AMF colonization in *Lotus japonicus* and its symbiosis interactions.

3. To develop the method to enhance the efficiency of AMF spore production under the substrate-based production system using LED light technology.

4. To explore the potential of incorporating fatty acids or organic compounds containing fatty acids for enhancing AMF spore production.

CHAPTER II

LITERATURE REVIEWS

2.1 Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal fungi (AMF), belonging to the phylum Glomeromycota (Schüβler et al. 2001), are commonly found soil microorganisms and are found in association with over 80% of vesicular plant species that form mycorrhizal symbiosis. Endomycorrhizae, a type of fungi located within plant roots, exhibit distinct characteristics that set them apart from other mycorrhizas, such as ectomycorrhiza. Notably, endomycorrhizae possess a specialized structure known as arbuscules, which resemble small trees within plant cells, along with vesicles. Arbuscules have a function in nutrients exchange by provide to their hosts (Kivlin et al. 2011), while the vesicles, where present are a storage organ. AMF also possess intraradical mycelium found inside the plant roots, and the collection of extraradical mycelium found outside the plant roots (Fellbaum et al. 2012). In plant-AMF symbiosis, the nutrient from soil translocated via extraradical hyphae of AMF to plant, in return AMF receive up to 30% of the host plant photosynthate (Drigo et al. 2010) and fixed organic carbon to the AMF (Lanfranco et al. 2018). This carbon supply is essential for the growth and survival of the fungi (Bonfante and Genre 2010). In addition to its role in nutrient uptake, the extraradical mycelium also plays an important part in spore formation and the initial stages of root colonization (Brundrett et al. 1996).

2.2 Plant-AMF interaction mechanism

The interaction between plants and mycorrhizal fungi is mediated by various factors, including plant strigolactones and mycorrhizal factor (myc factors) (Ruyter-Spira et al. 2011). This molecular crosstalk leads to the production of a penetration apparatus by the fungus (Oldroyd 2013). Strigolactones are plant hormones that play a crucial role in the establishment of mycorrhizal symbiosis (Brewer et al. 2013). They are involved in the signaling between plants and AMF, promoting the branching of fungal hyphae and the colonization of plant roots (Foo et al. 2013). Strigolactones

are also known to regulate shoot and root architecture, as well as plant development (Brewer et al. 2013). The production of strigolactones by plants is believed to be enhanced in order to encourage mycorrhizal symbiosis, a mutually beneficial association between plants and fungi which helps in the acquisition of phosphate from the soil (Brewer et al. 2013).

On the other hand, myc factors are signaling molecules produced by mycorrhizal fungi that play a role in the recognition and colonization of plant roots. These factors are recognized by specific receptors in the plant, leading to the activation of signaling pathways that promote the formation of mycorrhizal symbiosis. The interaction between myc factors and plant receptors is crucial for the successful establishment of mycorrhizal symbiosis (Gobbato et al. 2012). In addition to strigolactones and myc factors, other signaling molecules and pathways have also been implicated in the regulation of mycorrhizal symbiosis. For instance, phytohormones such as auxin and abscisic acid have been found to interact with strigolactones in the regulation of mycorrhizal symbiosis (Foo et al. 2013; Charpentier et al. 2014). Calcium signaling has also been shown to play a role in the recognition and response to mycorrhizal fungi (Kosuta et al. 2003) (**Figure 2.1**). Furthermore, the specificity of mycorrhizal symbiosis has been found to be influenced by various factors, including plant genetics and the phylogenetic distance between plant species. Some research studies have shown that mycorrhizal relationships can be stronger between plant types that are not closely related (Montesinos-Navarro et al. 2019). This suggests that the compatibility between plants and mycorrhizal fungi is influenced by genetic factors and evolutionary background.

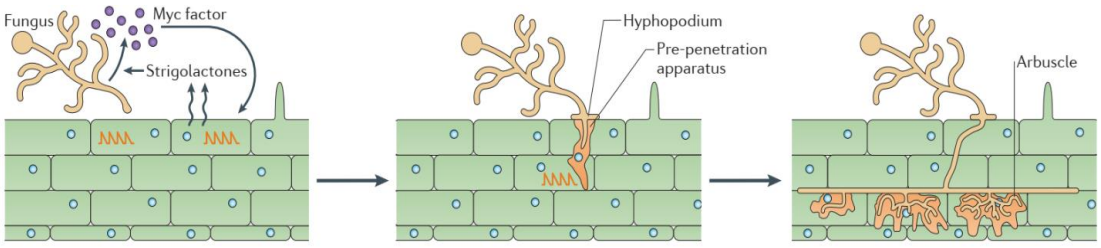

Figure 2.1 Plant release the root signals to arbuscular mycorrhizal fungi (AMF) in the rhizosphere (Oldroyd 2013).

2.3 Role of AMF in nutrients uptake

The hyphae of AMF extend into the soil, foraging for nutrients that are limiting to plant growth, such as nitrogen (N) and phosphorus (P) (van der Heijden et al. 2015). Phosphorus is a vital element for plant growth and development, and plants can absorb it in the form of phosphate ions (soluble form), such as monobasic orthophosphate ($H_2PO_4^{-1}$) and dibasic orthophosphate (HPO_4^{-2}) (Vessey 2003). AMF, with their efficient hyphae, aid in the absorption of phosphorus in the form of polyphosphate (Pi). It can also uptake nitrogen in form of ammonium (NH_4^+) and nitrate (NO_3^-) and transfer it to host plant. This is because AMF have a much larger surface area than plant roots, creating an external mycelial network and can also extend beyond the root zone (**Figure 2.2A**). AMF can also access nutrients that are bound to soil particles or organic matter, which plant roots cannot access (non-soluble form). They also acquire organically bound nutrients (van der Heijden et al. 2015). Moreover, AMF can uptake trace elements that are required by plants in smaller quantities, such as calcium (Ca), zinc (Zn), copper (Cu), and potassium (K), involved in a wide range of biochemical and physiological processes, from structural support to enzymatic reactions, photosynthesis, and nutrient uptake (Khan et al. 2022). In return, the plant delivers the AMF with carbohydrates (C) (**Figure 2.2B**), creating a mutualistic relationship (Judy et al. 2015).

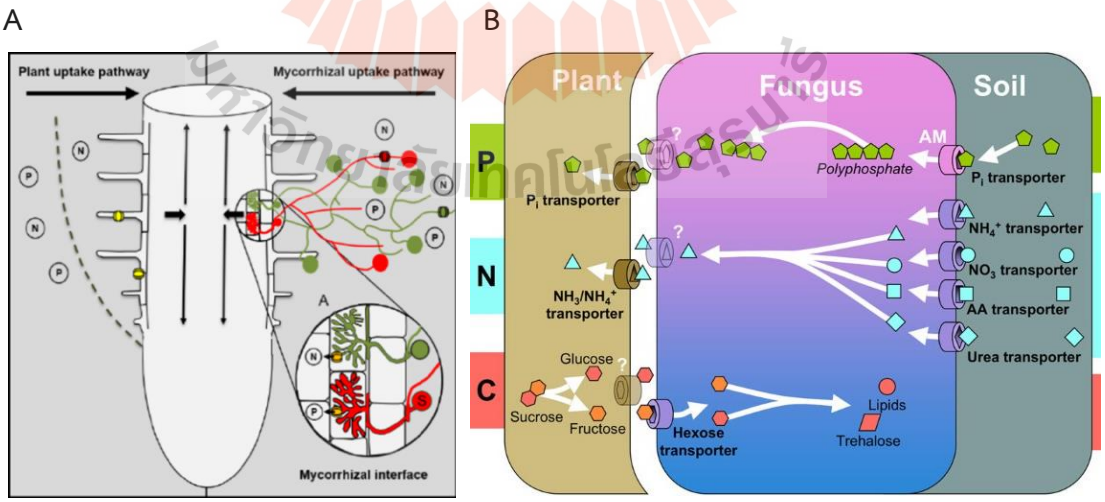

Figure 2.2 Nutrient uptake pathway between AM fungi in plant root (A) (Bücking and Kafle 2015) and nutrient transfer between plant and AMF (Bonfante and Genre 2010) during symbiosis.

2.4 Role of AMF in soil structure

AMF also play a key role in soil structure. AMF hyphae bind soil particles together, forming aggregates. These soil aggregates improve soil structure by buildup a macroporous structure. The presence of a macroporous structure enables the penetration of water and gases, consequently reducing the risk of soil erosion (Oehl et al. 2004). Additionally, AMF have been shown to improve soil formation and soil aggregation through their extensive network of hyphae, providing protection against soil erosion (Van Geel et al. 2018). Furthermore, AMF produce a variety of polysaccharides and proteins that contribute to soil aggregation (Wang et al. 2015). These compounds are known as glomalin, and they are very resistant to degradation. The production of glomalin by AMF is believed to indirectly contribute to soil aggregation (Řezáčová et al. 2021). Glomalin-related soil protein (GRSP), a glycoprotein synthesized by AMF, has the capacity to enhance the process of soil aggregate formation (Zhang et al. 2017). The external mycelia of AMF and their production of GRSP bind and enmesh soil particles together into larger aggregates, stabilizing the soil structure (Van Der Heijden et al. 2006). Glomalin also contributes to the preservation of organic carbon in the soil (Liang et al. 2015). It has been found that glomalin can facilitate the accumulation and preservation of soil organic carbon in tropical forests (Zhang et al. 2017). Additionally, glomalin has been shown to contribute to carbon and nutrient storage in deeper soils, with its association varying with different climates and soil properties (Wang et al. 2017). Therefore, the production of glomalin by AMF plays an important role in soil structure and the preservation of organic carbon in the soil (**Figure 2.3**).

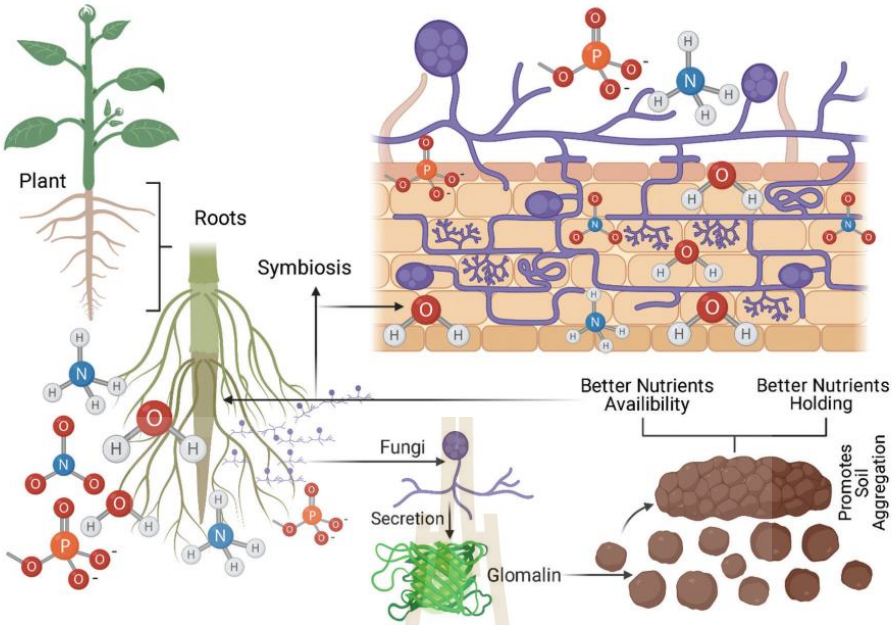

Figure 2.3 Regulation of nutrient and water in arbuscular mycorrhizal fungi (Van Der Heijden et al. 2006).

2.5 Role of AMF in soil structure

Plants can benefit from both abiotic and biotic stresses due to the AMF. Abiotic stress is stress caused on by non-living organisms such as drought, salinity, and heavy metals, whereas biotic stress is stress caused on by organisms such as pathogens (Jansa et al. 2008). Abiotic stresses such as salt stress (Evelin et al. 2009) drought stress (Wu et al. 2017), and heavy metal stress (Yang et al. 2015) may be made tolerant to plants by AMF. By stabilizing dehydrated enzymes and membranes and guarding biological components from desiccation damage, AMF can increase plant tolerance to salt stress (Evelin et al. 2009). AMF also contribute to sucrose and proline metabolisms under drought stress, enhancing plant tolerance to drought (Wu et al. 2017). Additionally, through enhancing resource supply, energy fluxes, and stress tolerance mechanisms, AMF (*Diversispora epigaea*, *Glomus aureum*, *Rhizophagus irregularis*, and *R. clarus*) could help reduce the deleterious effects of heavy metals in *Iris pseudacorus* (W**ęż**owicz et al. 2015).

In term of biotic stress such as bacterial pathogens, fungal pathogens, virus, nematodes and herbivores (Liu et al. 2014; Yang et al. 2015; Miozzi et al. 2020). AMF

can also induce changes in plant metabolism and gene expression, which leads to the production of defense-related compounds (Ricigliano et al. 2015; Miozzi et al. 2020) such as pathogenesis-related proteins and phytoalexins, which can inhibit or suppressed the pathogen growth. Moreover, AMF can stimulate the production of abscisic acid (ABA) in plant roots, which can suppress defense responses against biotrophic pathogens (Cameron et al. 2013), reduction of root damage caused by pathogens and nematodes (Liu et al. 2014). Therefore, the presence of AMF can help plants develop a stronger immune system, leading to resistance to both biotic and abiotic stress conditions, a phenomenon also known as priming (**Figure 2.4**).

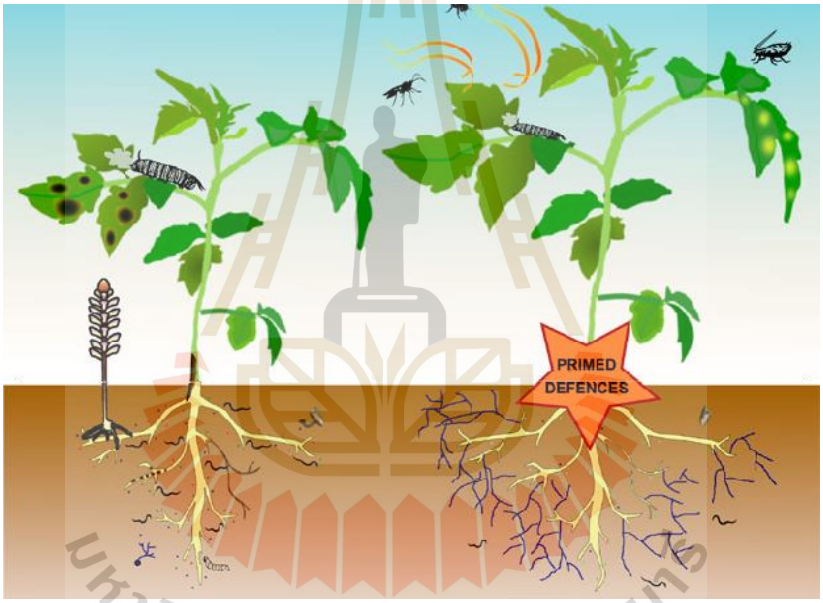

Figure 2.4 Arbuscular mycorrhizal fungi (AMF) induce plant priming against pathogens (Jung et al. 2012).

2.6 Mass production of AM fungal inoculum

Over the past decade, the utilization of AMF inoculations has gained prominence as a viable biotechnological approach in plant production. Consequently, a distinct sector has formed, characterized by its unique set of practices and considerations. There is a growing number of instances showcasing the positive influence of mycorrhiza on crop production, especially AMF. The estimation of company that produced AMF production, there are 15 companies (including a

small-medium company) in Europe producing mycorrhizal fungi (Mordor Intelligence, 2023). There are several supports for the development of AMF technology, driven by the growing demand for sustainability and organic products. The production of microbial inoculum is a multifaceted process that requires the acquisition of required biotechnological knowledge and the establishment of standardized protocols to ensure consistent quality of the inoculum. The establishment of globally acknowledged quality assurance protocols is a crucial step in achieving standardization of mycorrhizal products (Vosátka et al. 2012). The production systems of AMF have undergone significant evolution in recent years, transitioning from relatively simple technologies are more intricate and complex methods. Over the course of several decades, numerous technologies have been developed for the purpose of producing AMF on a large scale. There are two main systems for AMF production. A different system may have different advantages and disadvantages. Nowadays, the production system for AMF spore inoculum can be produced in both of *in vivo* and *in vitro* technologies (**Figure 2.5**) and both of them was performed *in planta*.

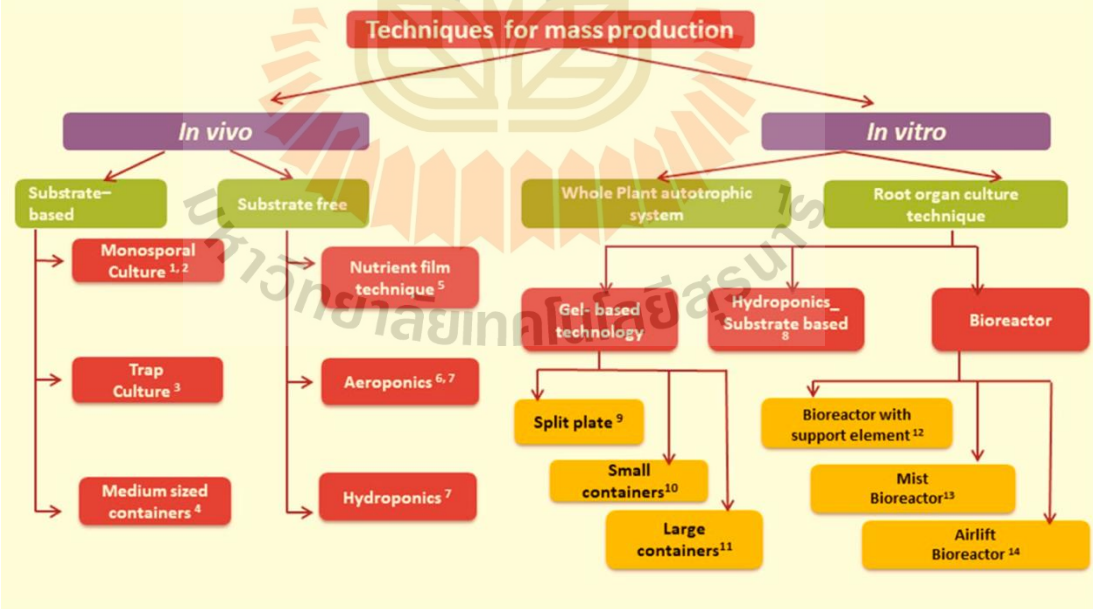

Figure 2.5 Classification of techniques used for mass production of AM fungi (Sudheer et al. 2023).

1) *In vivo* systems, the AMF inocula are grown under the greenhouse taken together with plant host. There are 2 sub-class of this technique by first substrate-based method; this method involved the cultivation of AMF using soil or other organic substrates in the pots. This system has been widely used for large-scale production of AMF (IJdo et al. 2011). It has been shown that the abundance and functioning of AM fungi can be limited in agricultural soils, and the application of fungal inoculum can reverse this (Verbruggen et al. 2013). Secondly, substrate free method without the use of soil substrates such as nutrient film, aeroponics, and hydroponic systems also can be used for mycorrhizal propagation (Hung and Sylvia 1988; Jarstfer et al. 1998; Mohammad et al. 2000). Based on the hyphthesis that nutrients spraying or nutrient film development enhances aeration for improved mass transfer, gaseous exchange, and mycorrhizal growth in *in planta* systems. However, on-farm production of AM fungal inoculum has also been considered feasible and cost-effective (Schwartz et al. 2006). Futhermore, the efficacy of AMF inoculum may exhibit variability contingent upon various aspects, including the specific plant species, soil attributes, and habitat conditions (Pellegrino et al. 2012).

2) *In vitro* systems, composted of whole plant autotrophic system and root organ culture technique (ROC). ROC technique has been wildly used to produce AMF inoculum because of this technique can provides a pure and contamination-free inoculum in a smaller space, making it more efficient than conventional pot culture multiplication methods (Tiwari and Adholeya 2003). It has been widely used in research on the biology and ecology of AMF, allowing for *in vivo* studies and the formulation of *in vitro* AMF inoculants was patented (ROMERO and Pastor 2007). At present, many methodologies have been employed by academic and corporate entities, including INVAM, Premier Tech, and GINCO, to facilitate the cultivation of mycorrhizal fungi on a global scale. Interestingly, the addition of certain fatty acids to asymbiotic cultures has been found to promote spore production in AMF. Due to the lack of fatty acid synthase in AMF, exhibit a deficiency in endogenous fatty acid synthesis. Notably, recent research by Sugiura et al. (2020) reported that the using of myristate (C14:0) added into asymbiotic cultures can significantly increase the mass production of *R. irregularis*. Additionally, previous studies have demonstrated that the addition of myristate as a carbon and energy substrate in asymbiotic cultures can

enhance the growth of *R. clarus* biomass (Tanaka et al. 2022). This technique has also been developed for spore production in *Rhizoglomus intraradices* (Tanaka et al. 2022). However, it is important to emphasize that this technique is primarily aimed at achieving a higher purity of AMF. Nonetheless, it comes with the limitation of producing smaller daughter spores compared to spore production in symbiotic culture with host plant.

The various systems can be characterized as follows: the first system is the *in vivo* manufacturing system, which represents a more advanced substrate-based production approach. This technology is extensively utilized and primarily serves as a cost-efficient method for the mass production of AMF inoculum that is suitable for large-scale application. Nevertheless, the spore production of AMF utilizing this particular approach has a low output, as indicated in **Table 2.1**. In addition, substrate-free production systems, including as nutrient film, hydroponic, and aeroponic systems, have been created for the purpose of generating AMF inoculum that is comparatively free of contaminants. One of the limitations associated with this technique was the increased expense. The utilization of production systems has primarily been confined to smaller-scale applications and research purposes (**Table 2.2**). In conclusion, the *in vitro* cultivation systems can be categorized into two main approaches: excised root organ cultures (ROC) and complete autotrophic plants, as well as an asymbiotic technique that does not include the presence of a plant. Although the present costs of these systems are considerable, they ensure the generation of pure AMF without any contaminants. (**Table 2.3**). However, the substrate-free cultivation systems and the *in vitro* cultivation systems are especially for use the large-scale arbuscular mycorrhizal production or in industry. So, the classical system can be applied to use in many countries form the small-scale to large-scale of AMF production.

Table 2.1 Spore production of substrate-based production system (IJdo et al. 2011).

Authors	Plant host	AM fungus	Main substrate[a]	Method	Production
Gaur and Adholeya 2002	*Zea mays*	Consortium of indigenous *Glomus*, *Gigaspora*, and *Scutellospora* spp. (inoculum used on all plant species)	Sandy loam/compost	Raised beds	100 IP/g/substrate
	Medicago sativa				105 IP/g/substrate
	Trifolium alexandrium				80 IP/g/substrate
	Avena sativa				70 IP/g/substrate
	Sorghum vulgare				110 IP/g/substrate
Gaur and Adholeya 2000	*Zea mays*	*Glomus intraradices* (DAOM181602)	Perlite	Pots	600 IP/100 ml substrate
			River sand		880 IP/100 ml substrate
			Charcoal		550 IP/100 ml substrate
			Coal marl		400 IP/100 ml substrate
			Clay-brick granules		880 IP/100 ml substrate
Douds et al. 2005	*Paspalum notatum*	Noninoculated	Vermiculite/compost	Raised beds	830 propagules/cm^{-3}
		Glomus mosseae			707 propagules/cm^{-3}
		Glomus etunicatum			465 propagules/cm^{-3}
		Glomus claroideum			365 propagules/cm^{-3}
		Glomus geosporum			2,150 propagules/cm^{-3}
		Glomus intraradices			950 propagules/cm^{-3}
		Gigaspora gigantea			465 propagules/cm^{-3}
		Gigaspora rosea			21 spores/cm^{-3}
Douds and Schenck 1990b	*Paspalum notatum*	*Acaulospora longula* (INVAM316)	Sandy soil	Pots	1,000 spores/cm^{-3} soil
		Scutellospora heterogama (INVAM117)			14 spores/cm^{-3} soil
		Glomus intraradices (INVAM208)			80 spores/cm^{-3} soil
		Gigaspora margarita (INVAM105)			49 spores//cm^{-3} soil
		Gigaspora margarita (INVAM185)			25 spores/cm^{-3} soil
		Gigaspora margarita (INVAM597)			6 spores/cm^{-3} soil
		Gigaspora margarita (INVAM680)			7 spores/cm^{-3} soil
Gryndler et al. 2003	*Allium ampeloprasum*	*Glomus claroideum* (BEG23)	Sand/Cambisol	Pots	2,010 spores/plant
	Plantago lanceolata	Mix of 3 *Glomus* spp	Sand/Cambisol		163 spores/g soil
	Lactuca sativa	*Glomus mosseae* (BEG25)	Sand/vermiculite		1,447 spores/plant
Millner and Kitt 1992	*Zea mays*	*Glomus etunicatum*	Sand	Pots/drip irrigation	341 000/spores per pot
		Glomus mosseae (INVAM156)			210 000/spores per pot
		Gigaspora margarita			64 800/spores per pot
Sylvia and Schenck 1983	*Paspalum notatum*	*Glomus clarum*	Limed loamy sand	Pots	55 spores/g soil
		Glomus mosseae			15 spores/g soil
		Glomus etunicatum			14 spores/g soil
		Glomus macrocarpum			31 spores/g soil
		Gigaspora margarita			20 spores/g soil
		Gigaspora heterogama			700 spores/kg soil
		Gigaspora gigantea			120 spores/kg soil

IP infectious propagules

[a] Substrate amendments and watering/fertilization regimes are not detailed in this table

Table 2.2 Spore production of *In vitro* production systems (IJdo et al. 2011).

