DETERMINANTS DERIVED FROM PGPR CAPABLE OF INCREASING SOYBEAN AND MUNG BEAN PRODUCTION VIA *Bradyrhizobium* INOCULATION

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ปัจจัยจาก PGPR ที่สามารถเพิ่มผลผลิตถั่วเหลืองและถั่วเขียวเมื่อใช้ร่วมกับแบรดี้ไรโซเบียม

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นายสุรชาติ สิบพลกรัง : ปัจจัยจาก PGPR ที่สามารถเพิ่มผลผลิตถั่วเหลืองและถั่วเขียวเมื่อใช้ร่วมกับแบรดี้ไรโซเบียม (DETERMINANTS DERIVED FROM PGPR CAPABLE OF INCREASING SOYBEAN AND MUNG BEAN PRODUCTION VIA *Bradyrhizobium* INOCULATION) อาจารย์ที่ปรึกษา : ศาสตราจารย์ ดร.หนึ่ง เตียอำรุง, 215 หน้า.

Plant growth promoting rhizobacteria (PGPR) เป็นกลุ่มแบคทีเรียที่มีอยู่ในดินตามธรรมชาติโดยอาศัยอยู่บริเวณรากพืช และเป็นประโยชน์ในการส่งเสริมการเจริญเติบโตของพืช การใช้หัวเชื้อร่วมกันระหว่างแบรดี้ไรโซเบียมและแบคทีเรียในกลุ่ม PGPR สามารถกระตุ้นการเจริญเติบโต และการสร้างปมในถั่วเหลืองได้ อย่างไรก็ตามอิทธิพลจากการใช้หัวเชื้อร่วม ต่อการส่งเสริมการเจริญเติบโตของถั่วเหลืองยังต้องการการศึกษาในเชิงลึก วัตถุประสงค์ของงานวิจัยนี้ เพื่อประเมินอิทธิพลจากการใช้หัวเชื้อร่วมกันระหว่างแบรดี้ไรโซเบียมกับแบคทีเรียในกลุ่ม PGPR และสารที่ปลดปล่อยจากแบคทีเรียในกลุ่ม PGPR ในการส่งเสริมการสร้างปม และการเพิ่มประสิทธิภาพการตรึงไนโตรเจนในถั่วเหลือง (*Glycine max*) สายพันธุ์เชียงใหม่ 60 และถั่วเขียว (*Vigna radiata*) สายพันธุ์ SUT4 จากการทดลองใช้หัวเชื้อร่วมกันระหว่าง *Bacillus* สายพันธุ์ S141 กับแบรดี้ไรโซเบียม สามารถส่งเสริมการสร้างปม เพิ่มขนาดปม เพิ่มประสิทธิภาพการตรึงไนโตรเจน และเพิ่มผลผลิตของถั่วเหลืองและถั่วเขียว นอกจากนั้นการใช้หัวเชื้อร่วมกันระหว่างแบรดี้ไรโซเบียมกับสารที่ปลดปล่อยจาก *Bacillus* สายพันธุ์ S141 ยังส่งเสริมการเจริญเติบโตของถั่วเหลืองและถั่วเขียวเช่นเดียวกับการใช้เซลล์ของ *Bacillus* สายพันธุ์ S141 อีกด้วย จากการศึกษาการแสดงออกของโปรตีนแสดงให้เห็นว่า *Bacillus* สายพันธุ์ S141 สามารถปลดปล่อยสารประกอบที่หลากหลายชนิด โดยสารประกอบเหล่านี้สามารถกระตุ้นกระบวนการเมตาบอลิซึม และการตอบสนองต่อสารเคมี ส่งผลให้เกิดการกระตุ้นการสร้างปม และเพิ่มประสิทธิภาพการตรึงไนโตรเจนของถั่วเหลืองและถั่วเขียว เพื่อให้เข้าใจถึงกลไกการทำงานร่วมกันที่ซับซ้อนระหว่างแบรดี้ไรโซเบียม แบคทีเรียในกลุ่ม PGPR และพืช จึงได้ทำการอ่านลำดับนิวคลีโอไทด์ทั้งหมดในจีโนมของ *Bacillus* สายพันธุ์ S141 สามารถจำแนกชนิดของ *Bacillus* สายพันธุ์ S141 เป็น *Bacillus velezensis*/*amyloliquafaciens* โดยลำดับนิวคลีโอไทด์ทั้งหมดนี้ มีลักษณะโครโมโซมเป็นวงกลม (Circular chromosome) และมีขนาด 3,974,582 นิวคลีโอไทด์ ประกอบด้วย 3,817 ยีน เมื่อทำการวิเคราะห์จีโนม พบยีนที่เกี่ยวข้องกับการสังเคราะห์ออกซิน และไซโตไคนินใน *B. velezensis* สายพันธุ์ S141 จากการทำลายยีนที่เกี่ยวข้องกับการสังเคราะห์ IAA พบปริมาณการสังเคราะห์ IAA ลดลงในสายพันธุ์กลาย S141Δ*dhaS*, S141Δ*yhcX* และ S141Δ*IPyAD* แสดงให้เห็นว่าแบคทีเรียสายพันธุ์ที่ถูกทำลายยีนที่เกี่ยวข้องกับการสังเคราะห์ IAAทั้งหมดเกี่ยวข้องกับกระบวนการสังเคราะห์ IAA นอกจากนี้การทดลองใช้หัวเชื้อร่วมกันระหว่างแบรดี้ไรโซเบียมสายพันธุ์ USDA110 กับ S141Δ*yhcX* ทำให้จำนวนปมที่มีขนาดใหญ่ลดลง รายงานนี้เป็นการรายงานครั้งแรกเกี่ยวกับยีน *yhcX* ซึ่งอาจจะมีบทบาทสำคัญต่อกระบวนการสังเคราะห์ IAA ใน *Bacillus velezensis* ที่ส่งผลกระทบหลักต่อการกระตุ้นการเจริญเติบโตของถั่วเหลือง จากการทำลายยีนที่เกี่ยวข้องกับการสังเคราะห์ไซโตไคนินที่ประกอบด้วยยีน *IPT* และ *IPI* เมื่อทำการทดลองใช้หัวเชื้อร่วมกัน