Authors	Host type	Host species	Fungal Code	Culture (weeks)	Method	Approximate production/unit
St-Arnaud et al. 1996	ROC	*Daucus carota*	DAOM 181602	16	Petri plate (split)	15,000/Petri plate
Jolicoeur et al. 1999	ROC	*Daucus carota*	Not coded	12	Airlift bioreactor	12,400 spores/bioreactor
Declerck et al. 2001	ROC	*Daucus carota*	MUCL 41833	15	Petri plate	8,400 spores/Petri plate
Douds 2002	ROC	*Daucus carota* (DC1)	DAOM 181602	28	Petri plate (split)[a]	65,000 spores/initial culture
Elsen et al. 2003	ROC	*Daucus carota*	MUCL 41833	18	Petri plate	16,800 spores/Petri plate
Gadkar et al. 2006	ROC	*Daucus carota* (DC2)	DAOM 181602	8	Container	No data on sporulation
Voets et al. 2009	Plant	*Medicago truncatula*	MUCL 41833	4	Petri plate[b]	7,300 spores/Petri plate
Voets et al. 2005	Plant	*Solanum tuberosum*	MUCL 43194	22	Petri plate	12,250 spores/Petri plate
IJdo et al. 2010	Plant	*Medicago truncatula*	MUCL 49410	12	Petri plate	7,200 spores/Petri plate

DC1, 2 Daucus carota transformed roots, clone 1 and clone 2

[a] Medium was regularly renewed

[b] Plants were preinoculated with AM fungus

2.7 Other factors affect AMF development *in planta*

2.7.1 Mycorrhizal Helper Bacteria (MHB)

To improve the efficiency of AMF inoculum production under substrate-based system, the approach of using some helper bacteria to facilitate AMF propagation has been proposed. Mycorrhiza helper bacteria (MHB) are a diverse group of bacteria that interact with mycorrhizal fungi and plants play a crucial role in promoting mycorrhizal establishment and functionality (Lladó et al. 2017). These Mycorrhizal helper bacteria (MHB) belonged to a wide range of genera *Burkholderia, Paenibacillus, Pseudomonas, Bacillus, Streptomyces* and *Bradyrhizobium* (Miozzi et al. 2020; Chitarra et al. 2016) They have been discussed in terms of both tropical and temperate ecosystems (Founoune et al., 2002). Furthermore, according to Duponnois and Plenchette (2003), the concept of "mycorrhiza helper bacteria" is not just applicable to the ectomycorrhizal symbiosis but also to the endomycorrhizal symbiosis. Interestingly, the functioning of mycorrhiza, a symbiotic association between plant roots and fungi, is modulated by MHB (Lladó et al. 2017). These bacteria have been shown to stimulate the formation of mycorrhizal associations with host plants (Xie et al. 2018). The positive effects of MHB on mycorrhizal fungi have been widely acknowledged (Bonfante et al. 2019). They interact with both ectomycorrhizal fungi and AMF (Bonfante et al. 2019). MHB have been shown to stimulate

mycelial growth and mycorrhiza formation (Scheublin et al. 2010). They promote the growth of ectomycorrhizal fungi and improve the quality of the substrate (Carrasco and Preston 2020). MHB also enhance the level of mycorrhization between plant roots and fungi. The stimulation of presymbiotic fungal growth by MHB leads to an increase in root-fungus contacts and colonization (Sundram et al. 2011; Mediavilla et al. 2016).

The mechanisms by which MHB promote mycorrhizal establishment and functionality are not fully understood. However, several proposed mechanisms have been suggested. MHB in a group of *Cellvibrio*, *Chondromyces*, *Flexibacter*, *Lysobacter*, and *Pseudomonas* may improve mycorrhizal root colonization and stimulate extraradical hyphal growth of *G. geosporum* and *Glomus constrictum* (Roesti et al. 2007). They may also facilitate AMF spore germination (Roesti et al. 2007). Some MHB have been found to solubilize poorly soluble calcium phosphates and whether silicates, which can benefit mycorrhizal fungi (Uroz et al. 2007). MHB can also induce differential gene expression in ectomycorrhizal fungi, leading to enhanced mycorrhizal symbiosis (Schrey et al. 2005). The interactions between MHB and mycorrhizal fungi have significant implications for sustainable agriculture (Johansson et al. 2004). In addition, *Bacillus amyloliquefaciens* can enhance the growth and biomass of host plants by promoting mycorrhizal associations (Xie et al. 2018). They can also improve the establishment and functionality of *G. mosseae* BEG12 symbiosis, leading to increased nutrient uptake and growth of *Medicago truncatula* (Pivato et al. 2009). MHB play a crucial role in facilitating this symbiotic interaction. However, there is no report about the group of chemical substances produced from MHB that stimulate these positive effects on plant-AMF symbiosis. Therefore, it is interesting to elucidate these effective substances to be used as mycorrhization helper molecules to support the plant-AMF symbiosis and the spore production.

2.7.2 Effect of light quality on plant growth and AMF growth

Plant can respond with sink stimulation of photosynthesis when colonized with AMF and other bacterial root symbionts. The carbohydrate as a carbon source of plant will be allocated to the microbes and microbes turn back of some nutrients to their plant host. In terms of light limitation, once plants cannot

extensively increase photosynthesis, it may also affect to microbes (Xie et al. 2018). Several studies reviewed that light is the main factor that has an important for plant growth and development including microbial responsive. For example; Wang and Folta (2013) investigation on green light and a change in the proportion of blue to green to red to green, green light may help plants adapt to growing beneath foliage. In 2013, Olle and Viršile conducted an experiment to see how LED lighting affected the growth, yield, and nutritional value of transplanted tomatoes, cucumbers, and sweet peppers. They discovered that far-red light is crucial for plant photomorphogenetic processes, including the encouragement of plant development (Olle and Viršile 2013). On the other hand, using microbiological preparations for growing plants may have a number of advantages. Recently, the researcher has been reported about quality on AM fungi and they found that under the blue light LED was inhibit the AMF spore formation. In contrast, the red light LED was stimulated AMF spore and colonization (Cruz 2016). Therefore, the light quality has major effects on plant by increasing the photosynthetic rate and needed for some microbial development such as AMF (Jiang et al. 2017; Luginbuehl et al. 2017; Cheeld et al. 2020). However, the factors of light intensity, species of AMF and plant may be influenced the interaction under LED light.

2.7.3 Role of fatty acid in AM fungal growth

Fatty acids play a crucial role in the growth and development of AMF. AMF are known to be fatty acid auxotrophs, meaning they rely on their host plants to supply them with fatty acids for their growth and survival (Jiang et al. 2017; Luginbuehl et al. 2017; Cheeld et al. 2020). The host plants synthesize fatty acids, which are then transferred to the fungi to sustain their colonization (Luginbuehl et al. 2017). Recent research has elucidated the significance of fatty acids in the growth and development of arbuscular mycorrhizal (AM) fungi. The study conducted by Jiang et al. (2017) provided evidence supporting the notion that AMF are dependent on their host plants for the provision of fatty acids, since they lack the ability to synthesize these compounds independently. This reliance on external sources of fatty acids is crucial for the growth and successful colonization of AMF. The findings of the study indicate that fatty acids produced inside the host plants are transported to the fungi in order to support mycorrhizal colonization. This implies that fatty acids

play a crucial role in the creation and maintenance of the symbiotic association between AMF and plants. In addition, Rillig et al. (2020) discovered that myristate, a distinct fatty acid, exhibits potential as a carbon and energy substrate for the asymbiotic proliferation of AMF. The research findings indicate that the application of external myristate stimulates the growth of presymbiotic hyphae in *Gigaspora* spp., resulting in the formation of numerous lateral branches along the major germ tubes. This observation suggests that myristate is involved in the initial phases of AMF development and branching.

Besides myristate, various additional fatty acids have also been involved in the formation of AMF. Rillig et al. (2020) reported the impact of two specific 2-hydroxy fatty acids, namely 2OH-C14:0 and 2OH-C12:0, on the formation of presymbiotic hyphae in *Gigaspora* spp. The fatty acids were found to stimulate the development of numerous lateral branches along the primary germ tubes. Nevertheless, no morphological alterations were observed in *R. irregularis*. This implies that distinct AMF may have diverse reactions to particular fatty acids. The involvement of fatty acids in the formation of AMF extends beyond their direct impact on fungal development. The study conducted by Trépanier et al. (2005) demonstrates the significant involvement of fatty acid metabolism in the obligatory biotrophism of AMF. According to the study conducted by Trépanier et al. (2005), It has been shown that the activity of fatty acid synthase in AMF is exclusively expressed within the intraradical mycelium. This finding suggests that the metabolic process of fatty acids plays a crucial role in the biotrophic lifestyle of AMF, as they rely on the plant host for the production of palmitic acid. Furthermore, the investigation of fatty acid synthesis and lipid metabolism has been conducted in the obligate biotrophic fungus *R. irregularis* during its establishment of a mutualistic symbiotic association with *Lotus japonicus* (Brands et al. 2018).

The study indicated that lipid metabolism, including storage lipid production and fatty acid profile, plays a crucial role in the symbiotic connection between AMF and plants. This finding provides additional evidence for the significance of fatty acids in the formation and development of AMF. Since fatty acid is very important for AMF life cycle, the organic material (that contain natural fatty acid) is the one kind of materials that can try to apply for stimulation AMF spore germination, colonization

and spore production in the production system. Therefore, the potential of fatty acids or the organic compound containing fatty acids on promoting AMF spore production were also investigated in this study.

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CHAPTER III

Secretion compounds from *Brevibacillus* sp. SUT47 promote spore propagation of *Acaulospora tuberculata* colonizing maize roots (*Zea mays* L. cultivar Suwan 5)

3.1 Introduction

AMF have been widely distributed to terrestrial plants and have a potential role to improve plant nutrients (de Marins and Carrenho 2017). AMF can be used as a biofertilizer to increase plant yield in eco-agricultural system (Berruti et al. 2016). The need of AMF inoculants has continuously increased due to their potential support on plant growth through water and mineral nutrient acquisition that is particularly suitable for the situation of growing plant under climate changes. Nevertheless, the bottle neck of AMF application is the insufficient supply of AMF inoculants due to difficult, laborious, and time-consuming process of AMF inoculant production. The most convenience with low-cost AMF propagation technique is the substrate-based system, which is normally used for farmers in developing countries. However, the propagation rate is usually low.

Previously, the mycorrhization helper bacteria (MHB) which are selective to the fungal species and adapted to live in the close vicinity of the mycorrhizal fungus and their function were reported to be involved in mycorrhiza formation (Bowen and Theodorou 1979; GARBAYE 1994; Deveau and Labbé 2016). MHB can promote the AMF infection in the different stages during tripartite of bacterium-fungus-plant interaction. For example, at pre-infection phase including spore germination and hyphal growth was enhanced by MHB (Bowen 1994; Schrey et al. 2005). The exudate of MHB is one of the factors capable of stimulating fungal spore germination. Many researchers reported that some rhizospheric bacteria and their culture filtrates were able to stimulate the spore germination and hyphal growth of *Glomus mosseae*, *G. fistulosum*, and *G. versiforme* (Mosse 1962; Mayo et al. 1986; Azcon-Aguilar et al. 1986; Gryndler and Vosátka 1996). Not only MHB but also some of plant growth

promoting rhizobacteria (PGPR) were reported to promote biomass of AMF (Frey-Klett et al. 2005). However, few reports have been focused on the application of these bacteria to promote the propagation of AMF spores for inoculant production.

Previously, Piromyou et al. (2011) reported an effective PGPR, *Brevibacillus* sp. SUT47 as a biofertilizer for forage corn (*Zea mays* L.), and this strain also played the role on the enhancement of the colonization and spore production of *Acaulospora tuberculata* in maize roots (*Zea mays* L. cultivar Suwan 5) under the substrate-based system (Yuttavanichakul et al. 2018). However, the drawback of this technique when applied for AMF inoculum production is the presence of *Brevibacillus* sp. SUT47 cells contaminated in AMF inoculum, and it may not be appropriate if the high purity of AMF spores is needed. To solve this problem, the aim of this present study is to develop the new methodology using the cell free culture of strain SUT47 (the secretion compounds) to increase the spore propagation of *A. tuberculata*, which has been widely used as a biofertilizer (Li et al. 2013; Dalli et al. 2019; DJOCGOUE et al. 2019; Ntengna et al. 2019) for a better quality of AMF inoculant production.

3.2 Objective

To develop the new methodology using the cell free culture of *Brevibacillus* sp. SUT47 (the secretion compounds) to increase the spore propagation of *A. tuberculata*, which has been widely used as a biofertilizer.

3.3 Materials and methods

3.3.1 Preparation of bacterial cells and secretion compounds

The starter culture of *Brevibacillus* sp. SUT47 was prepared in Luria-Bertani (LB) broth. Then, 0.1% (v/v) of the fresh culture (OD_{600} = 1.0; approximate cell number at 10^8 CFU/ml) was inoculated into 1000 ml LB broth and incubated on the shaker at 28°C, 150 rpm for 20 h before separating the bacterial cells and the supernatant (secretion compounds) using centrifugation 6,000 rpm for 5 minutes. The culture at 20 h after inoculation was in the late log phase of growth, which is the duration that cells may secrete and accumulate the highest amount of

metabolites in the medium while the cells are still active. The secretion compounds were filtered twice using Nalgene filter funnel (Thermo Scientific) combined with nylon membrane with pore size of 0.45 µm and 0.2 µm, respectively, to get rid of all bacterial cells. Then, the cell free-culture was subjected to freeze-drying process to concentrate the secretion compounds by taking out 100 ml of the culture and transferring to 500-ml CHRIST freeze-drying flasks. The program of freeze-drying machine (Christ/Gamma2-16 LSCplu, Germany) was set at the shelf temperature of −70°C with vacuum at 0.110 mbar for 28 h. The crude powder of the secretion compounds was collected into dry tubes and stored at −80 °C until use. The fresh LB broth medium was also freeze-dried and used as control while the bacterial cells were washed twice and dissolved in normal saline (0.85% NaCl (w/v)) by adjusting the bacterial cell density equal to 10^8 cells/ml and used for further experiment.

3.3.2 AMF inoculant production under substrate based system

The AMF inoculant production test was conducted using pot culture technique under the controlled greenhouse condition at Suranaree University of Technology (14°52021.400, E:102°1020.200) during Jan–Apr, 2018. Maize (*Zea mays* L. cultivar Suwan 5) was used as a host plant. The seeds were surface sterilized with 3% (v/v) of sodium hypochlorite (NaOCl) for 10 min. Then washed with sterilized water with several times and germinated in a sterilized paper towel. After 3 days, 1 seedling was transplanted into the pot (1 L) containing the mixture by volume of sterilized vermiculite:sand (1:1). Seedling was inoculated with 100 spores of *A. tuberculata* (FJ687415) (School of Biotechnology, Suranaree University of Technology, Thailand) per pot. One ml containing 10^8 cells of SUT47 was co-inoculated once with AMF as a positive control treatment while different concentrations (90 180 270 360 and 540 mg/ml/plant) of SUT47 secretion compounds were applied to determine the effect on promoting AMF spore propagation. The same amount of freeze-dried LB broth at each concentration was included in the experiment as a control. Each treatment was performed with 6 replications compared with AMF-inoculated plant. Plants were grown under the greenhouse with the light 14 h/10 h and temperatures at 30±4°C/28±4°C Day/night cycle. Plant nutrients of 400 ml were exactly irrigated every 3 days with a half strength of modified Hoagland solution with 100 µM Pi (Djocgoue et al. 2019).

3.3.3 Data collection

Experimental design using randomized completely block design (RCBD). Post inoculation at 3 months, 3 replications of each treatment were harvested to determine plant biomass, chlorophyll content, and histological analysis. The chlorophyll contents were measured using a spectrophotometer (SPAD502 Plus) on the third and fourth leaves (from top). The plant shoots and roots were harvested and dried at 70°C for 48 h (Shipleyl and Vu 2002). Mycorrhizal infection was observed using the trypan blue staining method revealed by Phillips and Hayman (Phillips and Hayman 1970) and the percentage of colonization was observed using gridline intersect method (Giovannetti and Mosse 1980). The remaining plants were further dried by stop watering for 1 month before collecting AMF spores.

3.3.4 AMF spore counting and statistical analysis

The AMF spores were extracted from the substrate in each pot using the wet-sieving method followed by sucrose gradient centrifugation (Jenkins 1964; Dandan and Zhiwei 2007). The statistical analyses were performed using SPSS 16.0 statistical software package (SPSS Inc., Chicago, IL, USA). Data analyses were carried out using Tukey's honest significance test with $p<0.05$ significance level to determine the significance of differences between the treatments.

3.4 Results

Plant biomass was determined at 3 months after inoculation, and the data were presented in **Table 3.1**. The plant growth according to the shoot dry weight and root dry weight was not significantly different among the treatments. Plant acquisition of nutrients revealed in root/shoot ratio and the chlorophyll content also showed no significant difference. Thus, there was no direct effect of nutrients from LB broth, secretion compounds as well as the application of whole cell strain SUT47 on plant growth. The fungal structure and root colonization efficiency were also determined in the maize roots. There was no significant difference in the AMF root colonization efficiency among all treatments (**Figure 3.1** and **Table 3.1**).

Then, the number of AMF spore was determined at 4-months-old plant. The results showed that co-inoculation of AMF with the cells of strain SUT47

produced the highest number of spore propagation. The number of AMF spores was 1.2 times higher than that of the control (AMF inoculation alone) (**Figure 3.2**). Interestingly, the co-inoculation of AMF with strain SUT47 secretion compounds also correlatively increased the number of AMF spores up to a certain concentration of the SUT47 secretion compounds. However, the number of AMF spores was lower than that of co-inoculation using SUT47 cells.

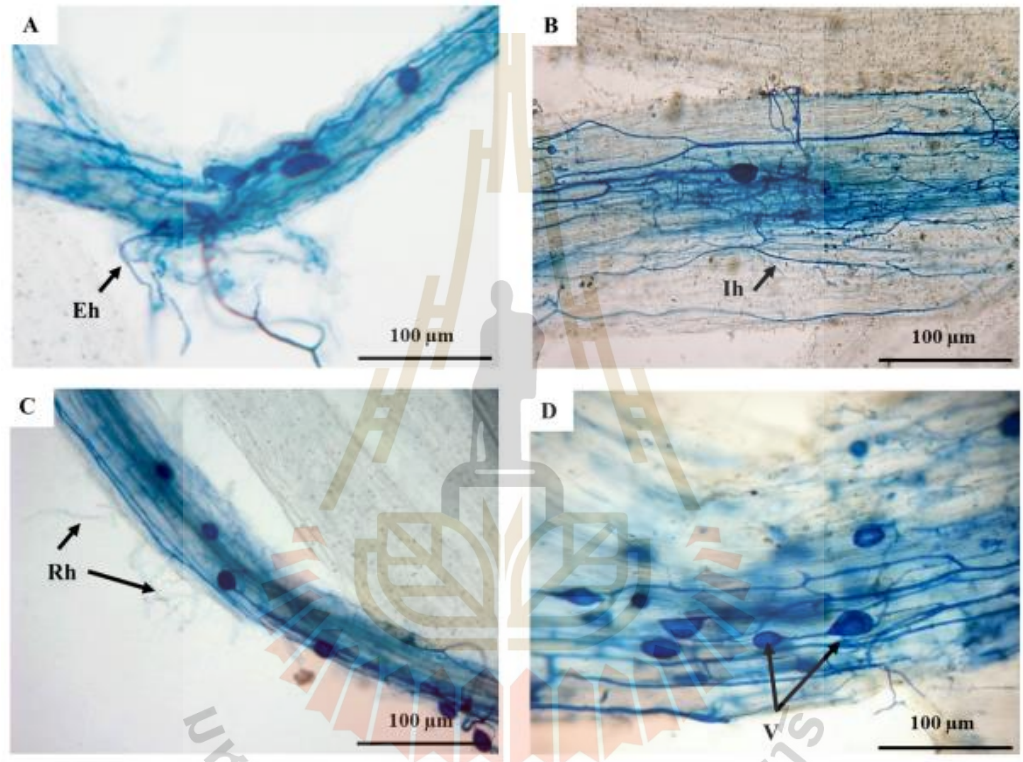

Figure 3.1 Trypan blue staining of the fungal colonization in maize roots by *A. tuberculata*. Trypan blue staining of the roots was used to visualize fungal structures at 90 dpi under compound microscope, (**A**) extracellular hyphae; Eh, (**B**) intracellular hyphae; Ih, (**C**) maize root hair; Rh, and (**D**) vesicle; V.

Then, the number of AMF spore was determined at 4-months-old plant. The results showed that co-inoculation of AMF with the cells of strain SUT47 produced the highest number of spore propagation. The number of AMF spores was 1.2 times higher than that of the control (AMF inoculation alone) (**Figure 3.2**). Interestingly, the co-inoculation of AMF with strain SUT47 secretion compounds also

correlatively increased the number of AMF spores up to a certain concentration of the SUT47 secretion compounds. However, the number of AMF spores was lower than that of co-inoculation using SUT47 cells. Based on the application of secretion compounds, the significant increase of AMF spores was found when applying the secretion compounds at 270 and 360 mg which increased the spore number of *A. tuberculata* up to 1,463 and 1,590 spores/plant, respectively. The co-inoculation of AMF with LB medium at different concentrations was also performed, and the result showed that all concentrations of LB medium significantly reduced the number of AMF spores when compared to the plant inoculated with AMF alone. Therefore, the AMF spore propagation may be inhibited by some composition contained in LB medium that may have an effect mainly on spore germination. Nevertheless, some concentrations of strain SUT47 secretion compounds could significantly increase the number of AMF spores when compared to control. This result revealed the effect of some compounds secreted from strain SUT47 that can increase the AMF spore propagation.

Table 3.1 Biomass production of maize Suwan 5 and fungal colonization at 3 months; inoculation with/without Luria Bertani medium and secretion compounds at the different concentrations.

Sample	Concentration (mg)	Plant biomass				Fungal colonization (%)
		Chlorophyll content (SPAD units)	Shoot DW (g)	Root DW (g)	R/S ratio	
AMF (−)	–	18.64 ± 2.42	18.91 ± 1.47	15.52 ± 0.86	0.74 ± 0.06	–
AMF (−) + SUT47	–	19.06 ± 0.73	20.23 ± 1.34	14.34 ± 0.51	0.71 ± 0.04	–
AMF (−) + Secretion	90	18.46 ± 0.50	18.42 ± 1.47	15.52 ± 0.94	0.75 ± 0.01	–
AMF (−) + Secretion	180	17.75 ± 0.72	17.75 ± 1.04	16.78 ± 0.17	0.95 ± 0.07	–
AMF (−) + Secretion	270	18.34 ± 0.78	18.34 ± 0.56	17.56 ± 0.99	0.96 ± 0.08	–
AMF (−) + Secretion	360	19.42 ± 0.79	19.42 ± 0.50	17.23 ± 1.60	0.89 ± 0.10	–
AMF (−) + Secretion	450	17.95 ± 1.32	17.90 ± 1.30	16.62 ± 0.76	0.82 ± 0.02	–
AMF (−) + Secretion	540	17.62 ± 1.24	17.62 ± 1.27	16.53 ± 1.42	0.94 ± 0.09	–
AMF (+)	–	20.83 ± 0.38	21.38 ± 0.48	14.60 ± 0.80	0.68 ± 0.04	87.00
AMF + SUT47	–	23.93 ± 1.52	21.40 ± 0.61	15.61 ± 0.63	0.73 ± 0.05	92.67
AMF + Luria Bertani	90	21.73 ± 0.28	23.21 ± 2.81	20.37 ± 1.11	0.88 ± 0.05	79.67
AMF + Luria Bertani	180	27.20 ± 2.77	23.21 ± 2.81	18.37 ± 2.27	0.80 ± 0.08	80.67
AMF + Luria Bertani	270	20.00 ± 0.65	25.71 ± 1.90	19.39 ± 1.28	0.76 ± 0.05	86.33
AMF + Luria Bertani	360	17.73 ± 0.68	21.76 ± 0.25	17.89 ± 1.23	0.82 ± 0.08	82.00
AMF + Luria Bertani	450	21.93 ± 1.60	23.31 ± 1.82	17.38 ± 0.69	0.75 ± 0.05	79.33
AMF + Luria Bertani	540	19.87 ± 0.75	24.01 ± 0.44	18.95 ± 2.52	0.79 ± 0.01	79.00
AMF + Secretion	90	21.20 ± 2.88	25.06 ± 1.18	15.25 ± 2.52	0.61 ± 0.02	81.67
AMF + Secretion	180	19.60 ± 2.15	20.81 ± 0.71	16.52 ± 1.11	0.79 ± 0.01	78.00
AMF + Secretion	270	19.33 ± 0.64	20.55 ± 1.07	15.63 ± 2.27	0.75 ± 0.06	90.67
AMF + Secretion	360	19.50 ± 0.57	20.71 ± 1.01	13.14 ± 1.28	0.63 ± 0.06	81.33
AMF + Secretion	450	19.50 ± 1.01	20.22 ± 0.36	15.64 ± 1.23	0.77 ± 0.03	81.33
AMF + Secretion	540	19.36 ± 0.33	20.27 ± 1.86	17.34 ± 0.69	0.86 ± 0.04	79.67

*Root and shoot ratio (R/S)

AMF (–), no AMF inoculation; AMF (+), inoculation with *A. tuberculata*; +SUT47, inoculation with SUT47 cells; +secretion, inoculation with SUT47 secretion compounds; +Luria Bertani, inoculation with Luria Bertani medium. The number corresponds with specific plant biomass. Data are presented as averages±standard error of mean (s.e.m.) from 3 biological replicates. DW indicates plant dry weight (g/plant).

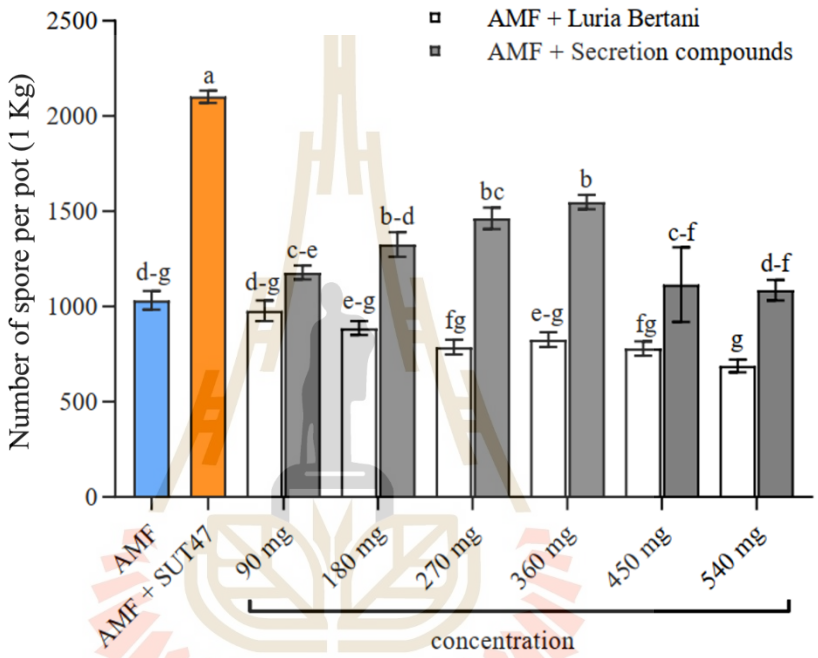

Figure 3.2 Number of AMF spore formation at 4 months; inoculated with AMF alone (blue bar), co-inoculated with strain SUT47 cell (orange bar), and co-inoculated with different concentrations of Luria Bertani medium (white bars) or strain SUT47 secretion compounds (grey bars). Extra-spore isolated from the substrate mixture of sand: vermiculite (1:1) contained in the pot. Data are reported as averages and standard error of mean (s.e.m.) in triplicate. Same letters above the bars indicate no significant difference among treatments. Statistical analysis was determined using Tukey's HSD test ($P<0.05$).

3.5 Discussion

In this study, a new technique using the secretion compounds from strain SUT47 was developed to promote the spore propagation of (*A. tuberculate*.) strain SUT47 was discovered to promote AM root colonization and spore number of *A. tuberculata* in maize. This bacterium also has an influence on the alteration of some plant defense-related compounds and antioxidative enzyme activities associated with an enhanced maize root colonization by AMF and may result in the increase of the spore production (Yuttavanichakul et al. 2018). Therefore, the strain SUT47 has potential to stimulate AMF spore propagation for the purpose of enhancing AMF inoculum production efficiency under substrate-based production system.

In this study, the activation of AMF spore number produced per plant using the secretion compounds from the strain SUT47 was focused to avoid the contamination of its bacterial cells in the AMF inoculum. It was found that at the specific concentration of secretion compounds of strain SUT47 in range of 270–360 mg/plant could promote the spore number of *A. tuberculata* (**Figure 3.2**). This result indicates that the secretion compounds of strain SUT47 contain some substances that may interact with plant or interact directly with AMF and activate the propagation of AMF spores. These interactions seem to be dose responses of the specific beneficial compounds. However, too much concentration of those substances could interfere this beneficial interaction. Nevertheless, the deep biochemical experiments are further required to identify the beneficial compounds and their interaction on AMF or plant, and the structure of main active compound is required to be investigated. However, the number of spores produced from maize treated with secretion compounds was still significantly less than that of plant treated with the living cells of strain SUT47. It could be possible that the colonization of living bacterial cells on maize root could benefit continuously secreted beneficial compounds at suitable amount along with plant growth which is probably better than one time treated with concentrated secretion compounds. Moreover, colonization of living cells could have more interactions with plant through several plant hormones produced from bacteria that may require the signaling from plant or AMF in tripartite interactions and may results in supporting the AMF spore production. Recently, the effective signaling compounds in broth culture of PGPR had potential to be a biostimulant by

promoting seed germination and plant growth at early stage (Backer et al. 2018). Several PGPR are known to excrete hormones such as IAA as well as cytokinin and gibberellin that can enhance plant growth (Ruzzi and Aroca 2015; Shailendra Singh 2015). However, this study showed that co-inoculation of AMF with strain SUT47 living cells or their secretion compounds did not affect plant growth. Although strain SUT47 has been reported to promote the growth of forage corn (Piromyou et al. 2011), this strain when applied together with AMF on maize did not promote plant growth (Yuttavanichakul et al. 2018). It is likely possible that these beneficial secretion compounds could directly affect AMF sporulation and spore propagation since there is no effect on the AMF colonization (**Figure 3.2**). It has been reported that inoculation of *Klebsiella pneumoniae* on sea oats (*Unicola paniculata*) increased spore germination and hastened branching of *Glomus deserticoloa* (Will and Sylvia 1990). Nevertheless, the active compounds from strain SUT47 secretion are interesting to identify. The crude proteins, fatty acids, and sugar secreted by the bacteria may be the target and may be separately used to identify the active fraction, which can be verified with plant-AMF symbiosis, and the active compounds may be identified using LC-MS. The identification of sugar or fatty acid profiles of bacterial secretion should also be characterized. The imitate of fatty acid or sugar composition profile as derived from bacterial secretion compound may support AMF growth.

3.6 Conclusion

This study showed the application of secretion compounds from SUT47 at the optimal concentration to stimulate spore production of *A. tuberculata* in maize roots. Although the spore number was lower than that of using living cells of SUT47, this technique may be appropriate for high quality AMF inoculant production to avoid the contamination of bacterial cells. However, the preference or the compatibility among plant host, AMF, and bacterial species could be an obstacle when using this technique to produce other species of AMF inoculant. Thus, the finding of specific compounds that commonly activate the colonization and spore production of various AMF species and their behind mechanisms still remains to be further investigated.

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CHAPTER IV

Unveiling the Tripartite Synergistic Enhancement of Plant-Arbuscular Mycorrhizal Fungus Symbiosis by endophytic *Bacillus velezensis* S141 in *Lotus japonicus*

4.1 Introduction

Plant growth promoting rhizobacteria (PGPR) are a group of beneficial bacteria that enhance plant growth and development (Oleńska et al. 2020; Vocciante et al. 2022; Bhat et al. 2023). These beneficial bacteria, including various species of *Alcaligens*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Pseudomonas*, *Klebsiella*, *Enterobacter*, *Burkholderia,* and *Bacillus*, are often found in association with plant roots (Figueiredo et al. 2010; Bashan et al. 2014; Singh et al. 2019), and some of them have been identified for their potential applications as biofertilizers. The growth-promoting effects contributed by these bacteria are mediated through diverse mechanisms, for example, nitrogen (N) fixation (Liu et al. 2019; Matse et al. 2019), phosphate solubilization (Prabhu et al. 2019; Amy et al. 2022), production of plant growth hormones, and biocontrol of plant pathogens (Odoh et al. 2019; Chauhan et al. 2021).

AMF also provide significant benefits to plant growth via their symbiotic associations with plant roots (Genre et al. 2018). The symbiotic relationship is established by a complex and sophisticated mechanism between AMF and the host plants. The interaction begins with the release of signaling molecules, such as chitooligosaccharides (COS) and lipochitooligosaccharides (LCO) from the AMF, which are then recognized by plant receptors on the root surface (Feng et al. 2019; Allen Rush et al. 2020). This recognition event activates symbiotic signaling pathways in the plant that result in the development of specialized fungal structures, called arbuscules, within the cortical cells of the plant root. Arbuscules contribute to the supply of nutrients, such as phosphorus (P) and N, to the plant (Smith 1988; George et al. 1995; Smith et al. 2011). AMF have also been shown to improve plant growth,

enhance plant resistance to environmental stress by assisting in osmolyte accumulation, ion uptake, and improvement in soil fertility via enhancement of water and mineral nutrition through their mycelial hyphae (Kempel et al. 2010; Mitra et al. 2021).

In the rhizosphere, synergistic interactions between PGPR and AMF have been observed to enhance plant growth and performance through an increase in AMF colonization, regulation of plant hormones, such as auxin and cytokinin, and inhibition of plant pathogens (Tsukanova et al. 2017; Emmanuel & Babalola 2020). For example, co-inoculation of soybean with *Bacillus amyloliquefaciens* and AMF led to an increase in AMF colonization, which is postulated to have contributed to improvements in plant growth and yield (Sheteiwy et al. 2021). It was also demonstrated that the application of PGPR *Brevibacillus* sp. SUT47 in combination with the AMF, *Acaulospora tuberculata*, to maize resulted in an increase in AMF colonization and spore production compared to a single inoculation of AMF (Yuttavanichakul et al. 2018; Kiddee et al. 2020). Recently, Begum et al. (2022) showed that *B. methylotrophicus* co-inoculated with AMF produced an increase in photosynthetic activity and mineral, osmolyte, and phytohormone content in tobacco. These increases positively affected secondary metabolite content and antioxidant system under drought stress conditions and resulted in enhanced tobacco growth.

B. velezensis is a recently characterized PGPR species that has shown potential as a biocontrol agent (Adeniji et al. 2019; Rabbee et al. 2019). For example, *B. velezensis* FZB42 has been reported to produce secondary metabolites with antimicrobial properties (Fan et al. 2018). The other strain S141 of *B. velezensis*, which were isolated from soybean rhizospheres in Thailand, efficiently controlled cercospora leaf spot in mung bean (Prakamhang et al. 2015; Songwattana et al. 2023). In soybean-*Bradyrhizobium* symbiosis, *B. velezensis* S141 colonized the nodule and root surface and induced the production of larger nodules and enhanced nitrogen-fixing efficiency by *B. diazoefficiens* USDA110 (Sibponkrung et al. 2020). *B. velezensis* S141 has a set of genes for auxin biosynthesis in its genome (Sibponkrung et al. 2017). After inoculating the disrupted strains of those genes into soybean-*Bradyrhizobium*, symbiosis attenuated the effects of *B. velezensis* S141 on increasing nodule size and

promotion of N-fixing activity. These findings suggest that this specific PGPR strain may play an important role in enhancing the symbiotic functionality between rhizobia and legumes through its phytohormone producing properties.

In contrast, it remains to be elucidated whether *B. velezensis* S141 exerts an influence on plant–AMF symbiosis. Phytohormones, including auxins, have been shown to modulate the development of the mycorrhizal symbiosis (Gutjahr 2014; Pozo et al. 2015; Liao et al. 2018). Etemadi et al. (2014) demonstrated that overexpression of miR393, an miRNA that specifically targets a plant auxin receptor gene, resulted in suppression of arbuscule formation, suggesting a necessary role for auxin perception in arbuscule development. In this situation, it was hypothesized that *B. velezensis* S141 could affect the symbiotic relationship between plants and AMF through its potential to produce auxins. To test this hypothesis, this investigated the effects of *B. velezensis* S141 and its mutant strains containing disrupted auxin biosynthesis genes on AMF development both intra- and extra-radically were investigated in addition to on host growth using a model mycorrhizal system with AMF *Rhizophagus irregularis* and the model legume *Lotus japonicus*. In addition, the gene expression of symbiosis-related genes in both AMF and host plant was analyzed.

4.2 Objective

To investigate the effects of *B. velezensis* S141 and its mutant strains containing disrupted auxin biosynthesis genes on AMF development both intra- and extra-radically in addition to on host growth using a model of mycorrhizal-plant system with AMF *Rhizophagus irregularis* and the model legume *Lotus japonicus*.

4.3 Materials and Methods

4.3.1 Biological materials and growth conditions

L. japonicus B-129 seeds were surface-sterilized with a 3% (w/v) sodium hypochlorite solution for 10 min and then thoroughly rinsed with sterile distilled water. The sterilized seeds were germinated on moist paper in a Petri dish and incubated at 26°C in the dark for two days followed by three more days under light.

Seedlings were transplanted into 50-ml centrifuge tubes (one plant per tube), each with a drainage hole at the bottom and filled with autoclaved river sand (particle size, 0.5–2.0 mm). The plants were inoculated with approximately 250 spores of *R. irregularis* DAOM197198 (Mycorise, Premier Tech, Rivière-du-Loup, Canada). The plant growth-promoting rhizobacterium *Bacillus velezensis* S141 (Sibponkrung et al. 2017) was cultured in Luria-Bertani (LB) broth at 28°C for 20 h and used as inoculum. Derivative mutant strains of *B. velezensis* S141 (*ΔIPyAD*, *ΔyhcX* and *ΔdhaS*), which have a reduced production of indole-3-acetic acid (IAA), were cultured under the same conditions but in the presence of antibiotics: (1) 1 mg L^{-1} erythromycin, (2) 10 mg L^{-1} kanamycin, and (3) 100 mg L^{-1} spectinomycin (Sibponkrung et al. 2020). Bacterial cells were separated by centrifugation and washed twice with 0.85% (w/v) NaCl. The cell density was then adjusted to OD_{600} = 1 (approximately 10^8 CFU/ml). The bacterial cells at different cell concentrations (10^5, 10^6, and 10^7 CFU in 50 ml) were then inoculated or co-inoculated with *R. irregularis* in soil. Non-inoculated control plants were also included in the experiment by adding them with 50 ml of 0.85% (w/v) NaCl. Plants were grown in a growth chamber at 28°C under a 16/8-hour light and dark cycle and a light intensity of 150 µmol m^{-2} s^{-1}. The plants were supplied with a half-strength Hoagland's solution containing a low concentration of KH_2PO_4 (100 µM) every two days. Plants were harvested at 60 days after inoculation (DAI) and separated into shoot and root parts. The plant parts were dried at 70°C for 48 h and weighed according to the method described by Shipley et al. (2002). At 30 and 60 DAI, plant height was measured above the ground level. Chlorophyll content was estimated in fully expanded leaves from the top as SPAD units using SPAD-502 Plus (Konica Minolta Optics, Japan). The experiment was designed using the randomized completely block design (RCBD) and the experiment was performed with four replicates for each treatment.

4.3.2 Analysis of plant P and N contents

Plant shoots were dried at 70°C for 48 h, ground, and then weighed. For phosphorus (P) analysis, the samples underwent digestion in an acid mixture (HNO_3+$HClO_4$), and P concentration was determined using the vanadomolybdate blue method (Watanabe and Olsen, 1965). For nitrogen (N) analysis, the plant samples were digested in a Kjeldahl digestion apparatus with the temperature gradually

increased to 200°C and then further raised to 350 to 375°C for 1 h until a clear solution was obtained. N concentrations were determined using the Kjeldahl method (Yash and Kalra, 1998).

4.3.3 Localization of bacteria in roots

L. japonicus were inoculated with *B. velezensis* S141 at a concentration of 10^5 CFU/plant or co-inoculated with both *B. velezensis* S141 and 250 spores/plant of *R. irregularis*. These plants were grown under the conditions described above. At 7, 15, 30 and 45 DAI, roots were harvested and thoroughly washed with sterilized water to remove sand particles. These roots were then cut into fine segments, each 1–2 cm in length. The segments were embedded in 5% agarose gel and sectioned into 40 µm thick slices using a Leica vibratome (VT1000S, Leica Microsystems, Germany). The sections were stained for 10 min with 5 µM SYTO 9 (Thermo Fisher Scientific, USA) to visualize live bacterial cells and with 0.01% (w/v) Calcofluor (Bonaldi et al. 2011) to highlight the cell walls of plant roots and AMF. The stained sections were washed with phosphate-buffered saline (PBS), mounted on a glass slide with 10% PBS-glycerol solution, and covered with a coverslip. The Calcofluor was excited at 405 nm, and its emission signal was detected using a 460–500 nm filter for image acquisition. SYTO 9 fluorescence was detected by exciting the samples with a 488 nm laser line and collecting the emission signal at 490 to 522 nm. Bacterial localization was observed using a Nikon inverted Eclipse Ti-E Confocal Laser Scanning Microscope (Nikon, Japan).

4.3.4 Enumeration of endophytic bacteria

The roots of *L. japonicus* inoculated with and without S141 (green fluorescent protein [GFP]-tagging strain) as described by Sibponkrung et al. (2020) were subject to surface sterilization by immersing roots in a 3% (w/v) NaClO solution for 5 min followed by a 5-min soak in 70% ethanol. The sterilized roots were then thoroughly rinsed with sterile distilled water at least five times (Pongdet et al. 2015). Plant roots were ground with 2 ml of 0.85% NaCl followed by preparing serial dilutions ranging from 10^0 to 10^4. Subsequently, 100 µl of each dilution was plated onto LB agar supplemented with 8 µg/ml phleomycin and incubated at 28°C for 20 h. The number of endophytic bacteria was calculated as colony forming units (CFU)/plant.

4.3.5 Assessment of AMF colonization

The root system of *L. japonicus* was collected and carefully cleaned by removing all soil particles. The cleaned roots were cut into 1–2 cm segments and washed with tap water. To remove the cytoplasmic content of the plant root cells, these root segments were treated with 10% (w/v) potassium hydroxide (KOH) at 90°C for 10 min followed by acidification with 2% (v/v) hydrochloric acid (HCl) for 5 min. The segments were subsequently stained with 0.05% trypan blue in lactic acid at 90°C for 10 min. The degree of AMF colonization was assessed using the method described by Trouvelot et al. (1986) with some modifications. Briefly, approximately 10 root fragments from each plant were mounted on glass slides and observed under a light microscope with a 10 × objective. The intensity of AMF colonization and the abundance of arbuscules in a field of view were categorized into six and three classes, respectively. Based on the scores obtained from 100 fields of view, five parameters of AMF colonization (F%, M%, m%, A%, and a%) were calculated according to Trouvelot et al. (1986). F% represents the frequency of mycorrhiza in the root system, M% represents the intensity of the mycorrhizal colonization in the root system, m% represents the intensity of the mycorrhizal colonization in the root fragments, A% represents the arbuscule abundance in the root system, and a% represents the arbuscule abundance in the mycorrhizal parts of the root fragments.

4.3.6 Quantification of AMF in roots by quantitative PCR

Inoculated root samples of *L. japonicus* were collected, and total DNA was extracted using the Dneasy® Plant Mini Kit (Qiagen, USA) according to the manufacturer's instructions. Quantitative real-time polymerase chain reactions (qPCR) were run using a StepOne Real-Time PCR System (Thermo Fisher Scientific) with approximately 50 ng of each DNA sample used to estimate fungal abundance. A primer set specific for *R. irregularis* was used for qPCR. The set consisted of a forward primer (CCCACCAGGGCAGATTAATC), a reverse primer (3'-TGGCTTTGTACAGGCAACAG-5'), and a TaqMan-MGB probe (197198P: 3'-FAM-CCCTGGAGTATCTG-MGBEQ-5' synthesized by Eurofins Genomics, Japan). This primer set was designed based on the single-copy *cox3-rnl* intergenic region in mitochondrial DNA (Badri et al. 2016). As an internal control, a primer set designed for genomic DNA of the *L. japonicus* ubiquitin-conjugating enzyme E2 gene (LotjaGi1g1v0401300.1) was used. The primer set for this

gene consisted of a forward primer (3'-AAATGGACGGCTCTTATCAAGGT-5'), a reverse primer (3'-GACCGGTCGAACATCTTACACA-5'), and a TaqMan-MGB probe (3'-FAM-TGCTGGCTAATATGC-MGBEQ-5'). Data were normalized relative to this internal control and analyzed according to the $2^{-\Delta\Delta}$Ct method (Livak and Schmittgen 2001).

4.3.7 Enumeration of *R. irregularis* spores in soil

AMF spores were extracted by wet sieving and decanting (Gerdemann and Nicolson 1963; Daniel and Skipper 1982). All soil contained in a 50 ml tube was collected and mixed with 100 ml of tap water in a 500 ml beaker. The soil sample was agitated vigorously to disperse the AMF spores and left to stand for 5 min to allow the heavier soil particles to settle to the bottom. The supernatant was decanted through standard sieves with pore sizes of 250, 106, 75, and 38 µm. The AMF spores and soil particles retained on the 106 75 and 38 µm sieves were collected in 15 ml centrifuge tubes and centrifuged at 4,000 rpm for 5 min. After removing the supernatant, 40% (w/v) sucrose solution was added and centrifuged at 5,000 rpm for 5 min. The supernatant was carefully removed and poured into sieves to retain the AMF spores. The spores on the sieves were rinsed with tap water and collected in Petri dishes. AMF spores were counted under a stereomicroscope.

4.3.8 Gene expression analysis

Total RNA was extracted using RNA Prep Pure Plant Plus Kit (TIANGEN Biotech, China). To remove DNA contamination, the RNA samples were treated with Dnase I (Thermo Fisher Scientific). cDNA was synthesized from the RNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) following the manufacturer's instructions. Quantitative RT-PCR was conducted using Luna Universal qPCR Master Mix (New England Biolabs, USA) and the CFX Opus 96 Real-Time PCR System (Bio-Rad, USA). The primers for this system are listed in **Table 4.1** and **4.2**. As an internal control for gene expression, the elongation factor 2 (*EF2*) was used in *L. japonicus* and *EF1β* was used in *R. irregularis* (Kobae et al. 2015). Three biological replicates were performed, each using approximately 5 ng of cDNA template. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Table 4.1 Primers of marker genes for *L. japonicus*.