ระหว่างแบรดี้ไรโซเบียมสายพันธุ์ USDA110 กับ เชื้อสายพันธุ์กลาย S141Δ*IPI* ทำให้จำนวนปมที่มีขนาดใหญ่ลดลง โดย *IPI* อาจจะมีบทบาทสำคัญต่อขนาดของปมถั่วเหลือง อย่างไรก็ตาม นอกจากผลของ IAA หรือ ไซโตไคนิน อาจจะมีผลจากสารอื่นที่ปลดปล่อยออกมาจาก *B. velezensis* สายพันธุ์ S141 ช่วยส่งเสริมแบรดี้ไรโซเบียม ทำให้เกิดการกระตุ้นการสร้างปมที่มีขนาดใหญ่ ส่งผลให้ประสิทธิภาพการตรึงไนโตรเนสูงขึ้น ดังนั้นการเพิ่มประสิทธิภาพการตรึงไนโตรเจนของถั่วเหลือง โดยใช้กลยุทธ์การใช้หัวเชื้อร่วมระหว่าง *B. velezensis* สายพันธุ์ S141 ร่วมกับแบรดี้ไรโซเบียมนั้น สามารถพัฒนาเพื่อใช้เป็นหัวเชื้อที่มีประสิทธิภาพสำหรับถั่วเหลืองและถั่วเขียวได้

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2561

ลายมือชื่อนักศึกษา_____
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SURACHAT SIBPONKRUNG : DETERMINANTS DERIVED FROM PGPR CAPABLE OF INCREASING SOYBEAN AND MUNG BEAN PRODUCTION VIA *Bradyrhizobium* INOCULATION. THESIS ADVISOR : PROF. NEUNG TEAUMROONG, Dr.rer.nat., 215 PP.

SOYBEAN/MUNG BEAN/CO-INOCULATION/*Bradyrhizobium*/PGPR

Plant growth promoting rhizobacteria (PGPR) are naturally occurring soil bacteria that aggressively colonize plant roots and benefit plants by providing growth promotion. Co-inoculation of soybean plants with PGPR strain and *Bradyrhizobium* altered plant growth parameters and significantly improved nodulation. However, the effects of co-inoculation need to be analyzed in more detail. The objectives of this research were to evaluate PGPR and its supernatant influence on promoting nodulation and N_2-fixing efficiency of soybean (*Glycine max* cv. CM60) and mung bean (*Vigna radiate* cv. SUT4) by co-inoculation with *Bradyrhizobium*. Co-inoculation between *Bacillus* sp. S141 and *Bradyrhizobium* with soybean and mung bean resulted in enhancing nodulation, size of nodule, N_2-fixing efficiency and increasing soybean and mung bean production. Besides, co-inoculation of supernatant of S141 with *Bradyrhizobium* produced the same results by using cells of S141. SDS-PAGE and protein identification suggested that S141 might facilitate the induction of numerous compounds which were strong attractants stimulating chemotactic response and metabolism, resulting in the enhanced nodulation and N_2-fixing efficiency. In order to better understand these complex *Bradyrhizobium*-PGPR-plant interactions, the whole genome of the typical S141 was analyzed. The genome of S141 is categorized in *Bacillus velezensis*/*amyloliquefaciens* and comprises a 3,974,582 bp long circular chromosome that consists of 3,817 protein-coding genes. Based on genomic analysis, auxin and cytokinin functionally related genes in the genome of S141 were