Gene	Primer sequence (5'-3')	Reference
LjEF2	GGTGGCTGCTGCTGGAATTA	Nguyen et al. 2021
	ACGGGTATCAGTCATACGGACAT	
LjSbtM1	TGTATGCTGCTGCTGAAAAAAACAACT	Takeda et al. 2009
	CTTCTTGACCTTTTGCAATAAATGGGATTC	
LjRAM1	TGGAGGAAGATCATGGAAGG	Handa et al. 2015
	CAACCTGTACCCATCACACG	
LjHA1	TGGTAAAGCAACGAACTGCATT	Nguyen et al. 2021
	GCGGCCTGACGAGTTTCTC	
LjPT4	TCCGGGCTCTCCTTTGG	Kojima et al. 2014
	AGAAGCATAGCGTTCCCATCA	
LjAMT2;2	ACACATGCTTGCACTGCTACC	Guether et al. 2009
	CTGCCCATCCTTGAACAACCC	
LjFatM	TGAATCATCAGCAGCTACTACCACTA	This study
	TCTGGCGTTGGCCACTCT	
LjRAM2	GCCCGTAGCCATGGTGAA	Nguyen et al. 2021
	CCCTCTTGCAGTGGTTCCA	
LjSTR	CTATATTGGTGACGAGGGAAGG	Kojima et al. 2014
	GTCCTGAGGTAGGTTCATCCAG	

Table 4.2 Primers of marker genes for *R. irregularis*.

Gene	Primer sequence (5'-3')	Reference
RiEF1β	CCCATGCAGCTCGATGGTA	Kobae et al. 2015
	TGCCAGGAAGTGAAGAAAATGA	
RiH2	GCGTAAAGGAGCAAAAGCCATA	This study
	GTCCTGCCTTAACGCTTTTTGT	
RiH3	GGAAAAGCTCCCCGCAAA	This study
	AGCTGGAGCGCTTTTTCGT	
RiPolδ	GGAGAGAGCTCAAAACCTTGGTT	This study
	TTCGGAAGTCAGCGCATTT	
RiCDK1	GCTTGCCATTGATTTACTTTCGA	This study
	GCGCTTGGCAGAGATTCG	
RiPCNA	CGCGTTGGTATCTCTTTTGCT	This study
	CCTCGGTCACATCGGTATGG	
RiRNR	GCTGAACGTCTTGTAGCGTTTG	This study
	ACGCGAAAGCTCCAGAAAAG	
RiKIF	CACCCACGGTGGAAAAGC	This study
	GAATGCCTTGCTACTTCCTGTTC	
RiPPN1	GAAAGATTTAACAATACCGAGTTGGAT	Nguyen et al. 2021
	TGCGCTATTAAGTCCATCATTAACA	
RiPPN2	TCGACCACCTAATGCTTTTGG	Nguyen et al. 2021
	ATCGCAATTATATTCTCCCCATTTT	
RiPPN3	CCGAATAGTGACCCATCCAAA	Nguyen et al. 2021
	CATTCCGATTGTGGAGTTCCA	
RiPPN4	GGTTTATTTGACGGGTCACGTT	This study
	GCCAATCGTCCGTATCTTCTATAAC	
RiVTC1	GAGAATCAGGTCCTTATGATGATAGGT	Nguyen et al. 2021
	CACCCACGAGGAAAAAGCA	
RiVTC2	GCACGACAGACGTACAAGTTACAA	Nguyen et al. 2021
	AATGGGCACGCGTTCAAA	
RiVTC4	CATGGTGTCGCAACACTTATGG	Nguyen et al. 2021
	GTGGAAGCCAGAAAGGAAAAAG	
RiMST2	CCACGTTGGTTAGTTGATCATGA	This study
	GGATCGTAAATTTGCCAATACTGTT	

4.3.9 Statistical analyses

Statistical analyses were conducted using R software (version 4.2.2). Turkey's honestly significant difference (HSD) test was used for multiple comparisons. The effects of single inoculation with *R. irregularis* and co-inoculation with *R. irregularis* and *B. velezensis* S141 were compared using the Student's *t*-test. All data were visualized with GraphPad Prism (v.9.1.1).

Table 4.3 Growth of *L. japonicus* at 60 DAI under four inoculation conditions: non-inoculation (NI), inoculation with *B. velezensis* S141 (S141), inoculation with *R. irregularis* (AMF), and co-inoculation with both *R. irregularis* and *B. velezensis* S141 (AMF+S141).

	Plant height (cm)	Plant biomass (mg/plant)	
		Shoot DW[a]	Root DW
NI	18.1±1.2 cd	157±16 b	69±14 c
S141(10^5)[b]	16.0±0.8 d	155±19 b	67±7 c
S141(10^6)	19.1±0.6 c	170±8 b	68±5 c
S141(10^7)	20.1±0.3 bc	329±30 a	78±12 bc
AMF	17.9±0.3 cd	232±32 ab	109±9 abc
AMF+S141(10^5)	23.5±0.6 a	245±20 ab	141±18 ab
AMF+S141(10^6)	22.7±0.6 ab	308±41 a	162±23 a
AMF+S141(10^7)	19.7±0.5 bc	331±27 a	151±13 a

[a]DW: dry weight.

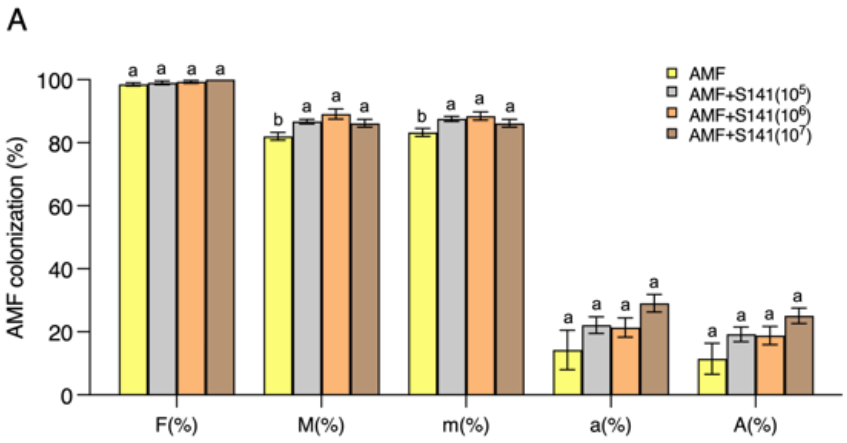
[b]10^5, 10^6 and 10^7 in parentheses represent the cell density of *B. velezensis* S141 (CFU/plant).

Values are means ± s.e.m. (n = 4). The same letter after the number in each column is not significantly different at $p<0.05$ on Turkey's HSD test.

4.4 Results

4.4.1 *B. velezensis* S141 enhances AMF abundance in the interior and exterior of *L. japonicus* roots

To investigate whether S141 could enhance the development of *R. irregularis* during its symbiosis with *L. japonicus*, the AMF abundance in roots was assessed and quantified spore production in soil. Co-inoculation AMF with S141 using different cell densities on *L. japonicus* and was measured the extent of AMF colonization at 60 DAI. The presence of S141 caused a slight but significant increase in the intensity of AMF colonization (M% and m%) compared to the absence of S141 (**Figure 4.1A**). This enhancement was consistently observed across all S141 inoculum cell densities. In contrast, the frequency of AMF colonization (F%) and arbuscular abundances (A% and a%) were not affected by inoculation with S141. AMF abundance in roots was further quantified using a hydrolysis probe-based qPCR assay specifically targeting *R. irregularis* DAOM197198 mitochondrial DNA as described by Badri et al. (2016). The relative copy number of the *cox3-rnl* intergenic region consistently increased more than 2-fold in the co-inoculation treatments compared to AMF single inoculation (**Figure 4.1B**). Notably, S141 also promoted AMF spore production in soil (**Figure 4.1C**). Regarding the inoculum cell density of S141 at 10^5, 10^6, and 10^7 CFU/plant, the co-inoculation produced an increase in the number of spores produced in soil by 39%, 19%, and 27%, respectively when compared to single inoculation with *R. irregularis*. These results show that *B. velezensis* S141 significantly promoted AMF development both inside and outside the roots.



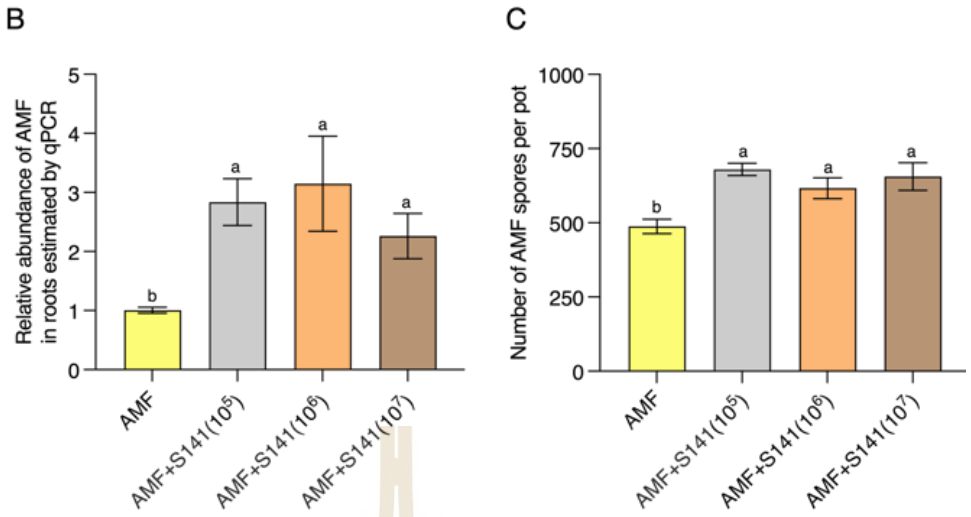


Figure. 4.1 The abundance of *R. irregularis* at 60 days after inoculation (DAI). *L. japonicus* was either inoculated with *R. irregularis* alone (AMF) or co-inoculated with both *R. irregularis* and *B. velezensis* S141 at different cell densities: 10^5 (AMF+S141(10^5)), 10^6 (AMF+S141(10^5)), 10^7 (AMF+ S141(10^5)) CFU/plant. (**A**) AMF colonization in *L. japonicus* roots. F(%), the frequency of mycorrhiza in the root system; M(%), the intensity of the mycorrhizal colonization in the root system; m(%), the intensity of the mycorrhizal colonization in the root fragments; a(%), the arbuscule abundance in the mycorrhizal parts of the root fragments; and A(%), the arbuscule abundance in the root system. (**B**) The relative abundance of *R. irregularis* in *L. japonicus* roots estimated by quantitative PCR (qPCR) using a hydrolysis probe designed from the *cox3-rnl* intergenic region in *R. irregularis* mitochondrial DNA. The abundance of *R. irregularis* in roots is normalized based on the copy number of the genomic region of the *L. japonicus* ubiquitin-conjugating enzyme E2 gene and expressed relative to that in plants singly inoculated with *R. irregularis* (AMF). (**C**) The number of *R. irregularis* spores produced in the soil. Values are means±s.e.m. (n = 4). Bars marked with the same letter are not significantly different at $p<0.05$ on Turkey's HSD test.

4.4.2 *B. velezensis* S141 affects mycorrhizal plant growth

The observed enhancement of AMF development in the presence of S141 may be due to the direct stimulation of plant growth by S141 thus increasing the benefit AMF receives from the plant. To determine the direct effect of S141 on *L. japonicus* growth, the plant was inoculated with different densities (10^5, 10^6, and 10^7 CFU/plant) of the bacteria in the absence of AMF. At 60 DAI, S141 did not affect the plant growth of *L. japonicus*. However, the bacterial cells at a density of 10^7 CFU/plant produce a significant increase in the shoots' dry weights by 2.1-fold compared to the non-inoculated plants (**Table 4.3**). Even in the presence of AMF, S141 produced virtually no significant growth promotion in *L. japonicus*. When the bacteria were inoculated at a cell density of 10^5 CFU/plant, the plant height increased significantly compared to the single AMF inoculation condition.

The 60-day growth period was probably too long to evaluate *L. japonicus* growth in a 50-ml pot. Therefore, the assessed the plant growth at 45 DAI was assessed. A density of 10^5 CFU/plant was selected for inoculation with *B. velezensis* S141, a density that was shown to be effective in enhancing plant height at 60 DAI (**Table 4.3**). Even at 45 DAI, a modest increase in AMF colonization, M% and m%, due to co-inoculation (**Figure 4.2A**) was found. No direct effect of S141 on *L. japonicus* growth was observed at 45 DAI (**Figure 4.2B, C**). Conversely, the effect of AMF inoculation was pronounced, namely plant heights and dry weights of AMF-inoculated plants were significantly greater than those of non-inoculated plants. In particular, the co-inoculation of S141 and *R. irregularis* led to a maximal shoot dry weight approximately 1.2-fold higher than that of the single AMF inoculation (**Figure 4.2C**). In addition, shoot nitrogen (N) and phosphorus (P) concentrations significantly increased with co-inoculation yielding 1.7- and 1.5-fold increases in N and P, respectively, relative to the single AMF inoculation (**Figure 4.2D**). These results indicate that S141 promotes plant growth and nutrient acquisition when AMF is present.

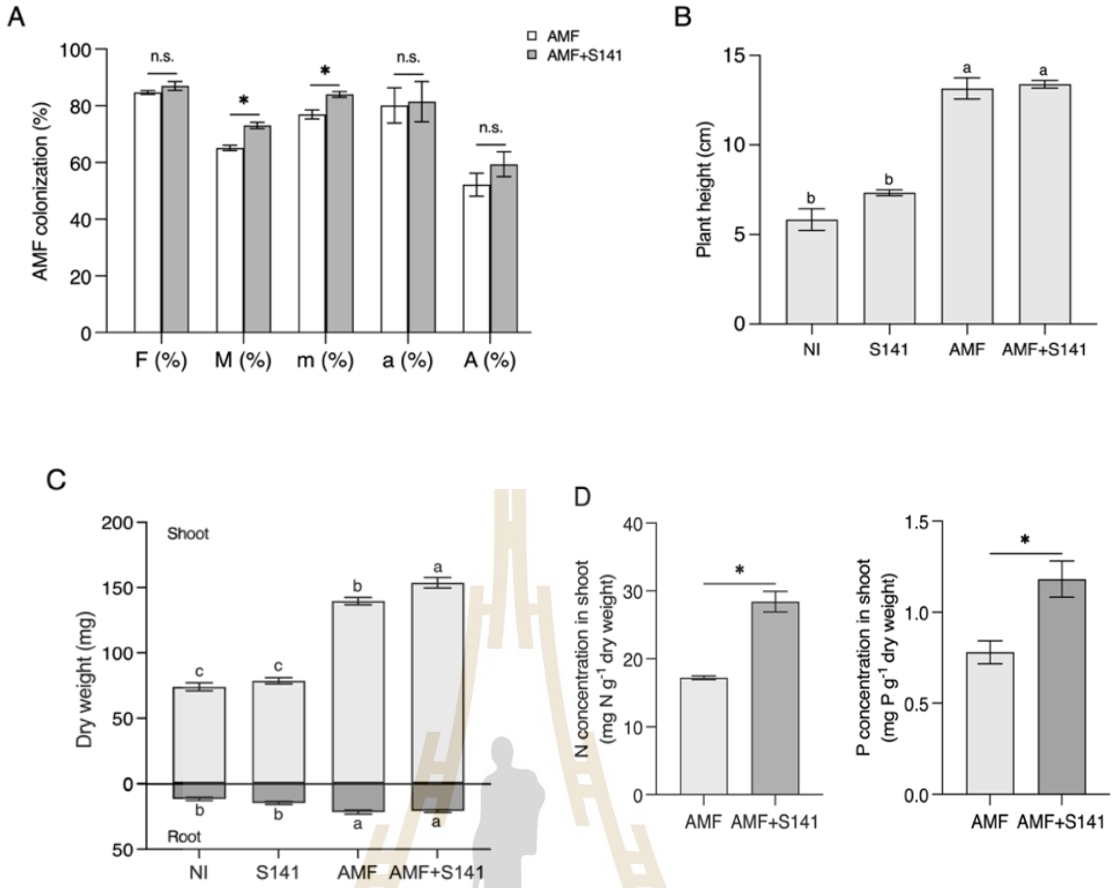


Figure 4.2 *R. irregularis* abundance and *L. japonicus* growth at 45 days after inoculation (DAI) under four inoculation conditions: non-inoculation (NI), inoculation with *B. velezensis* S141 (S141), inoculation with *R. irregularis* (AMF), and co-inoculation with both *R. irregularis* and *B. velezensis* S141 (AMF+S141). Cell density of *B. velezensis* S141 was 10^5 CFU/plant. (**A**) AMF colonization in *L. japonicus* roots. F(%), the frequency of mycorrhiza in the root system; M(%), the intensity of the mycorrhizal colonization in the root system; m(%), the intensity of the mycorrhizal colonization in the root fragments; a(%), the arbuscule abundance in the mycorrhizal parts of the root fragments; and A(%), the arbuscule abundance in the root system. Plant height (**B**) and dry weight (**C**) of *L. japonicus*. (**D**) The concentration of nitrogen (N) and phosphorus (P) in *L. japonicus* shoot. The values presented are means ± s.e.m. (n = 3). Student's t-test (n.s., not significant; *, p<0.05) was conducted in (**A**) and (**D**). Bars marked with the same letter are not significantly different at p<0.05 on Turkey's HSD test in (**B**) and (**C**).

4.4.3 B. velezensis S141 stimulates root elongation at early growth stage

To explore the possibility of a direct growth-promoting effect from S141 on *L. japonicus* during the early growth stages, the shoot and root elongation at 7 and 15 DAI. S141 produced no influence on shoot elongation (**Figure 4.3**). However, the length of tap roots inoculated with the bacteria was approximately double than that of non-inoculated plants at 15 DAI.

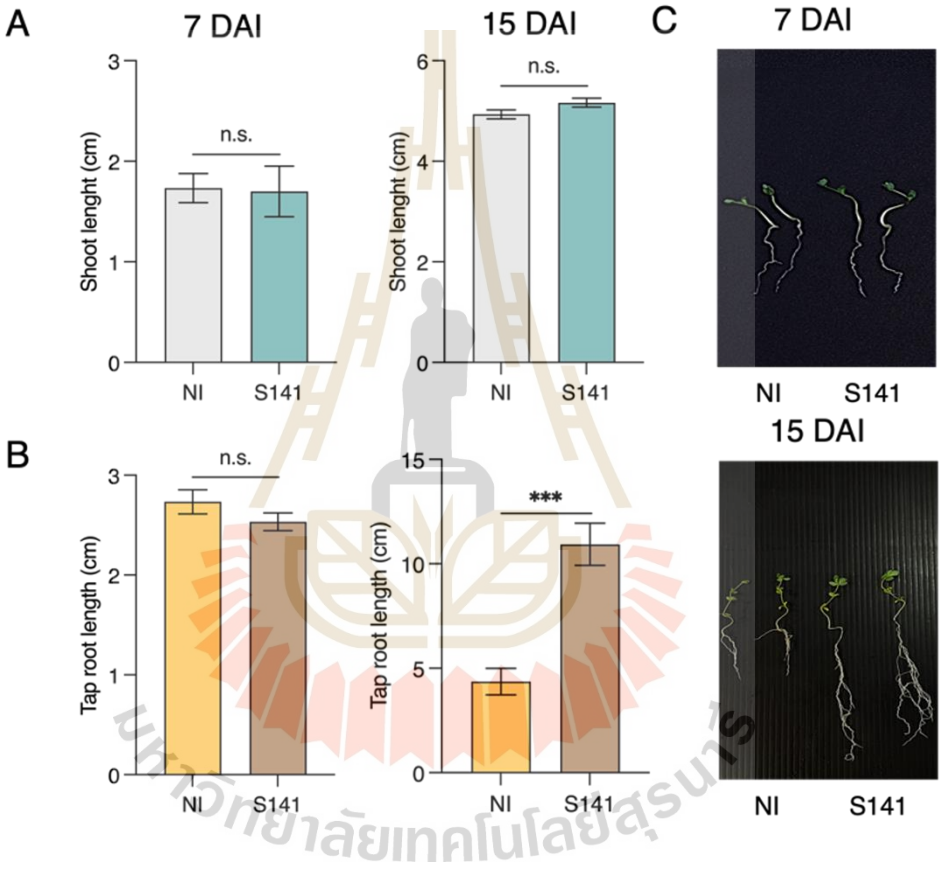


Figure. 4.3 Growth of *L. japonicus* inoculated with 10^5 CFU/plant of *B. velezensis* S141 (S141) or not inoculated (NI) at 7 and 15 days after inoculation (DAI). Graphs show shoot length (**A**) and tap root length (**B**). Values are means±s.e.m. (n = 4). The statistical analysis was performed using Student's t-test (n.s., not significant; ***, p<0.001). (**C**) Appearance of *L. japonicus* roots. Scale bars: 1 cm.

4.4.4 IAA production-related genes in *B. velezensis* S141 are not involved in promoting tap root elongation and AMF colonization

Given that S141 produces IAA (Sibponkrung et al. 2020), and this IAA could stimulate root elongation in *L. japonicus* and consequently, AMF colonization. Therefore, it was investigated whether the IAA produced by S141 supported root elongation using derivative mutants of S141 (***Δ****dhaS*, ***Δ****yhcX*, and ***Δ****IPyAD*) that yielded lower amounts of IAA than the wild type (Sibponkrung et al. 2020). The lengths of shoot and tap root of the plants that were inoculated with these mutant strains were comparable to those of plants inoculated with the wild type at both 7 and 15 DAI, indicating that the S141 *dhaS*, *yhcX*, and *IPyAD* genes do not significantly affect root and shoot elongation (**Figure 4.4A, B**). In addition, the abundance of AMF when co-inoculated with these mutant strains was evaluated. It was observed no significant differences in AMF colonization between inoculations of the wild type and mutant strains at 60 DAI (**Figure 4.4C**). Overall, IAA produced by *dhaS*, *yhcX*, and *IPyAD* of S141 does not appear to be a major factor in promoting tap root elongation and AMF colonization in *L. japonicus*.

4.4.5 *B. velezensis* S141 affects gene expression in both *L. japonicus* and *R. irregularis* during symbiosis

The changes in gene expression associated with the symbiotic response of *L. japonicus* and *R. irregularis* after inoculation with S141 were investigated. *L. japonicus* marker genes involved in the regulation of AMF accommodation within roots and nutrient exchange were assessed at 45 DAI. The expression of the subtilisin-like serine protease gene, *SbtM1*, which participates in the early phases of AMF colonization (Takeda et al. 2009), was enhanced 6-fold after co-inoculation with S141 compared to the single AMF inoculation (**Figure 4.5A**). The expression of the transcription factor gene *RAM1*, which is mainly responsible for arbuscule formation, was doubled. The mycorrhiza-specific H^+-ATPase *HA1*, the phosphate transporter (*PT4*) and the ammonium transporter (*AMT2;2*), all of which are involved in P and N uptake in arbuscule-containing cortical cells (Javot et al. 2007; Guether et al. 2009; Krajinski et al. 2014; Wang et al. 2014), showed elevated expression levels in the presence of S141. For genes implicated in fatty acid biosynthesis and lipid transport in arbuscular mycorrhizal symbiosis, S141 produced enhancement of expression of the glycerol-3-phosphate acyltransferase gene *RAM2* (Wang et al. 2012) while the

acyl-ACP thioesterase *FatM* (Bravo et al. 2017; Brands et al. 2018) and the ABC transporter gene *STR* (Zhang et al. 2010) did not undergo enhancement.

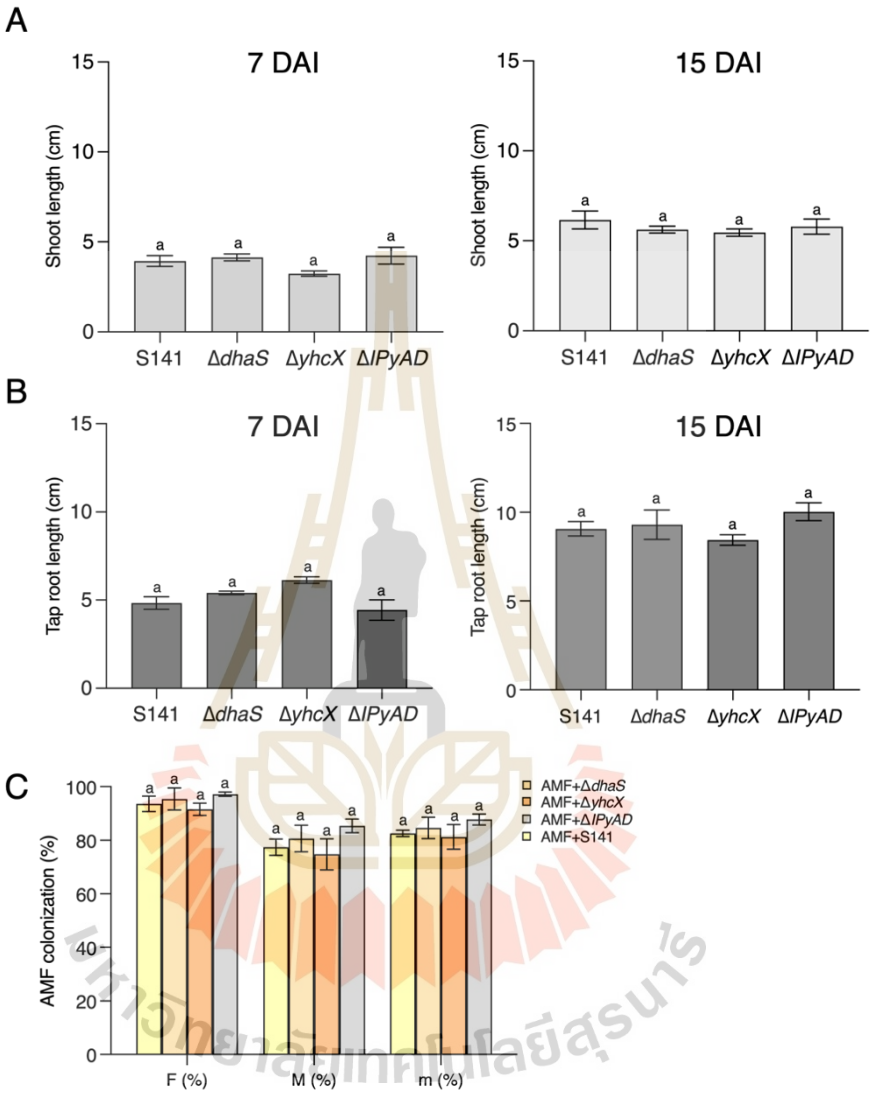


Figure. 4.4 The growth promotion of *L. japonicus* at 7 (**A**) and 15 (**B**) days after inoculation (DAI) when inoculation with *B. velezensis* S141 wide type (S141) and its derivative mutants (**Δ***dhaS*, **Δ***yhcX*, and **Δ***IPyAD*) decreases in the indole-3-acetic acid (IAA) production. (**C**) The measurement of AMF colonization at 60 DAI (AMF root colonization was defined as F(%), the frequency of mycorrhiza in the root system, M(%), the intensity of the mycorrhizal colonization in the root system, and m(%), the intensity of the mycorrhizal colonization in the root

fragments). Values are means ± s.e.m. (*n* = 4). Bars marked with the same letter are not significantly different at $p<0.05$ on Turkey's HSD test.

The transcript levels of cell cycle-related genes and phosphorus (P) and carbon (C) nutrition-related genes in *R. irregularis* colonizing *L. japonicus* roots were also analyzed. Five out of the seven cell cycle-related genes tested showed significantly higher expression in roots with S141 than in those without the bacteria (**Figure 4.5B**). For genes involved in P nutrition in *R. irregularis*, it was focused on those related to polyphosphate synthesis and degradation (Ezawa and Saito 2018). The expression levels of genes encoding the polyphosphate-synthesizing enzymes, VTC1, VTC2, and VTC4 (Nguyen et al. 2022), were found to have decreased upon co-inoculation with S141. On the other hand, the transcript levels of the endopolyphosphatase genes *PPN1–4* (Nguyen & Saito, 2021) showed no significant differences. The expression of the monosaccharide transporter gene *MST2*, which plays a crucial role in sugar uptake in AMF, was reduced in the presence of S141.

4.4.6 *B. velezensis* S141 is an endophytic bacterium

The localization of S141 was examined using confocal microscopy to confirm the presence of the bacteria in *L. japonicus* roots during the symbiotic association with AMF. No bacterial cells were observed in the longitudinal root sections of non-inoculated plants at 7 and 45 DAI (**Figure 4.6A, C**). Intriguingly, bacterial cells were observed within root cells at 7 DAI (**Figure 4.6B**) although they did not colonize within intraradical hyphae and arbuscules of AMF (**Figure 4.6D**). The amount of living endophytic bacteria was detected at 30 DAI at a density of approximately 10^3 and 10^4 CFU/plant in mycorrhizal and non-mycorrhizal plant roots, respectively, using a plate count technique.

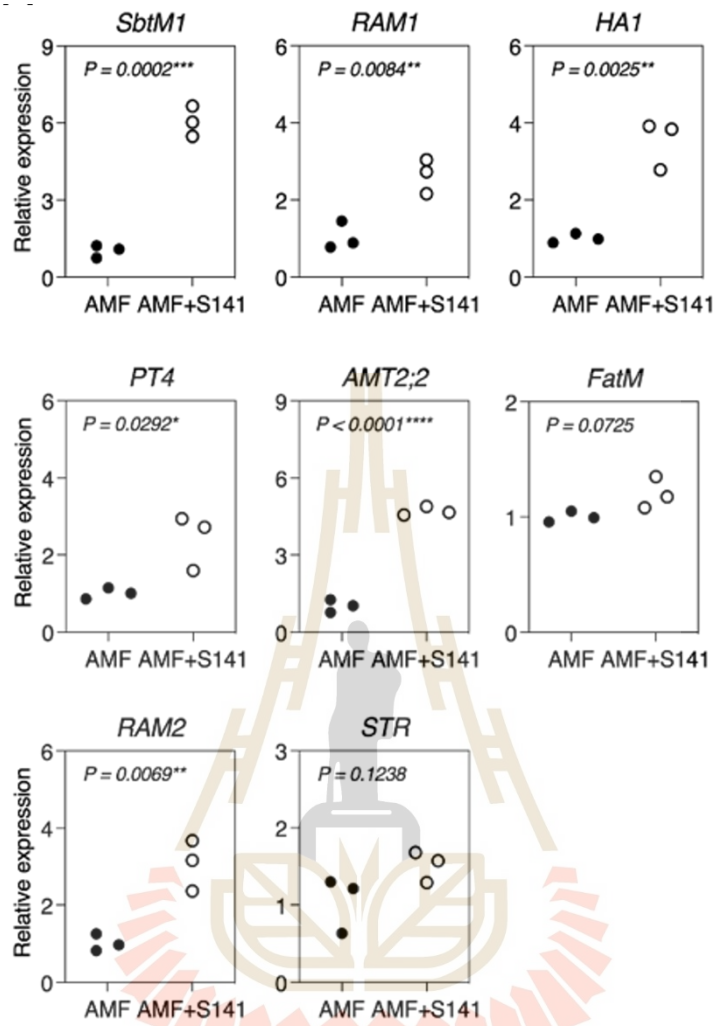


Figure 4.5 The gene expression profiles of *L. japonicus* at 45 days after inoculation. Plants were inoculated with *R. irregularis* (AMF) or with both *R. irregularis* and *B. velezensis* S141 (AMF+S141). *P*-values were calculated based on the Student's *t*-test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; and ****, $p<0.0001$). *SbtM1*: subtilisin-like serine protease, *RAM1*: GRAS transcription factor, *HA1*: H^+-ATPase, *PT4*: phosphate transporter, *AMT2;2*: ammonium transporter, *FatM*: acyl-acyl carrier protein thioesterase, *RAM2*: glycerol-3-phosphate acyltransferase, *STR*: ABC transporter.

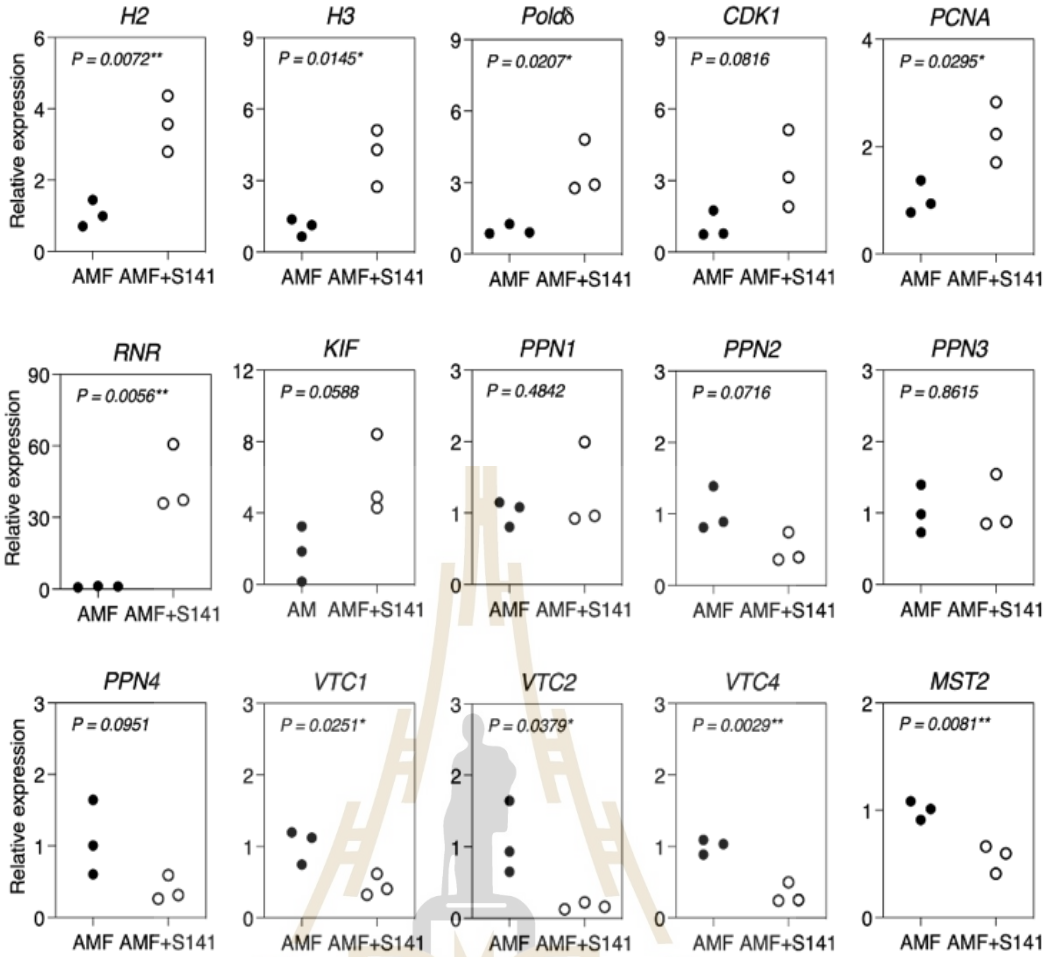


Figure 4.6 The gene expression profiles of *R. irregularis* at 45 days after inoculation. Plants were inoculated with *R. irregularis* (AMF) or with both *R. irregularis* and *B. velezensis* S141 (AMF+S141). *P*-values were calculated based on the Student's *t*-test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; and ****, $p<0.0001$). *H2* and *H3*: histones, *Pol*δ: DNA polymerase delta subunit 4, *CDK1*: cyclin-dependent kinase, *PCNA*: proliferating cell nuclear antigen, *RNR*: ribonucleotide reductase, *KIF*: kinesin, *PPN1-PPN4*: endopolyphosphatases, *VTC1*, *VTC2*, and *VTC4*: vacuolar transporter chaperones, *MST2*: monosaccharide transporter.

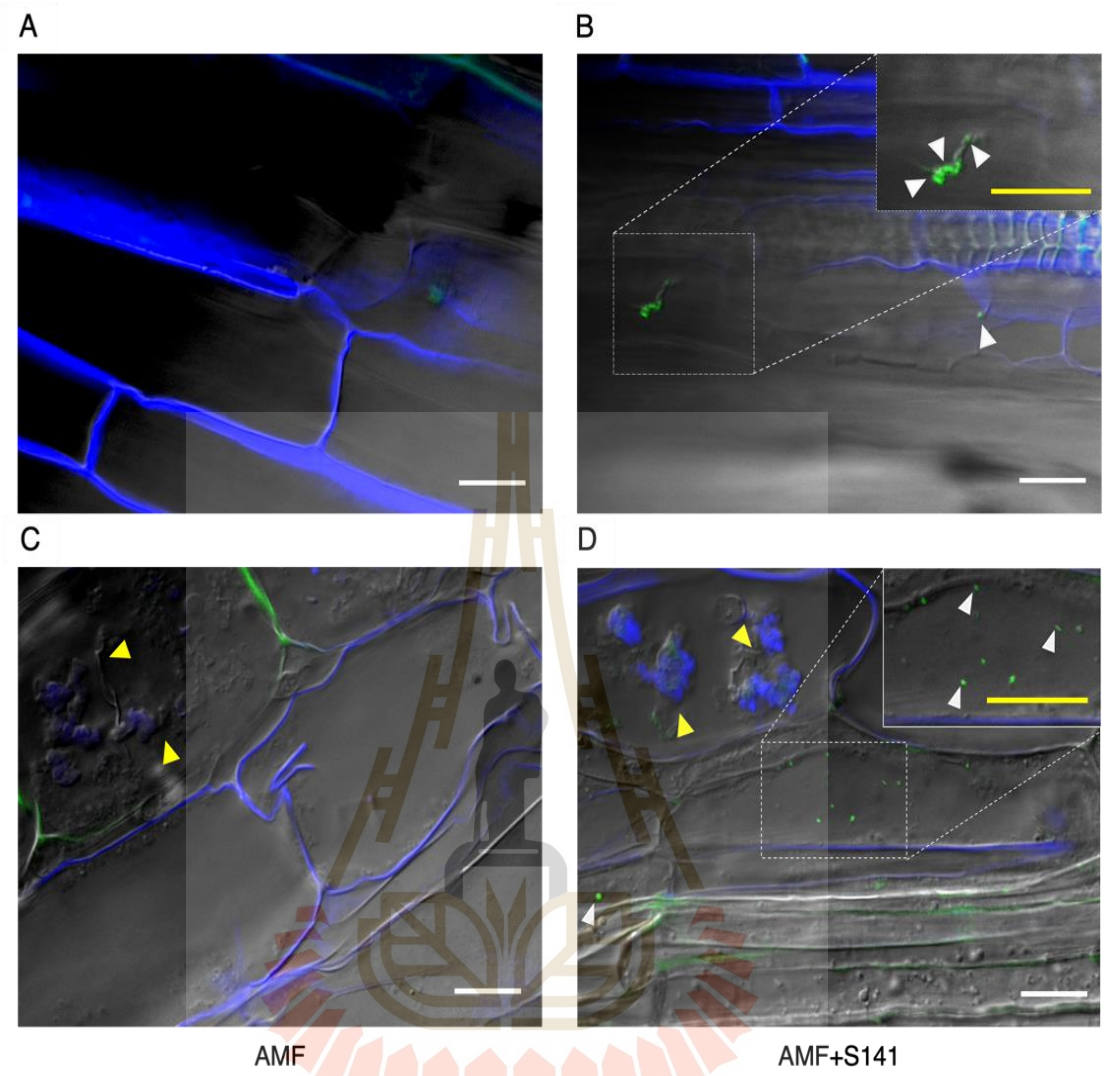


Figure 4.7 Confocal laser scanning microscopy images illustrating the bacterial localization in *L. japonicus* roots at 7 (**A**, **B**) and 45 (**C**, **D**) days after-inoculation (DAI). (**A, C**) Single inoculation of *R. irregularis*; (**B, D**) Co-inoculation of *R. irregularis* with *B. velezensis* S141. Longitudinal sections depict mycorrhizal roots stained with calcofluor (blue) to visualize cell walls of plants and arbuscular mycorrhizal fungi, and with SYTO 9 (green) to detect live bacterial cells (white arrowheads). Arbuscules of *R. irregularis* are highlighted by the yellow arrowhead. Scale bars denote 10 µm.

4.5 Discussion

PGPRs and AMF can coexist in roots, and certain combinations of these microorganisms produce enhancement of both AMF abundance and plant growth (Meyer & Linderman 1986; Yuttavanichakul et al. 2018; Kiddee et al. 2020; Sheteiwy et al. 2021). In this study, it was demonstrated that the endophytic PGPR S141 produced an increase in AMF colonization in *L. japonicus* roots and promoted spore production (**Figure 4.1**). Although S141 was originally isolated as a PGPR for soybean, cell concentrations of 10^5 and 10^6 cells/plant showed no effect on the biomass production of *L. japonicus*. However, in the presence of AMF, S141 augmented the plant biomass in addition to shoot N and P concentrations (**Figure 4.2**). This finding suggested that S141 can exert its PGPR effect in *L. japonicus* when coexisting with AMF. At 15 days after inoculation with S141, the root length more than doubled compared to that without the bacterial strain (**Figure 4.3**). Similar plant growth promotion at the seedling stage has also been observed in peppers inoculated with *B. velezensis* BBC047 (Stoll et al. 2021). This accelerated initial root elongation may cause enlargement of the zone of AMF colonization in the early phase, leading to greater AMF abundance in roots. The increase in AMF colonization due to the co-inoculation could lead to enhancement in biomass and N and P concentrations of *L. japonicus*. Alternatively, the synergistic effects of S141 and AMF in the roots might have altered the gene expression patterns in both the plant and AMF, resulting in activation of plant and AMF growth.

Auxin is involved in the initiation of arbuscular mycorrhizal symbiosis and the development of arbuscules (Ho-Plágaro & García-Garrido 2022). S141 produces auxins, and disruption of the *IpyAD*, *yhcX*, and *dhaS* genes reduces auxin production to 4% to 30% of the wild-type strain (Sibponkrung et al. 2020). Using these mutant strains, the potential role of auxins from S141 with respect to producing an increase in AMF abundance was tested, but it was found that the AMF colonization was similar to wild-type inoculation (**Figure 4.4**). Such levels of auxin reduction might not affect AMF abundance, possibly due to compensatory effects from other auxin-producing genes. Another explanation for this finding could be that the auxin-producing capability of S141 is not a primary mechanism for promoting plant growth or AMF

colonization. Nevertheless, S141 might employ other mechanisms that facilitate AMF–plant symbiosis, and these mechanisms should be further investigated.

S141 enhanced mycorrhizal formation in *L. japonicus*, a finding that is consistent with the activation of plant marker genes (*SbtM1* and *RAM1*) associated with mycorrhizal symbiosis (**Figure 4.5**). Similarly, the expression of the *HA1*, *PT4*, and *AMT2:1* genes, all of which are essential for nutrient uptake during mycorrhizal symbiosis, was also upregulated by inoculation with S141. This finding mirrors the observed increase in shoot N and P concentrations. These results suggest that the presence of endophytic S141 amplifies the benefits of mycorrhizal symbiosis. In contrast, the expression of genes involved in lipid transfer from plants to AMF (except for *RAM2*) remained unchanged after S141 inoculation. The different gene expression responses related to carbon supply and symbiotic nutrient uptake when exposed to S141 might influence the cost-benefit balance in mycorrhizal symbiosis.

Expression of AMF cell cycle-related genes is related to AMF proliferation in roots in response to environmental changes (Sugimura and Saito, 2017). AMF cell cycle-related genes were found to be downregulated during the suppression of AMF colonization in roots caused by high phosphate exposure. In our experiments, the presence of S141 led to an increase in the expression of cell cycle-related genes in *R. irregularis* (**Figure 4.6**). This finding is consistent with the increase in abundance of *R. irregularis* in *L. japonicus* roots after inoculation with S141, thus supporting the occurrence of activation of AMF proliferation by the PGPR strain in terms of gene expression. The gene expression related to P metabolism, an important function in AM symbiosis, as an effect of the coexistence of S141 on AMF was analyzed. Polyphosphate synthesized in AMF is an important source of P for plants, and the amount of polyphosphate in AMF was thought to be regulated through a metabolic balance between polyphosphate synthesis by the vacuolar transporter chaperone (VTC) complex and degradation by endopolyphosphatases (PPNs) (Ezawa et al. 2018). Based on the gene expression analysis, the amount of PPN transcripts in AMF was not affected by S141 inoculation, but VTC transcripts had decreased (**Figure 4.6**). Although polyphosphate levels were not determined, polyphosphate may progress in the direction of degradation in intraradical hyphae and arbuscules of AMF. Since short-chain polyphosphate acts as a pool of P supply to the plant (Takanishi et al.

2009), the higher shoot P content of plants inoculated with S141 may be related to modulation of polyphosphate metabolism genes in AMF. In addition, the expression of *MST2*, which is involved in sugar transport to AMF cells, was downregulated after co-inoculation with S141 (**Figure 4.6**). These results shed light on the complex mechanisms involved in P and sugar acquisition and delivery in AMF and highlight the potential role of co-inoculation with an endophytic PGPR involved in these processes, which should be further studied.

4.6 Conclusion

To summarize, this study sheds light on the beneficial effects of S141 on the plant-AMF symbiosis. The results suggested that S141 leads to enhancement of root growth in the early stages and later promotes the abundance of AMF. However, the production of a plant growth hormone, IAA, is not the key mechanism used by S141 to promote mycorrhizal symbiosis. Surprisingly, strain S141 was identified as an endophyte in *L. japonicus*, and this strain had the capability of inducing the expression of plant marker genes mainly involved in mycorrhizal formation and nutrient uptake. Moreover, induction of the expression of marker genes related to the cell cycle of *R. irregularis* when co-inoculated with S141 strongly supports its potential for promoting fungal growth. These results highlighted the importance of exploring the potential of beneficial microbes in enhancing AMF growth and promoting plant growth through the nutrient uptake pathway.

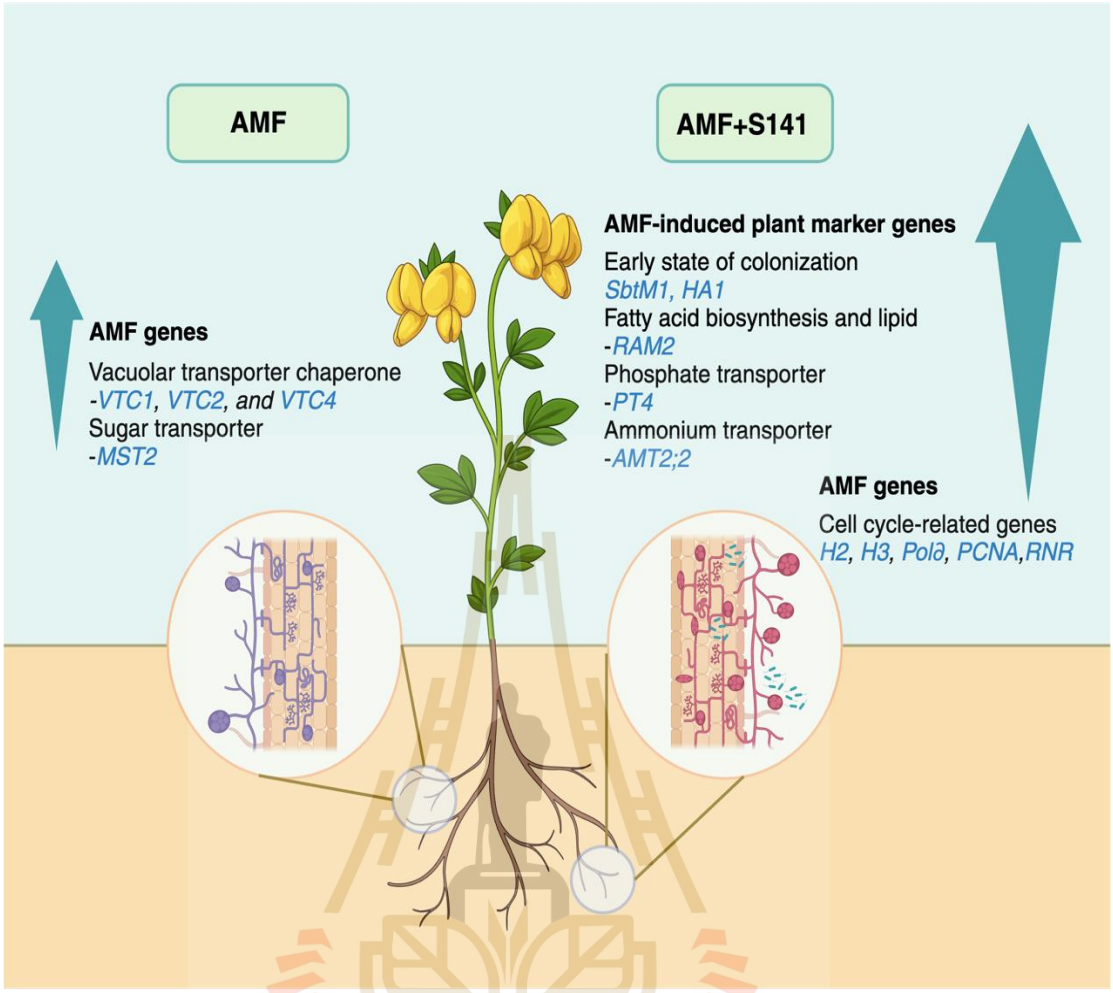


Figure 4.8 The schematic of AM fungal colonization in roots at 45 DAI, with and without *B. velezensis* S141. Hyphae sprout from spores and create a hyphopodium on the root epidermis, followed by intraradical cells. The bacterial cells of *B. velezensis S141* (green rod shape) are endophytic localized in the proximity of the arbuscular cells and around the root. The green arrow showed the upregulation of genes in their respective marker gene expression groups during the symbiotic relationship.

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CHAPTER V

Improving Inoculum Production Efficiency of Arbuscular Mycorrhizal Fungi in *Zea mays* using Light Emitting Diode (LED) Technology

5.1 Introduction

Light is one of the most important factors that affects plant growth and development (De Wit et al. 2016). LED lights, which emit specific wavelengths of light, have been found to be highly effective in promoting plant growth and development (Bula et al. 1991; Paradiso & Proietti, 2022; Y. Xu et al. 2016). Red and blue LED lights have been extensively studied due to their ability to stimulate various physiological processes in plants. Red light is essential for photosynthesis and is particularly effective in promoting plant growth and flowering, while blue light regulates plant growth and development, including stem elongation, leaf expansion, and the formation of chloroplasts (Kim et al. 2004; Ohtake et al. 2018; Samuolienė et al. 2010). The use of LED lighting systems has numerous advantages over traditional lighting methods, including increased energy efficiency, lower heat production, and the ability to customize the spectral output to the specific needs of the plants being grown.

Arbuscular mycorrhizal (AM) fungi are a type of soil fungi that form mutually beneficial relationships with the roots of most plants (Kivlin et al. 2011; Lee et al. 2018). These fungi play an essential role in plant growth and ecosystem functioning majority of plants under conditions of P-limitation, nutrient uptake and plant productivity (Jeffries et al. 2003; Mei et al. 2019; Prasad et al. 2012). Traditionally, the production of AMF inoculant has relied on substrate-based systems, where AMF are grown in a substrate, such as vermiculite or sand, and the spores are harvested from the substrate and used as inoculum (Ijdo et al. 2011; Sayeed Akhtar et al. 2014). The AMF spore production under substrate-based system depends on the plant

compatibility and plant growth efficiency that could affect AMF colonization and propagation (Genre & Bonfante, 2005; Pepe et al. 2018; Zuccaro et al. 2014).

In recent years, there has been growing interest in the use of light-emitting diodes (LEDs) to enhance the growth and development of various plant species. The use of light to stimulate biological processes is a well-established practice in many fields, including agriculture and horticulture (He et al. 2019; Viršile et al. 2017). LEDs emit specific wavelengths of light that can be tailored to meet the specific needs of plants, such as promoting photosynthesis, influencing flowering, and enhancing nutrient uptake (J. Xu et al. 2021; Zhou et al. 2019). LEDs have demonstrated positive effects on the growth and development of AMF, even increasing spore production. Specifically, the application of red and blue LED lights with wavelengths of 630 nm and 450 nm has been shown to stimulate spore production in *Gigaspora margarita* within Bahiagrass. This approach, utilizing LEDs to enhance AMF spore production in substrate-based methods, was elucidated by Cruz (2016). By offering specific wavelengths of light that promote AMF growth and development, LEDs hold the potential to increase spore production, reduce the need for substrate materials, and mitigate contamination risks.

5.2 Objective

To evaluate the potential advantages of employing LED lights for enhancing *Rhizophagus irregularis* fungal spore production in maize (*Zea mays*) under substrate-based system and the mechanism of AMF spore production enhancement was proposed through investigation the plant growth, plant nutrients, and expression of some plant and fungal genes involved in metabolisms and plant-AMF symbiosis in response to LED lights.

5.3 Materials and Methods

5.3.1 Plant growth and condition

Maize (*Zea mays* L. cultivar Suwan 5) seeds were subjected to surface sterilization by immersing them in 95% ethanol for 30 seconds, followed by a 12-minute treatment with a 3% (v/v) sodium hypochlorite (NaOCl) solution.

Subsequently, the seeds underwent six thorough rinses with sterilized water and were left to soak for overnight on an incubator shaker operating at 200 rpm in sterilized water. After this soaking period, the seeds were once again washed with sterile water, carefully placed on sterilized paper towels, and maintained in darkness for a period of two days. Each pot was prepared by introducing 0.5 kg of sterilized river sand, featuring particle sizes ranging from 0.2 to 5 mm. Following this, 250 spores of *Rhizophagus irregularis* DAOM197198 (Mycorise, Premier Tech, Rivière-du-Loup, Canada) were inoculated into each plant. The plants were cultivated in a greenhouse environment as traditional condition in comparison with the LED lighting condition which controlled a light-dark cycle of 12 hours of light and 12 hours of darkness. To support the growth of AMF, a half-strength Hoagland's solution, supplemented with 100 ml (100 µM) of KH_2PO_4, was supplied every two days and the randomized completely block design (RCBD) was used in this experiment with 3 replications.

5.3.2 Optimizing the conditions of light emitting diodes (LEDs) for promoting maize growth and fungal abundance

Transplanted maize, whether inoculated with AMF or not, were exposed to LED conditions. The intensity of red and blue light was assessed using the LIGHTSCOUT quantum light meter (Spectrum Technology, Inc., USA). The primary objective was to optimize maize growth by manipulating the ratio of red and blue light (R:B) at four conditions: 80:20, 60:40, 40:60, and 20:80 (Songsaeng et al. 2022). All treatments maintained a consistent light intensity of 300 µMol/m^2/s. The measurement of plant height, chlorophyll content using a SPAD502 Plus, (Spectrum Technologies, Inc.), and biomass were conducted at 45 days after inoculation (DAI), with shoots and roots separated and subsequently dried at 70°C for 48 hours before recording their respective dry weights (Shipleyl & Vu, 2002). Then, the optimal light ratios were employed to investigate the optimum LED light intensity at 200, 300, and 400 µMol/m^2/s for enhancing AM spore production.

5.3.3 Gene references and primers design

This study was conducted a comprehensive investigation into the genes associated with plant photosynthesis, utilizing the genetic of *Zea mays* L. reference genome Zm-B73 (GCF_902167145.1) obtained from the NCBI database. These genes

were selected based on their relevance to the specific function of interest. Furthermore, the primer was designed in order to determine the gene expression in maize and *R. irregularis* that have been reported to be involved in plant-AMF symbiosis, following the methodology outlined in the study by Sugimura et al. 2022. The primer design process was carried out using Clone Manager 9 Professional Edition. The primers used for the amplification of photosynthetic genes, AMF induced genes in *Zea mays* and the symbiosis marker genes for *R. irregularis* were described in Table 5.1, 5.2, and 5.3, respectively.

5.3.4 Assessment of AMF colonization

The harvested roots underwent a rigorous preparation process to remove pigments. Initially, they were subjected to a 10% (w/v) potassium hydroxide (KOH) solution and boiled at 90°C for 10 minutes, followed by multiple rinses with sterile water. Subsequently, the roots were acidified with a 2% (v/v) HCl solution. Afterward, the cleared roots were stained with a solution containing trypan blue (0.05%) in lactic acid at 90°C for 10 minutes. Post-staining, the roots went thorough rinsing to eliminate excess stain and were then mounted on microscope slides in lactoglycerol solution. To visualize the extent of AMF colonization within the roots, a light microscope with 10X magnification was employed. The percentage of root length colonized by AMF was quantified using a scoring method (Trouvelot et al. 1986).

5.3.5 Isolation of *R. irregularis* spore from soil

After transplanting, maize at 45 days after inoculation (DAI) was stopped watering and provided LED lighting to the plants until 90 DAI. AMF spores were collected from each soil sample using wet sieving and decanting methods (Daniels & Skipper, 1982; Gerdemann & Nicolson, 1963). The isolation procedure followed by (Gerdemann and Nicolson 1963; Daniel and Skipper 1982). The AMF spores were subsequently counted using a stereomicroscope.

5.3.6 RNA Extraction and qRT-PCR

RNA extraction was performed using the RNA prep Pure Plant Plus Kit (TIANGEN Biotech (Beijing) Co., Ltd.), and DNA contamination was eradicated by treating the RNA samples with DNase I solution (Thermo Fisher Scientific). Subsequently, cDNA was synthesized following the manufacturer's instructions,

employing the High-Capacity cDNA Reverse Transcription Kit (iScript, Bio-Rad, USA). For quantitative real-time PCR (qRT-PCR) analysis, the CFX Opus 96 Real-Time PCR System (Bio-Rad, USA) was utilized in conjunction with Luna Universal qPCR Master Mix (NEB, Biolabs Inc). The primer sequences are provided in Table 5.1, 5.2 and 5.3. As an internal control for gene expression, was utilized 18S rRNA (Bezrutczyk et al. 2018) for chloroplast-encoded photosynthetic genes, and beta actin (*ZmACT1*) (Lu et al. 2013) for AMF-induced genes in maize. For transcript normalization in *R. irregularis*, was employed the translation elongation factor 1 α (*RiTEF*). To validate the gene expression level, three biological replicates were carried out, each using approximately 10 ng of cDNA template. Relative gene expression levels were determined using the $2^{-\Delta\Delta_{CT}}$ method (Livak & Schmittgen, 2001).

Table 5.1 Primers used for the amplification of photosynthetic genes in *Zea mays*.

Gene	Primer sequence (5'-3')	Reference
Zm18S	CCATCCCTCCGTAGTTAGCTT	Bezrutczyk et al. 2017
	CCTGTCGGCCAAGGCTATATA	
ZmrbcL	CGTTTCGTCTTTTGTGCCGA	This study
	TTTGCGGTGAATCCTCCTGT	
ZmatpB	CGTATTTGGCGGAGTAGGGG	This study
	CCGGCGGTTCATTCATTTGG	
ZmatpE	GCGGTTCTGTGGAGTGGTTT	This study
	TCAAGTGCCTGTTGAGCTTCT	
ZmpetA	CGGGAAGCAACTGGACGAAT	This study
	AGCACCCACATTCAACCCTC	
ZmpetD	GACCAAAGGTGTCTCCGCAT	This study
	GATGACATGGTTGGTGGGGT	
ZmpsaA	TGAATGGTGATGTAGGCGGG	This study
	CAATTTGGGAGCGGCTTTGT	
ZmpsbA	CTACTTCTGCGGCGATAGGG	This study
	CGCATACCCAGACGGAAACT	

5.3.7 Statistical analyses

The statistical analyses were conducted using R software (version 4.2.1). To assess variations in plant physiology (height and chlorophyll content), dry biomass and AMF abundance in the different LED light ratios, was employed Tukey's HSD-test for multiple comparisons. A Student's *t*-test was employed to compare AMF spore number between LED light and greenhouse conditions. Visualization of the data was carried out using GraphPad Prism (v. 9.1.1).

Table 5.2 Primers listed of arbuscular mycorrhizal fungi (AMF)-induced genes in *Zea mays*.

Gene	Primer sequence (5'-3')	Reference
ZmACT1	GATTCCTGGGATTGCCGAT	Lu et al. 2013
	TCTGCTGCTGAAAAGTGCTGAG	
ZmRAM1	CTTCGCCTTCTTCTCACCAG	This study
	ACTGTCCTGCGTAGTACAAG	
ZmRAM2	CGTACTTGTCATGCGACTTG	This study
	CGGATAGGTGACAGGATTTC	
ZmFatM	GCCCAAGCGAAGTGATCTG	This study
	AATAAGACGAGCCGGCAAAC	
Zmpht6	AGCTCGTCTTCGGCTACTTC	This study
	AGAAGCGGAAGAAGCAGAGC	
Zmpht11	TCGGCCGGAAGAAGTTCTAC	This study
	GGCGTACTCGGACATGATGG	
ZmAMT3	ATGAGCCTCCGACAAACAC	This study
	GGGCTACCAGATGTCCTTCG	

Table 5.3 Primers of marker genes for *R. irregularis*.

Gene	Primer sequence (5'-3')	Reference
RiTEF	ATTGGTCACGTCGATTCTGG	This study
	CCAAGTTCTGCGGCTTCTTT	
RiH2	GCGTAAAGGAGCAAAAGCCATA	This study
	GTCCTGCCTTAACGCTTTTTGT	
RiH3	GGAAAAGCTCCCCGCAAA	This study
	AGCTGGAGCGCTTTTTCGT	
RiPolδ	GGAGAGAGCTCAAAACCTTGGTT	This study
	TTCGGAAGTCAGCGCATTT	
RiCDK1	GCTTGCCATTGATTTACTTTCGA	This study
	GCGCTTGGCAGAGATTCG	
RiRNR	GCTGAACGTCTTGTAGCGTTTG	This study
	ACGCGAAAGCTCCAGAAAAG	
RiKIF	CACCCACGGTGGAAAAGC	This study
	GAATGCCTTGCTACTTCCTGTTC	
RiPPN1	GAAAGATTTAACAATACCGAGTTGGAT	Nguyen et al. 2021
	TGCGCTATTAAGTCCATCATTAACA	
RiMST2	CCACGTTGGTTAGTTGATCATGA	This study
	GGATCGTAAATTTGCCAATACTGTT	

5.4 Results

5.4.1 Selection of blue and red LED light on maize seedling growth and biomass

In this study was investigated the optimal red and blue (R:B) light ratios for promoting maize growth during the seedling stage at 7 and 14 days (**Figure 5.1**). This finding suggest that the utilization of R:B ratios of 60:40 and 80:20 can enhance maize height compared to ratios of 20:80 and 40:60. Conversely, a higher blue light to red light ratio promoted maize growth in terms of chlorophyll content at 7 days. Additionally, a higher red light to blue light ratio (80:20) significantly affected maize shoot elongation compared to other treatments but exhibited lower chlorophyll

content at 14 days (**Table 5.4**). Furthermore, the red to blue light ratios at 60:40 and 80:20 significantly increased shoot dry weight compared to ratios of 80:20 and 40:60, respectively. Moreover, treatments with a higher red-light ratio also demonstrated increased root dry weight compared to treatments with higher blue light ratios. Consequently, the R:B ratio of 60:40 was selected further to optimize the light intensity to promote fungal abundance.

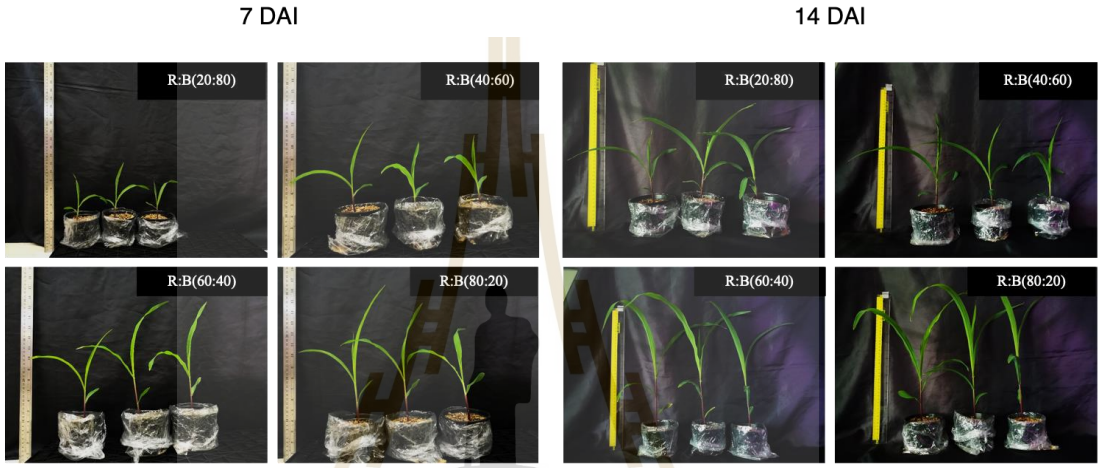

Figure 5.1 Evaluating the impact of Red:Blue light ratios on the growth of maize (*Zea mays* L. Suawan 5) at 7 and 14 days after exposure (DAE), with a total light intensity of 300 µMol/m^2/s.

5.4.2 Optimization of LED light intensity effect on fungal abundance

In this study was applied the selected R:B ratio to examine the impact of LED light at different total intensities of 200, 300, and 400 µMol/m^2/s, represented as RB200, RB300, and RB400 respectively, on fungal abundance. Specifically, LED light was focused on the colonization and spore production of *R. irregularis* (AMF), in comparison to a greenhouse (GH) condition. This finding indicated that 45 days after inoculation (DAI) AMF, LED light treatments at different intensities resulted in a substantial increase in AMF infection in the roots of maize when compared to conditions in a greenhouse environment (**Table 5.5**).

Table 5.4 The data illustrate the influence of various blue:red light ratios on the physiological responses of Suwan5 maize after 7 and 14 days of exposure (DAE). Four distinct treatments were implemented, the different combinations of red and blue light ratios at an approximate total intensity of 300 μMol/m^2/s.

	Light ratios	Plant height (cm)	Chlorophyll content (SPAD uint)	Plant biomass (g/plant)	
				Shoot DW	Root DW
7 DAI	R:B (20:80)	13.83 ± 0.6 b	25.86 ± 0.09 a	-	-
	R:B (40:60)	11.63 ± 0.75 b	25.37± 0.09 a	-	-
	R:B (60:80)	19.67 ± 2.09 a	23.63 ± 0.28 b	-	-
	R:B (80:20)	21.17± 1.01 a	23.44 ± 0.26 b	-	-
14 DAI	R:B (20:80)	16.00 ± 0.96 c	32.74 ± 0.81 a	0.22 ± 0.02 b	0.33 ± 0.03 ab
	R:B (40:60)	16.67 ± 0.59 c	29.04 ± 0.05 b	0.14 ± 0.02 b	0.22 ± 0.01 b
	R:B (60:80)	31.13± 1.14 b	26.49 ± 0.32 bc	0.36 ± 0.02 a	0.44 ± 0.03 a
	R:B (80:20)	36.93 ± 0.79 a	27.38 ± 0.13 c	0.41 ± 0.02 a	0.38 ± 0.04 a

DW: dry weight.

The values are presented as means ± standard error of the mean (n = 5). Statistically significant differences (p<0.05) were observed when comparing results within the same day with different letters following the number in each column, as determined by Tukey's Honestly Significant Difference (HSD) test.

Table 5.5 The growth of maize and AMF colonization at 45 days after inoculation (DAI) across four different conditions: non-inoculation under greenhouse (GH) and LED light (LED), and inoculation with *R. irregularis* under greenhouse (GH+AMF), and under LED light (LED+AMF).

	Plant height (cm)	Chlorophyll content (SPAD uint)	Plant biomass (g/plant)		AM colonization (%)
			Shoot DW	Root DW	
non-GH	43.43 ± 1.5 c	24.72 ± 0.1 b	0.82 ± 0.07 b	0.85 ± 0.01 ab	-
AMF+GH	50.10 ± 0.7 b	26.07 ± 0.4 b	1.26 ± 0.03 a	0.87 ± 0.01 ab	68 ± 1.70 b
non-LED	54.13 ± 0.9 b	27.50 ± 1.2 b	0.90 ± 0.06 b	0.83 ± 0.01 b	-
AMF+LED	62.80 ± 0.2 a	31.77 ± 0.3 a	1.34 ± 0.09 a	0.90 ± 0.02 a	81 ± 2.70 a

DW: dry weight.

The values were calculated as means ± standard error of the mean (n = 3). There are statistically significant differences (p<0.05) observed indicated with different letters after the number in each column when comparing the results using Turkey's Honestly Significant Difference (HSD) test and the Student's *t*-test.

The augmentation in AMF colonization showed an increased at 45 DAI (**Figure 5.1**). It is remarkable that the observations indicate an increased vesicle density on maize roots when LED light is applied, particularly at a total light intensity of 300 µMol/m^2/s, in comparison to the greenhouse (**Figure 5.2A and B**). In addition, the use of LED light not only facilitated the occurrence of AMF infection but also resulted in a significant enhancement of AMF spore numbers in the soil, reaching up to 250 spores per plant with an intensity of 300 µMol/m^2/s (**Figure 5.2C**). This consequence was observed to decrease under intensities of 400 and 200 µMol/m^2/s, and similar to the conditions existing in the greenhouse (**Figure 5.2C**). Based on the results, the specific LED light intensity of 300 µMol/m^2/s (RB300) was determined to be the optimal condition for stimulating the development of arbuscular mycorrhizal fungi (AMF) spores in maize roots.

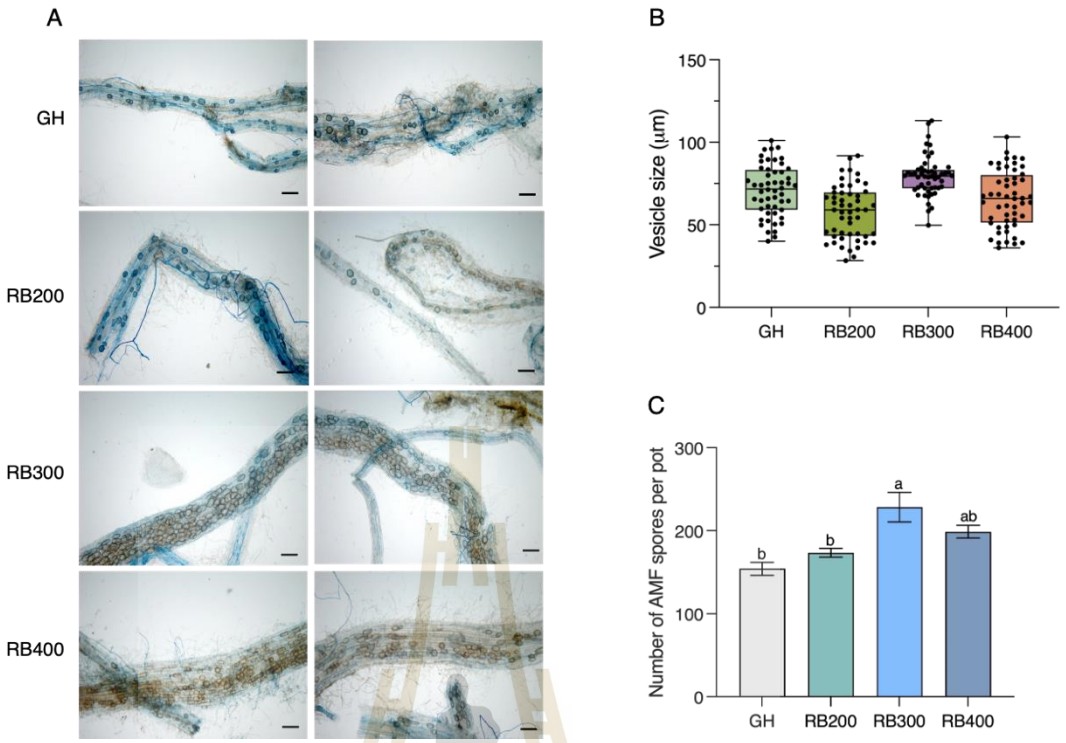

Figure 5.2 Impact of LED light on AMF colonization, vsicle size, and spore number of *R. irregularis* in maize roots at 45 DAI. (**A**) illustrates the four treatments for colonization, including greenhouse (GH), red:blue (RB) light ratio at 60:40, and different light intensities at 200, 300, and 400 µmol/m²/s. (**B**) Displays the average vesicle size for each treatment with the scale bars 100 µm, while (**C**) presents the AMF spore count per plant. Values are represented as means±s.e.m (n=3). Bars with different letters indicate significant differences at a *p*-value <0.05 based on the Tukey HSD test.

5.4.3 Red and blue LED light promote the growth, biomass, nutrient concentration in maize and enhance AMF colonization and spore number production

The data on plant physiology were collected to investigate the correlation between plant growth and AM fungal abundance over a short time period, aiming to accelerate the AMF spore production under specific LED light intensities at 45 and 90 DAI. Firstly, the experiment was assessed the effects of LED light under the optimal condition on maize growth at 45 days after inoculation (DAI)

in comparison with the green house (GH) condition. This findings revealed that the AMF inoculation under LED light (LED+AMF) provided the best plant height, chlorophyll content, and total dry weight of maize (**Figure 5.3A**). No significant differences were observed in plant height and chlorophyll content between the non-AMF groups in both of greenhouse and LED light condition while significantly was observed under the LED light when compared with greenhouse conditions (**Figure 5.3BC**). Furthermore, the LED+AMF treatment also exhibited a significant increase in shoot and root dry weight when compared to other treatments. Conversely, lower dry biomass was observed in the non-AMF treatment under greenhouse conditions, while the non-AMF under LED light did not differ from the GH+AMF treatment (**Figure 5.4A**).

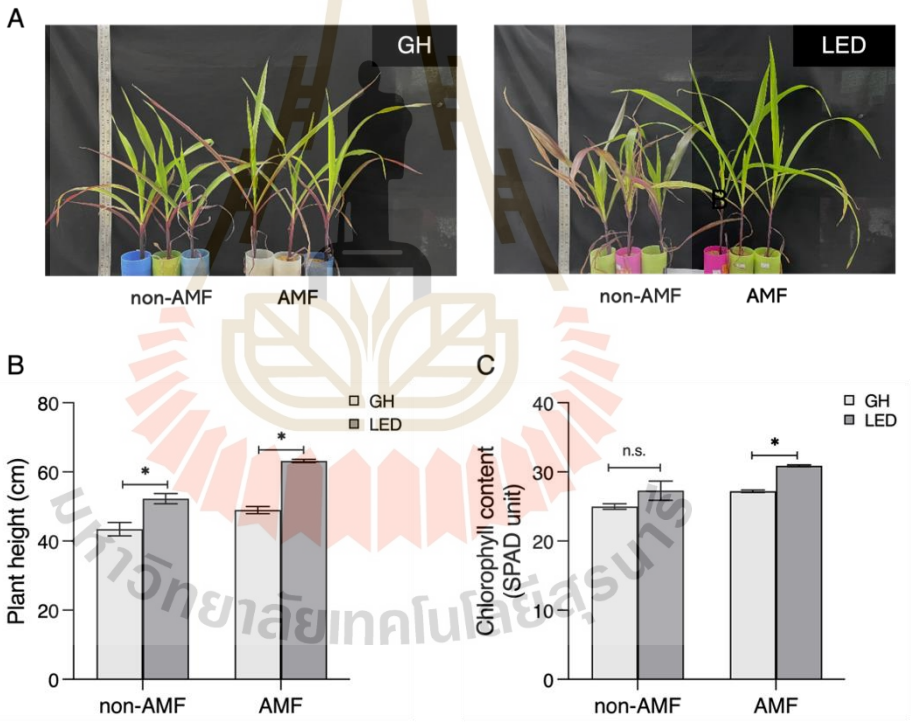

Figure 5.3 Plant Physiology of Suwan 5 Maize Growth at 45 days after inoculation (DAI) in a Greenhouse and LED Light. Under both greenhouse and LED light conditions (**A**), two forms of inoculation were conducted: one without *R. irregularis* (non-AMF) and one with *R. irregularis* (AMF). The height and chlorophyll content of maize are displayed in (**B**). Significant differences are indicated by distinct letters with the standard error bars (s.e.m., n=3). The statistical analysis using a Student *t*-test at $p<0.05$.

Additionally, phosphorus (P) and nitrogen (N) concentrations was analyzed, both of nutrients which are essential nutrients transferred during AMF symbiosis with maize roots. At 45 days after inoculation (DAI), the results indicated significant differences in root phosphorus (P) nutrition between the LED+AMF treatment and the GH+AMF treatment. However, there were no significant differences observed in the nitrogen (N) concentration in both the shoot and root of maize (**Figure 5.4B**).

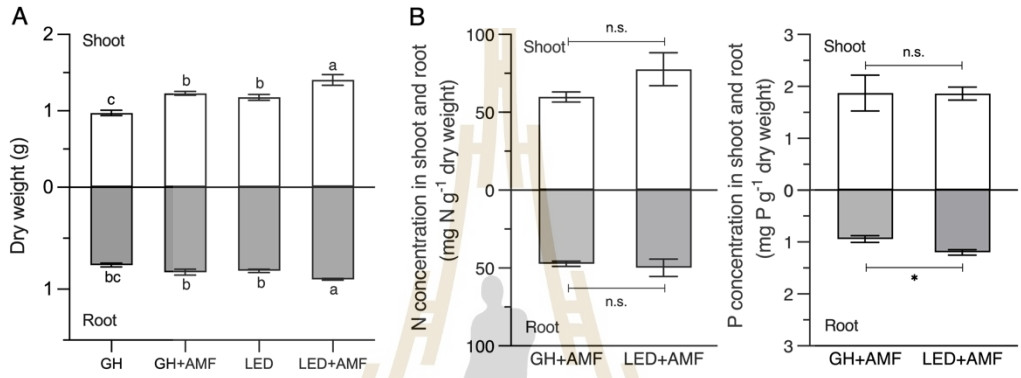

Figure 5.4 Dry Biomass and plant tissue nitrogen (N) and phosphorus (P) concentrations at 45 days after inoculation (DAI). Average concentrations of nitrogen (N) and phosphorus (P) (mg g^{-1} dry weight) in the shoot and root of Suwan 5 maize are presented with standard error bars (± s.e.m; n=3). Different letters indicate significant differences in N and P concentrations

The root infection was also randomly determined at 45 DAI. The fungal abundance, in terms of colonization, in the treatment supplied with LED light showed a significant increase in the frequency of AMF colonization (F%), intensity of colonization (M%), and arbuscule abundance (A%) when compared with the greenhouse condition. In contrast, the intensity (m%) and arbuscule abundance (a%) did not show significant differences (**Figure 5.5A**). Surprisingly, the quantification of AMF spores produced in the soil increased by up to 1.5 times in the treatment performed under LED light conditions compared to the greenhouse condition (**Figure 5.5B**). The number of AMF spore reached to 1,500 spores under LED light at 90 DAI,

while less than 1,000 spores were produced under GH condition (**Figure 5.5C**). This result demonstrates that LED light conditions have the potential to promote AMF development and spore production in conjunction with the plant host and it could accelerate the process of spore production in maize remain to 90 days in comparison with 120 days under the traditional method.

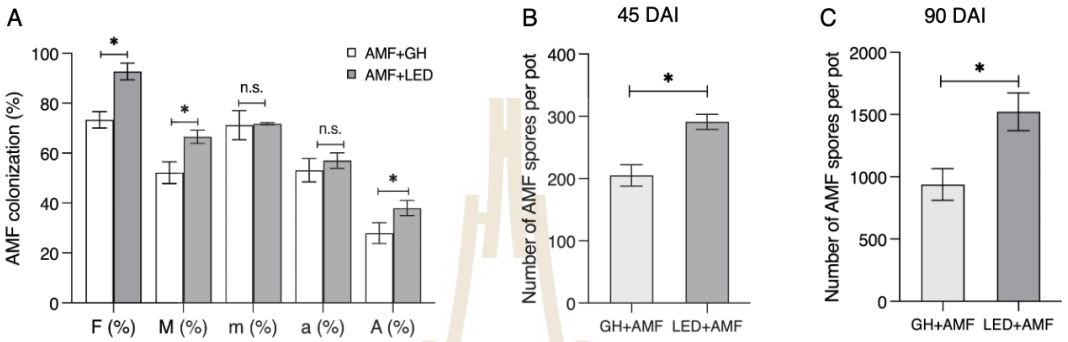

Figure 5.5 The abundance of *R. irregularis* at 45 days after inoculation (DAI). Maize was inoculated with *R. irregularis* (AMF) under the greenhouse (GH) and LED light conditions. (**A**) AMF colonization in maize roots. F(%), the frequency of mycorrhiza in the root system; M(%), the intensity of the mycorrhizal colonization in the root system; m(%), the intensity of the mycorrhizal colonization in the root fragments; a(%), the arbuscule abundance in the mycorrhizal parts of the root fragments; and A(%), the arbuscule abundance in the root system. (**B**) The number of *R. irregularis* spores produced in the soil at 45 DAI and (**C**) at 90 DAI. Values are means ± s.e.m. (n = 3). Bars marked with the same letter are not significantly different at $p<0.05$ on Turkey HSD test.

5.4.4 The gene expression in maize and R. irregularis during symbiosis in response to the growth under LED light and green house conditions

In this study was investigated changes in gene expression, focusing on plant photosynthesis-related genes and marker genes involved in the symbiotic response between maize and *R. irregularis*. The investigation was conducted in both a greenhouse (GH) and under LED light conditions. Data collection was performed in

root systems at 45 DAI. The transcript levels of genes related to plant photosynthesis consist of complex constituents of the photosynthetic electron transport (PET) process, including rubisco (large subunit) *rbcL*, as well as *psaA* and *psbA*, which are integral components of photosystem I and II respectively, in the electron transport chain. Notably, *petA* and *petD* are components of the cytochrome b_6f complex subunit IV. Additionally, *atpB* and *atpE* are integral components of the ATPase subunit β and ε within the stromal region, responsible for ATP production. All of these genes exhibited significant upregulation, surpassing around 1.5- to 40-fold increase, under LED light conditions compared to greenhouse conditions (**Figure 5.6**).

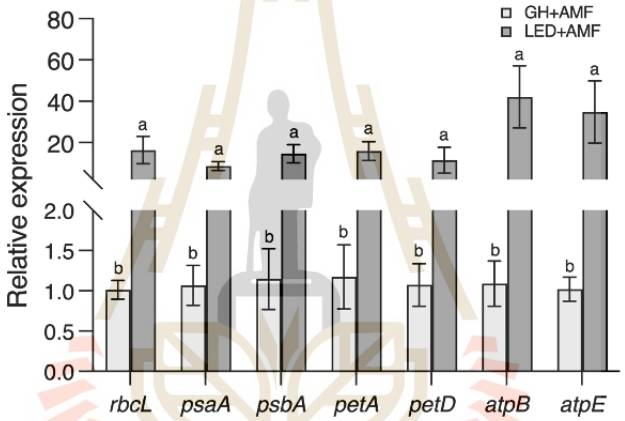

Figure 5.6 Maize photosynthetic related gene at 45 days after inoculation. Chloroplast-encoded photosynthetic genes: *rbcL*: rubisco (large subunit), *psaA* and *psbA*: electron transport (photosystem I and II), *petA* and *petD*: electron transport (cytochrome *f* and subunit IV of cytochrome b_6f complex), and *atpB* and *atpE*: ATP synthesis. There are tree replication and the letter above bar indicates significance at p value < 0.05 using a Student's t-test with a significance different at $p<0.05$.

This study also examined the transcript levels of five genes induced by *R. irregularis* in maize roots. The expression of the transcription factor gene *RAM1*, primarily responsible for arbuscule formation, showed no significant change. Likewise, the phosphate transporter *pht6* and ammonium transporter *AMT3*, involved in P and

N uptake in arbuscule-containing cells within the specialized tree-like structures in the cortical cells, exhibited no significant differences between LED light and greenhouse conditions. However, the expression of the glyceral-3-phosphate acyltransferase gene *RAM2*, which involved in AMF colonization increased 2-fold under LED light compared to the greenhouse, while the acyl-ACP thioesterase *FatM* showed no significant difference (**Figure 5.7A**).

In addition, the transcript levels of cell cycle-related genes and their response to changes in phosphorus (P) and carbon (C) nutrition during the symbiotic interaction between *R. irregularis* and maize, analyzing a total of seven genes. The results revealed that three out of the seven genes exhibited significantly higher upregulation in gene expression under LED light compared to greenhouse condition (**Figure 5.7B**). This focused on the transcript levels in *R. irregularis* in response to variable P nutrition, particularly concerning polyphosphate synthesis and degradation processes. Genes responsible for the synthesis of polyphosphate enzymes, namely VTC1, VTC2, and VTC4 (Nguyen et al. 2022), were downregulated under LED light condition. Conversely, the gene associated with endophosphatases, *PPN1*, showed robust upregulation in response to LED light conditions, whereas *PPN2*, *PPN3*, and *PPN4* exhibited downregulation (Nguyen & Saito, 2021). Additionally, the observed an increase in the expression of the monosaccharide transporter gene *MST2*, which plays a pivotal role in sugar uptake in *R. irregularis*, under LED light compared to greenhouse condition.

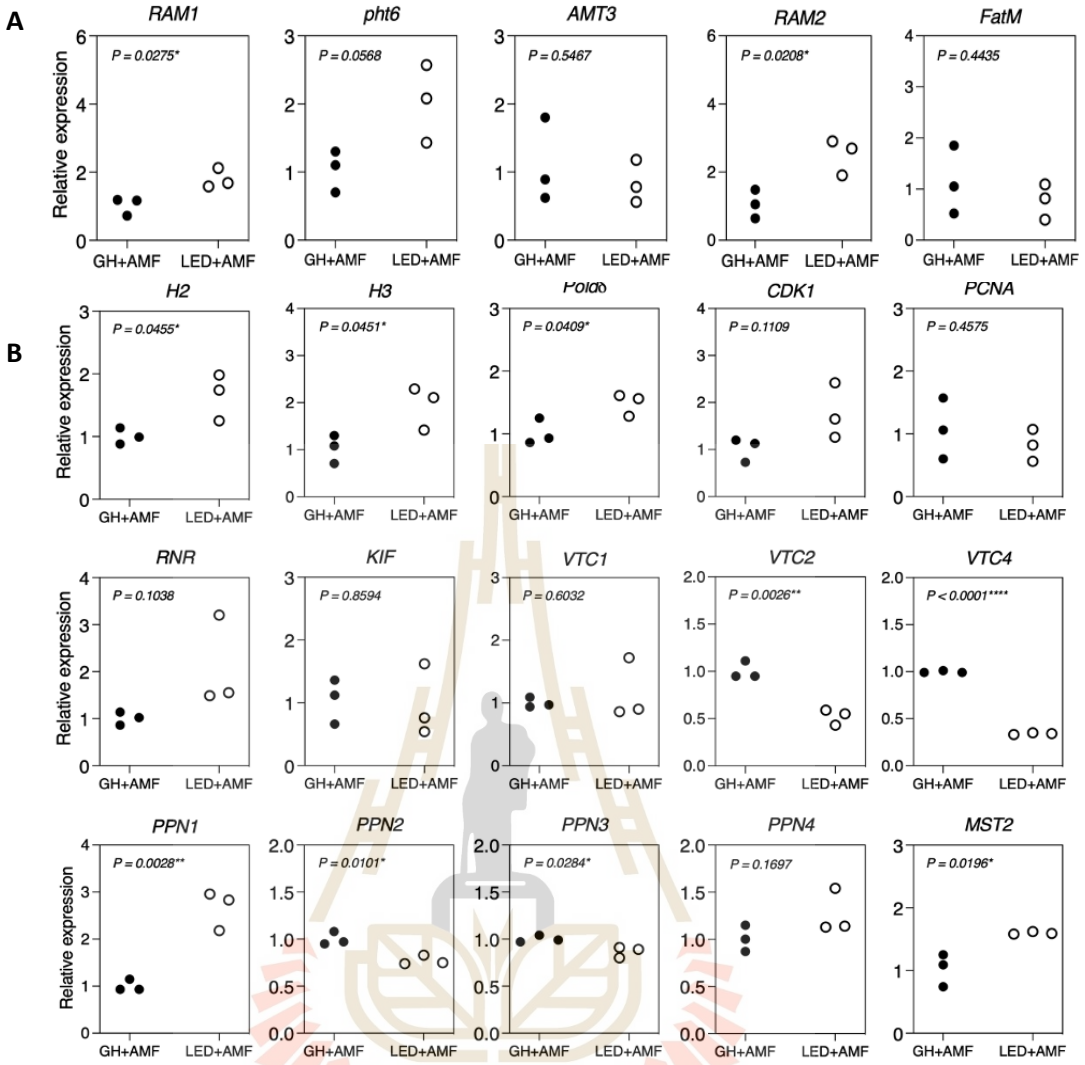

Figure 5.7 The gene expression profiles of maize (**A**) and *R. irregularis* (**B**) at 45 days after inoculation. Plants were inoculated with *R. irregularis* (AMF) and grown under the greenhouse (GH) and LED light conditions. *P*-values were calculated based on the Student's *t*-test (*, $p<0.05$; and **, $p<0.01$). (**A**) *RAM1*: GRAS transcription factor, *Pht6*: phosphate transporter, *AMT3*: ammonium transporter, *FatM*: acyl-acyl carrier protein thioesterase, *RAM2*: glycerol-3-phosphate acyltransferase (**B**) *H2* and *H3*: histones, *Pol*δ: DNA polymerase delta subunit 4, *CDK1*: cyclin-dependent kinase, *PCNA*: proliferating cell nuclear antigen, *RNR*: ribonucleotide reductase, *KIF*: kinesin, *PPN1-PPN4*: endopolyphosphatases, *VTC1*, *VTC2*, and *VTC4*: vacuolar transporter chaperones, *MST2*: monosaccharide transporter.

5.5 Discussion

Plants require light for photosynthesis, which is the process by which they convert light energy into chemical energy to fuel their growth and development. Different wavelengths of light have varying effects on plant growth and physiology. Especially, red and blue light, play important roles in plant growth (Xu et al. 2019; Kitazaki et al. 2018; Ouzounis et al., 2014). In this study was demonstrated that red and blue LEDs light enhanced maize growth with and without AMF in terms of plant height and chlorophyll content (**Figure 5.3**). Similarly, the exposing cucumber plants in different percentages of blue and red light also enhanced leaf photosynthetic capacity, net photosynthetic rate, stomatal conductance, and chlorophyll content (Zhou et al. 2019). Here, the implementation of red and blue LED light could promote maize growth at 45 DAI when compared with the conventional system or greenhouse condition. Furthermore, LED lighting with the specific red and blue light ratio, could be used to provide the optimal light condition for promoting plant growth and maximizing productivity.

This findings revealed that a red and blue light (R:B) ratio of 60:40, combined with a total intensity of 300 µMol/m²/s, effectively promoted maize growth during the early stages of seedling development (**Figure 5.1 and Table 5.4**). However, when the intensity range was deviated from this intensity range by reducing it to 200 µMol/m²/s or increasing it to 400 µMol/m²/s, observed a decrease in AMF colonization and spore numbers in the soil (**Figure 5.2**). Similarly, the utilizing of red and blue light could stimulate the hyphal growth of *Gigaspora margarita* in sand material (Cruz, 2016). However, the ratio of red and blue LED light supplied varied depending on the plant species (Ma et al. 2021; Songsaeng et al. 2022; Stamford et al. 2023). These findings suggest that different light ratios and intensities can have varying effects on enhancing plant growth and ultimately promoting AMF development.

The dry biomass of maize significantly increased when co-inoculated with R. irregularis under LED light conditions, both in the shoot and the root, compared to greenhouse conditions (**Figure 5.4**). This experiment also included an analysis of nutrient accumulation in the maize shoot and root, focusing particularly on phosphorus (P) and nitrogen (N), which play a significant role in nutrient uptake by

AMF during symbiosis (Campos-Soriano et al., 2011). The results revealed no significant difference in N accumulation in both the shoot and root of maize at 45 days after inoculation (DAI). However, a noteworthy promotion of P accumulation in the root was observed under the LED light treatment compared to the greenhouse treatment (**Figure 5.4B**). Nevertheless, this experiment indicated a lower biomass of maize than usual. It is possible that the nutrients supplied with the Hoagland solution and the intensity of LED light were insufficient to promote fully plant growth and development, resulting in a reduced synthesis of important compounds and lowering the concentrations of essential plant nutrients, especially nitrogen (N) and phosphorus (P). Additionally, maintaining a consistent environmental conditions between the greenhouse and LED light conditions are very important to confirm whether the LED light could directly affect plant growthand arbuscular mycorrhizal fungus (AMF) spore production. These findings suggest that LED lighting, with a specific light intensity, has the potential to enhance maize growth and development. Moreover, the presence of the fungal partner improves plant nutrient uptake, facilitating the transfer of phosphorus and nitrogen to the plant host (Wipf et al. 2019).

The specific application of red and blue light has been observed to impact AMF propagules, with red light followed by blue light promoting AMF colonization, while the use of only red light can stimulate AMF colonization and lead to increased spore production, particularly in glass bead materials. Conversely, the use of blue light alone suppressed AMF colonization and reduces spore numbers (Cruz, 2016). Similarly, the results demonstrate that a combination of optimal red and blue light can significantly enhance AMF colonization (**Figure 5.5A**), and spore production in both 45 and 90 DAI in sand materials (**Figure 5.5BC**). These findings are corresponded with previous studies suggesting that LED lighting can stimulate the proliferation of hyphal branches, expand the root area, thereby promoting AMF colonization and resulting in the production of numerous AMF spores in the soil (Cruz, 2016; Harris, 2008; Yu & Fischer, 2018).

Light serves as a pivotal regulator of photosynthetic gene expression in plants, instigating significant reprogramming of gene expression and thereby influencing various metabolic processes (Petrillo et al. 2014). In this study was investigated genes

involved in plant photosynthesis, including those responsible for carbon fixation (e.g., *rbcL*), the electron transport chain (e.g., *psaA*, *psbA*, *petA*, and *petD*), and ATP synthesis (e.g., *atpB* and *atpE*), in response to different environmental light conditions. The results revealed that all of the examined genes responded to light stimulation by upregulating their expression levels, showing increases ranging from 1.5 to 40-times under LED light conditions compared to greenhouse conditions, where natural sunlight was the primary light source. This substantial response to LED light suggests that an augmented carbon flux may occur, possibly triggering the activation of other genes involved in plant photosynthesis. This phenomenon, as reported by Fellbaum et al. (2012), could stimulate phosphorus uptake and transfer within the AMF symbiosis. These findings emphasized the more efficient of LED light in promoting the growth and biomass of horticultural and field crops through the stimulate of photosynthesis (Songsaeng et al. 2022; He et al. 2019; Viršile et al. 2017), and point out the potential benefit of using LED light for enhance AMF development and symbiosis and stimulate their spore production.

It was found that enhancing of AMF formation in maize under LED light correlates with the significant induction of the plant marker gene, *RAM1*, during the symbiotic process (**Figure 5.7A**). Interestingly, the observed that the expression of genes involved in plant nutrient uptake, such as nitrogen (N) and phosphorus (P), did not significant difference in the expression of *AMT3* and *pht6* under LED light conditions. In contrast, there was an upregulation in the expression of genes involved in lipid transfer from the host to AM fungi, particularly *RAM2*. Hence, this finding suggest that during the AMF-plant symbiosis under LED light, the promotion of AMF does not primarily occur through the nutrient uptake pathway. Instead, LED light appears to act as an activator of lipid biosynthesis in the plant host, facilitating its transfer to AM fungi for growth and development.

The expression of cell-cycle genes in AMF is closely linked to AMF proliferation in plant roots. The role of phosphate metabolism and nutrient uptake during AMF symbiosis has been described by Kiddee et al. (unpublished data). Their research revealed that the use of plant growth-promoting rhizobacteria (PGPR), specifically *Bacillus velezensis* S141, can stimulate genes within this group while downregulating genes involved in polyphosphate biosynthesis via vacuolar transporter chaperones

(VTCs) and endopolyphosphatases (PPNs). Interestingly, the transcript levels of sugar transporter *MST2* were upregulated. This study also showed that three of seven marker genes related to the cell cycle of *R. irregularis* were upregulated. Furthermore, phosphate metabolism genes were generally downregulated, with the exception of *PPN1*. Conversely, monosaccharide transporter genes exhibited upregulation (**Figure 5.8**). These results shed light on the intricate mechanisms by which LED light conditions can promote plant growth in terms of carbon supply (C), which is important for fungal fatty acid biosynthesis, and facilitate the return of nutrient uptake to the host plant during plant-AMF symbiosis by enhancing nutrient accumulation.

LED light application is used to supplement light for plants during periods or seasons when low natural light levels. Plants require sufficient light to prevent chlorosis or impaired leaf development (Hao & Papadopoulos, 1999; Jones, 2018). Therefore, in this study, LED lighting primarily affects plant growth by providing the necessary light conditions. Consequently, it is important to develop light condition optimized specifically for maize, taking into account the local natural lighting environment. Simultaneously, this may accelerate the AMF life cycle by reducing the time required to generate spore numbers over a shorter time period. Therefore, LED light has potential to reduce the time needed for AMF fungi production by stimulate its life cycle, which closely related to the growth of their host plant.

5.6 Conclusion

In summary, this study investigated the impact of red and blue LED light on various facets of maize growth, biomass, and fungal abundance. It was found that a red-to-blue light ratio of 60:40, combined with a total light intensity of 300 μMol/m^2/s, provided the optimal conditions for enhancing multiple key parameters of maize growth and development. Furthermore, this specific LED light intensity significantly promotes the colonization and spore production of *R. irregularis* when compared to traditional greenhouse conditions. The gene expression analysis unveiled the molecular mechanisms underpinning these improvements. LED light conditions upregulated crucial photosynthesis-related genes, increasing the photosynthetic net sugar production (C). Additionally, LED light stimulated the

expression of genes involved in the symbiotic relationship between maize and *R. irregularis*, along with genes associated with regulating the fungus's cell cycle. These findings demonstrate the potential of using a combination of red and blue LED light as an effective tool for enhancing maize growth, biomass, and nutrient uptake through the promotion of arbuscular mycorrhizal symbiosis.

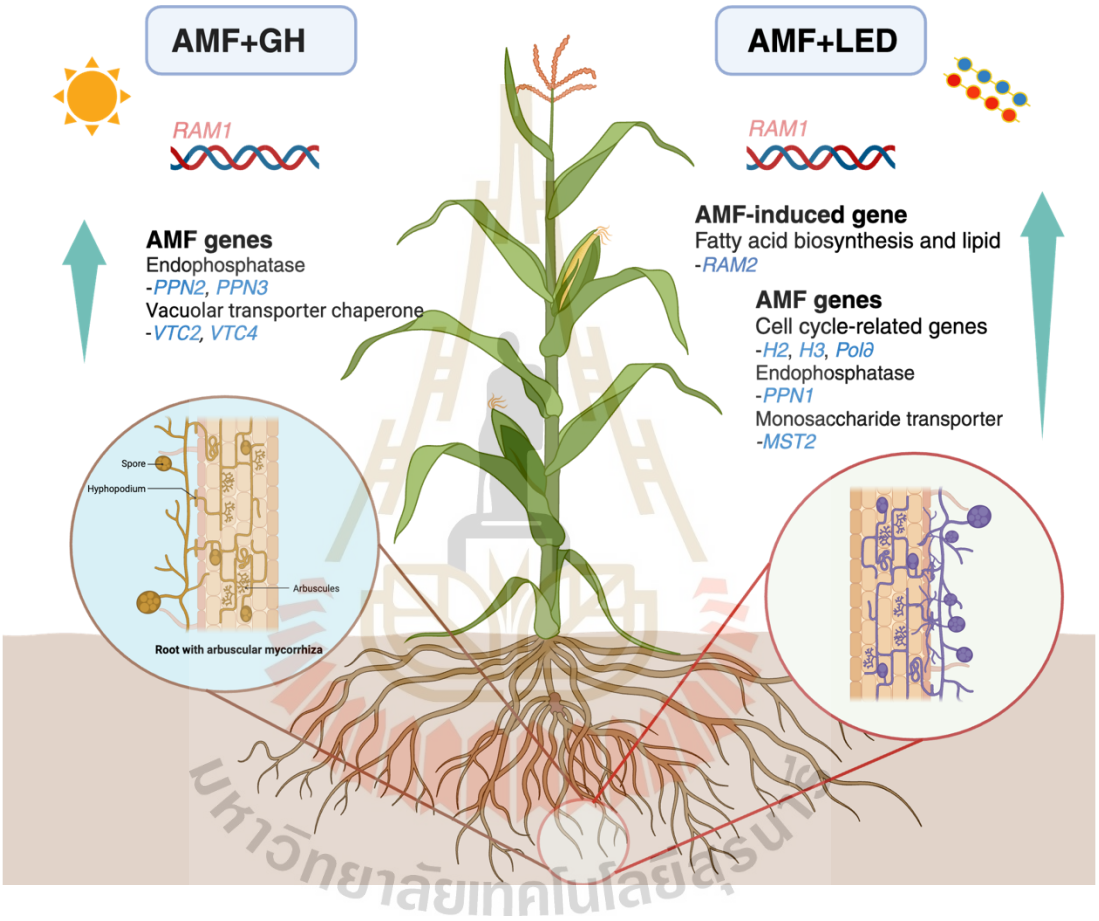

Figure 5.8 The schematic of gene expression under substrate-based production system with additional LED light (AMF+LED) compared to greenhouse (AMF+GH) condition for producing AMF spores in maize.

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CHAPTER VI

Investigation of Fatty Acid-Mediated AM Fungal Spore Production

6.1 Introduction

AMF play a pivotal role in facilitating nutrient acquisition and exchange between plants and the soil, establishing a mutualistic symbiosis with a diverse array of terrestrial plant species, ranging from trees to crops (Phillips et al. 2013). Their significance in enhancing plant development and aiding nutrient absorption, particularly in environments with limited nutrient availability, has been well established (Devi et al. 2021). Within this context, the role of specific fatty acids, including myristic acid, palmitic acid, and palmitoleic acid, has emerged as a crucial component influencing the interaction between AM fungi and their host plants.

Previous research have revealed the reliance of AM fungi on their plant hosts for the production of palmitic acid, a saturated fatty acid, with the fatty acid synthase activity confined primarily to the intraradical mycelium, emphasizing the substantial contribution of fatty acid metabolism to the obligatory biotrophic nature of these fungi (Trépanier et al. 2005; Luginbuehl et al. 2017). In addition, current research has suggested the deficiency of type I fatty acid synthase in AMF, indicating their inability to biosynthesize specific fatty acids (Luginbuehl et al. 2017). Notably, the presence of specific fatty acids, such as palmitoleic acid (C16:Δ9Z) can induced more secondary spores number of *R. irregularis* (Kameoka et al. 2019) and myristic acid (C14:0), has been also found to significantly influence the development of AMF, promoting their growth and serving as a source of carbon and energy for their asymbiotic growth (Sugiura et al. 2020; Shao et al. 2023). Tracer experiments using ^{13}C-labeled myristic acid have demonstrated its utilization in the synthesis of crucial components, including triacylglycerol and cell wall constituents within AMF, further highlighting its importance (Sugiura et al. 2020). Additionally, myristic acid has been effectively

utilized in host-free culture systems for the large-scale production of AMF, emphasizing its integral role in their life cycle (Tanaka et al. 2022).

Furthermore, specific fatty acids such as 16:0 and 16:1ω5, encompassing palmitic acid, have been identified as reliable indicators for assessing AM fungal development and biomass in different soil and root environments (Olsson and Johansen 2000; Van Aarle and Olsson 2003). Moreover, the synthesis of palmitoleic acid by the host plant has been established as crucial for the growth and survival of AMF, emphasizing the intricate relationship between fatty acids and the symbiotic association (Luginbuehl et al. 2017). Thus, the significance of fatty acids in the symbiotic relationship between AMF and their host plants extends beyond mere involvement, pointing toward a complex interplay with numerous implications.

6.2 Objective

To investigate some specific fatty acids, based on the fatty acid profile of arbuscular mycorrhizal fungi, on enhancement spore production of *R. irregularis* in the symbiotic conditions with maize using a substrate-based production system.

6.3 Materials and Methods

6.3.1 Plant growth and condition

Maize (*Zea mays* L. cultivar Suwan 5) seeds were surface-sterilized by immersion in 95% ethanol for 30 seconds, followed by a 12-minute treatment with a 3% (v/v) sodium hypochlorite (NaOCl) solution. The sterilized seeds underwent six thorough rinses with sterile water and were left to soak for overnight in sterilized water on an incubator shaker operating at 200 rpm. After this soaking period, the seeds were washed 1 time with sterile water, carefully positioned on sterilized paper towels, and maintained in darkness for two days. Each pot was prepared by incorporating 1 kg of a mixture of sterilized river sand and vermiculite in a 1:1 ratio. Subsequently, 100 spores of *Rhizophagus irregularis* DAOM197198 (Mycorise, Premier Tech, Rivière-du-Loup, Canada) were inoculated into each plant. The plants were cultivated in a greenhouse under traditional conditions for 3 months, with watering ceased in the final month. To support the growth of AMF, a half-strength Hoagland's

solution supplemented with 100 ml (100 µM) of KH_2PO_4 was supplied every two days.

6.3.2 Fatty acid preparation and inoculation

The fatty acid pellets, including myristic acid (C14:0) (Sigma-Aldrich), palmitoleic acid (C16:0) (Sigma-Aldrich), and palmitic acid (C16:0) (Sigma-Aldrich), were dissolved in 99% (w/v) ethanol, with different fatty acid concentrations, in a range of 10-10,000 µg, and each was adjusted to a total volume of 5 ml. Subsequently, the fatty acid solution was evenly distributed into the pot containing a mixture of sand and vermiculite using a pipette before the transplanting and AMF inoculation. Following this, 300 ml of distilled water was added to the pot to dilute the ethanol concentration.

6.3.3 AMF spore extraction

Soil samples were collected from each pot, and subsequently passed through a 250 µm mesh sieve to eliminate debris and obtain the fraction containing AMF spores (Daniels & Skipper, 1982; Gerdemann & Nicolson, 1963). The sieved soil was then mixed with distilled water, creating a slurry which was centrifuged at 5000 rpm for 5 minutes to facilitate the separation of soil particles from the spores. The resulting supernatant was discarded, and the sediment was resuspended in a 40% (w/v) sucrose solution. This suspension underwent an additional centrifugation at 5000 rpm for 5 minutes (Jenkins, 1964; Dandan and Zhiwei, 2007), enabling the AMF spores to float due to their lower density. Subsequently, the spores were meticulously collected from the gradient layers using a pipette and transferred into a Petri dish containing distilled water. Spore enumeration was conducted under a stereomicroscope.

6.3.4 Statistical analyses

Statistical analyses were performed using the SPSS 16.0 statistical software package (SPSS Inc., Chicago, IL, USA). Turkey's Honestly Significant Difference (HSD) test, with a significance level set at $p < 0.05$, was applied for multiple comparisons. The effects of supplementation with different fatty acids and concentrations were compared to the effects of AMF inoculation without fatty acid supplement alone. Data were subsequently visualized using GraphPad Prism (v.9.1.1).

6.4 Results

Based on fatty acid profile of AMF, this study aimed to examine the varying concentrations of pure fatty acids, specifically myristic acid, palmitic acid, and palmitoleic acid, which were the most composition fatty acid in organic oil and inoculated to maize plants for 120 days under symbiotic conditions. The results indicated that the presence of myristic acid at concentrations ranging from 10 µg to 10000 µg resulted in the maximum number of AMF spores, in comparison to the presence of palmitic acid and palmitoleic acid. However, no significant difference was observed when comparing the results of single inoculation with *R. irregularis* alone. Furthermore, it was observed that the presence of both palmitic acid and palmitoleic acid resulted in a decrease in the number of AMF spores. However, the addition of palmitoleic acid led to a significantly greater reduction in spore number compared to the use of AMF alone (**Figure 6.1**).

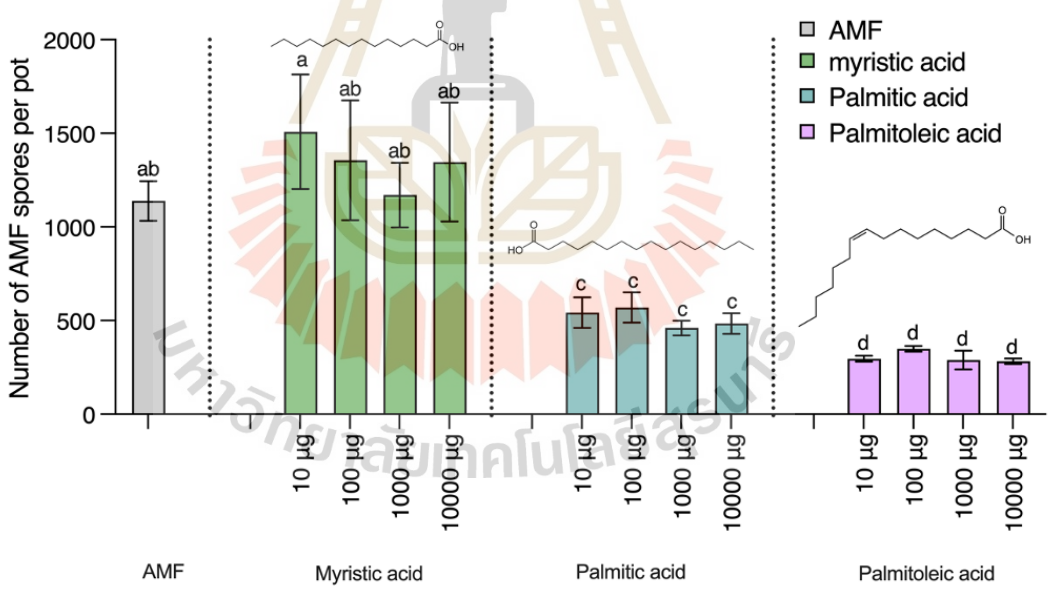

Figure 6.1 The impact of varying concentrations of fatty acid on the promotion of *R. irregularis* spore numbers is examined 120 DAI in the symbiotic conditions with maize using a substrate-base production system.

6.5 Discussion

Myristic acid, palmitic acid, and palmitoleic acid play important roles in the growth and development of arbuscular mycorrhizal fungi (AMF). These fatty acids are synthesized in the host plants and transferred to the fungi to sustain mycorrhizal colonization (Jiang et al. 2017). Lipid transfer from plants to AMF is crucial for their growth and development, as they are fueled not only by sugars but also by the transfer of lipids from the host plants (Keymer and Gutjahr 2018). The signature fatty acid 16:1**ω**5 has been identified as a promising tool for estimating AM fungal biomass in soil and roots (Olsson and Johansen 2000). AMF cannot produce the basic fatty acid palmitate (palmitic acid) in the absence of their host plants. However, fatty acid elongation and desaturation can occur independently of the plant (Rich et al., 2017). Studies by Tanaka et al. (2020) have shown that supplying fatty acids such as myristic acid and palmitoleic acid can support the host-free culture of AMF. Lipid metabolism in AMF, including the synthesis and response to various abiotic stresses, is an important aspect of their biology (Feng et al. 2020).

Myristic acid (C14:0) has been identified as a common organic acid in plant root exudates and can support the life cycle of AMF in the absence of a host when supplied in various formulations (Rillig et al. 2020). Another study reported that myristic acid can be used as a carbon and energy source for the asymbiotic growth of AMF (Sugiura et al. 2020). Similarly, application of myristic acid as a carbon and energy source in the symbiotic condition also promoted *R. irregularis* spore numbers up to 1,800 spores per plant when compared with other fatty acids produced in their AMF. Palmitic acid (C16:0) is one of the major fatty acids found in their fungal lipids (Brands et al. 2020). The fatty acid composition of AMF distinguishes them from other fungi and can be used as a marker for estimating the degree of AMF colonization (Sakamoto and Kaji 2017). The lack of de novo fatty acid synthesis in AMF may contribute to their obligate biotrophy and dependence on lipid transfer from host plants (Brands et al., 2018).

Furthermore, in the symbiotic environment, the application of fatty acids alongside the plant host introduces several factors that can affect the growth and development of AMF, including high temperatures, intense light exposure, and the

use of certain fatty acid forms that are not preferred by AMF. Supplying either low or high concentrations of these fatty acids resulted in a reduction in fungal spore numbers over the cultivation period. Therefore, it is possible that inoculation of one-time fatty acid to plant may not enough to provide the appropriate concentration of fatty acids since it might drain out every time when supplied with Hoagland solution. Moreover, it is possible that under greenhouse conditions, there is a higher risk of contamination by bacteria that can also use the fatty acid. Therefore, in this experiment, it is necessary to supply the fatty acid several times, as it may promote AMF spore production. In addition, fatty acids at a suitable concentration might enhance AMF spore production. AMF could absorb the fatty acids and utilize them as a carbon and energy source for growth, as well as for the biosynthesis of cellular components, especially AMF spores and vesicles as evidenced by the results observed in this study.

6.6 Conclusion

The results of this study indicate that myristic acid plays a key role in promoting the growth of arbuscular mycorrhizal fungi (AMF) spores in maize plants. This highlights the significance of understanding the specific fatty acid composition in plants and its impact on the symbiotic interactions with AMF. However, this approach may not be economically appropriate for substrate-based production system unless the natural fatty acid sources could be used instead. Furthermore, it is imperative to conduct further research on the potential inhibitory effects of palmitic acid and palmitoleic acid, as their negative impact on AMF spore counts reveals the need for future investigation. The findings presented in this study offer valuable insights for the production of AMF inoculum, contributing to the enhancement of agricultural practices and the augmentation of crop productivity.

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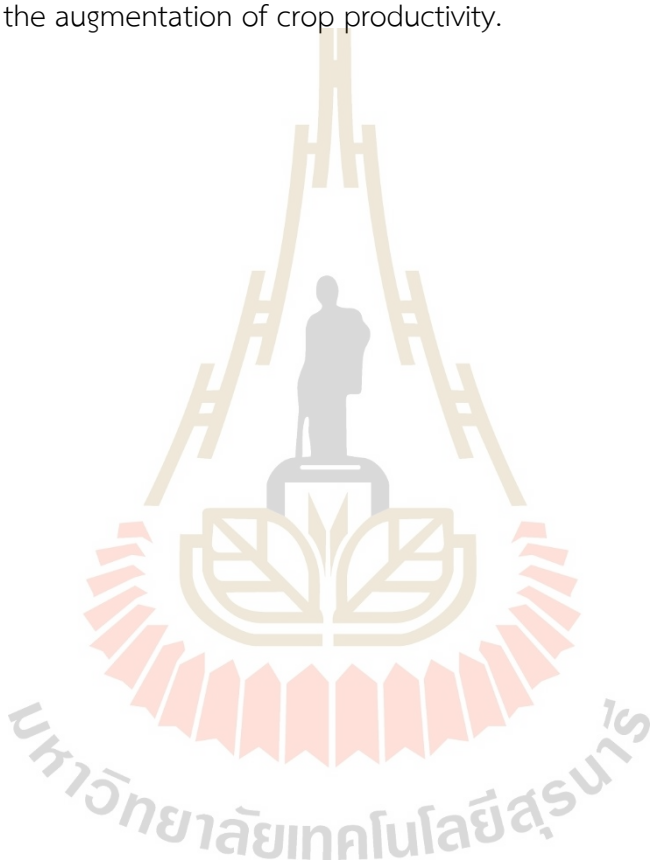
CHAPTER 7
CONCLUSION

This study demonstrated the application of biotic and abiotic approaches to AM fungal spore production under a substrate-based production system. Regarding the biotic approaches, firstly, the results of applying secretion compounds from SUT47 at an optimal concentration stimulated A. tuberculata spore production in maize roots. Secondly, the beneficial effects of B. velezensis S141 on the plant-AMF symbiosis were observed. The findings indicated that B. velezensis S141 not only enhanced early root growth but also promoted the abundance of AMF. Surprisingly, strain B. velezensis S141 was recognized as an endophyte in L. japonicus, inducing the expression of plant marker genes primarily involved in mycorrhizal formation and nutrient uptake. Moreover, the induction of marker genes associated with the cell cycle of R. irregularis upon co-inoculation with B. velezensis S141 strongly supported its potential for promoting fungal growth.

On the other hand, the abiotic approach was investigated by using LED light and supplemented fatty acids. The results showed that the optimal conditions of a red-to-blue light ratio of 60:40 and a total light intensity of 300 µMol/m2/s significantly enhanced crucial parameters of maize growth and development. Additionally, this specific LED light intensity notably stimulated R. irregularis colonization and spore production compared to conventional greenhouse conditions. The LED light also stimulated the expression of genes involved in the symbiotic relationship between maize and R. irregularis, along with genes regulating the fungus's cell cycle. Furthermore, when myristic acid was used with maize under symbiotic conditions, it promoted the growth of AMF spores in maize plants, emphasizing the importance of understanding the specific fatty acid composition in plants and its impact on symbiotic interactions with AMFi. However, the inhibitory effects of palmitic acid and palmitoleic acid were noted, as they negatively influenced the AMF spore count.

The use of biotic and abiotic approaches contributes to understanding beneficial microbial interactions, as the biotic approaches promote mycorrhizal symbiosis and highlight the importance of exploring the potential of beneficial microbes in enhancing AMF growth and plant nutrient uptake pathways. Furthermore, in biotic approaches, the promotion of plant growth and AM fungal inoculum production was correlated with their usage. Therefore, this study provides valuable insights for the production of AMF inoculum, contributing to the enhancement of agricultural practices and the augmentation of crop productivity.



APPENDIX

Growth media

Appendix A.1 Luria-Bertani (LB) medium (Bertani, 1951)

Constituents per liter.

1.	Tryptone	10.0 g
2.	NaCl	10.0 g
3.	Yeast Extract	5.0 g
4.	Distilled Water	up to 1 liter

Adjust the pH to 7.0 with 5 N NaOH.

Appendix A.2 Half-strength of Hoagland solution (Nguyen and Saito, 2021)

Constituents of Stock solution per 50 mL

Stock A

1.	500 mM KNO_3 (MW = 101.11)	2.528 g
2.	500 mM $Ca(NO_3)_2 \cdot 4H_2O$ (MW = 236.15)	5.904 g

Stock B

1.	200 mM $MgSO_4 \cdot 7H_2O$ (MW = 246.47)	2.465 g

Stock C

1.	100 mM KH_2PO_4 (MW = 136.08)	0.680 g

Stock D

1.	200 mM K_2SO_4 (MW = 174.26)	1.743 g

Stock E

1.	1 g Fe L^{-1} Fe (III)-EDTA (MW = 421.09)	0.377 g

Stock F

1.	0.5 g B L^{-1} H_3BO_3 (MW = 61.83)	0.143 g
2.	0.5 g Mn L^{-1} $MnCl_2 \cdot 4H_2O$ (MW = 197.90)	0.090 g
3.	0.05 g Zn L^{-1} $ZnSO_4 \cdot 7H_2O$ (MW = 287.56)	0.011 g
4.	0.02 g Cu L^{-1} $CuSO_4 \cdot 5H_2O$ (MW = 249.68)	0.004 g
5.	0.01 g Mo L^{-1} $Na_2MoO_2 \cdot 2H_2O$ (MW = 241.95)	0.00126 g

Half-strength Hoagland solution (for mycorrhiza) per liter

Stock A	5 mL
Stock B	5 mL
Stock C	1 mL (100 μM Pi)
Stock E	2.5 mL
Stock F	0.5 mL
Distilled Water	up to 1 liter
Adjust the pH to 6.0	

*When it is necessary to change concentrations of Pi in stock C, a concentration of K^+ should be adjusted by adding stock D

BIOGRAPHY

Mister Sutee Kiddee was born on January 5th, 1992, in Nan, Thailand. He attended Ban Wangyao School for primary education and Ban Luang School for high school. In 2014, he graduated from Suranaree University of Technology with a Bachelor's degree in Crop Production Technology with 1st class honor. In 2014, he received a scholarship from the Thailand Research Fund (TRF) under the Royal Golden Jubilee Ph.D. Programme (RGJ) to pursue a combined Master- Doctoral degree in the School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, under the Supervised of Assoc. Prof. Dr. Panlada Tittabutr. During his enrollment in the doctoral degree program, he presented his research work titled "Effect of co-inoculation between plant growth promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi on the growth promotion of rice growing under the system of rice intensification (SRI)" in a poster presentation at the 4th, Asian Conference on Plant- Microbe Symbiosis & Nitrogen Fixation held in Penang, Malaysia from October 16 to 19, 2016. He also participated in the 2021 National RGJ and RRI conference, Theme "Driving Thailand by Researchers and Research Topic Challenges in the New Era" Thailand in June 14th, 2021 for poster presentation in the topic "*Bacillus velezensis* S141 promote biomass of *Rhizophagus irregualris* DAOM197198 in *Lotus japonicus* B-129". Moreover, he obtained a scholarship for his one year research position at Shinshu University of Agriculture and Technology in Japan. This scholarship was generously supported by Thailand Research Fund (TRF) under the Royal Golden Jubilee Ph.D. Programme (RGJ) from June 3rd, 2019 to May 30th, 2020. His research work in the topic of "Secretion compounds from *Brevibacillus* sp. SUT47 promote spore propagation of *Acaulospora tuberculata* colonizing maize roots (*Zea mays* L. cultivar Suwan 5)" has been published in ScienceAsia 2020.