discovered. The disruption of putative genes related to IAA biosynthesis pathway revealed the IAA reduction in S141Δ*dhaS*, S141Δ*yhcX* and S141Δ*IPyAD* suggested that all of S141 IAA related mutant strains are related with IAA biosynthesis. Moreover, co-inoculation of *Bradyrhizobium diazoefficiens* USDA110 with S141Δ*yhcX* also reduced nodule number of L size nodules. This was the first time it was reported that *yhcX* may play a major role in IAA biosynthesis in *Bacillus velezensis* as well as provide a major impact on soybean growth promotion. The disruption of genes related to cytokinin biosynthesis pathway including: *IPT* and *IPI* genes and co-inoculation of USDA110 with S141Δ*IPI* reduced the nodule number of VL size nodules, it appears that *IPI* might play an important role in nodule size of soybean-*Bradyrhizobium* symbiosis. However, it was possible that not only IAA and cytokinin but also some other substrates secreted from S141 facilitate *Bradyrhizobium* to trigger bigger nodule formation resulting in the higher efficiency of N_2-fixation. Therefore, the efficiency to enhance soybean and mung bean N_2-fixation by S141 with *Bradyrhizobium* co-inoculation strategy could be developed for supreme soybean and mung bean inoculants.

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LIST OF ABBREVIATIONS

°C	=	degree Celsius
µm	=	micrometer
µg	=	microgram
µl	=	microliter
ABA	=	Abscisic acid
ACC	=	1-aminocyclopropane-1-carboxylic acid
AM	=	Arbuscular mycorrhiza
ANOVA	=	Analysis of variance
AON	=	Autoregulation of Nodulation
ARA	=	Acethylene Reduction Assay
ATP	=	Adenosine triphosphate
BNF	=	Biological Nitrogen Fixation
bp	=	base pair
CFU	=	Colony-forming unit
DAI	=	Days after inoculation
DNA	=	deoxyribonucleic acid
dNTP	=	deoxynucleotide 5’ triphosphate
DOA	=	Department of Agricultural
EPS	=	extracellular polysaccharides
ER	=	Endoplasmic reticulum
et al.	=	Et alia (and other)
FAO	=	Food and Agriculture Organization

LIST OF ABBREVIATIONS (Continued)

g	=	gram
GA	=	Gibberellic acids
GFP	=	Green Fluorescent Protein
h	=	hour
ha	=	Hectare
IAA	=	Indole-3-acetic acid
IAH	=	indole-3-acetaldehyde
IAM	=	Indole-3-acetamide
IAN	=	indole-3-acetonitrile
IAOx	=	indole-3-acetaldoxime
IBA	=	Indole-3-butyric acid
ICA	=	Indoel-3-carboxylic acid
iPA	=	isopentenyl-adenine
IPA	=	Indole-3-pyruvic acid
ISR	=	Induced Systemic Resistance
IT	=	infection thread
JA	=	Jasmonic acid
Kb	=	kilobases
l	=	litre
LB	=	Luria Bertani broth
LCO	=	Lipo-Chitin Oligosaccharides
LSD	=	Least significant difference
M	=	Molarity

LIST OF ABBREVIATIONS (Continued)

mg	=	milligram
Mg	=	Magnesium
min	=	minute
ml	=	milliliter
mM	=	millimolar
MT	=	metric tons
MY	=	Marketing Year
N	=	Nitrogen
NCBI	=	National Center for Biotechnology Information
NF	=	Nod factors
ng	=	nanogram
NifTAL	=	Nitrogen fixation for Tropical Agricultural Legumes
nm	=	nanometer
OM	=	Organic matter
PBM	=	Peribacteroid membrane
PCR	=	polymerase chain reaction
PGPB	=	Plant Growth Promoting Bacteria
PGPR	=	Plant Growth Promoting Rhizobacteria
PHB	=	polyhydroxybutyrate
PM	=	Plasma membrane
PSM	=	Phosphorus solubilizing microorganisms
RNA	=	ribonucleic acid
RNS	=	Root Nodule Symbiosis

LIST OF ABBREVIATIONS (Continued)

rpm	=	revolution per minut
rRNA	=	ribosomal ribonucleic acid
RT-PCR	=	Reverse Transcription Polymerase Chain Reaction
SA	=	Salicylic acid
SD	=	Standard Deviation
SUT	=	Suranaree University of Technology
TEM	=	Transmission Electron Microscopy
tRNA	=	transfer ribonucleic acid
USDA	=	United States Department of Agriculture
v/v	=	volume per volume
w/v	=	weight per volume
WAI	=	Weeks after inoculation
YEM	=	Yeast Extract Mannitol
ZT	=	zeatin

CHAPTER I

INTRODUCTION

1.1 Significance of this study

With around 18,000 species, Leguminosae are considered as the largest plant family and play an important role in the earth ecosystem. By developing nitrogen-fixing root nodules, legumes have established a symbiotic relationship with specific soil bacteria are known as rhizobia. Rhizobia are Gram-negative soil bacteria belonging to genera *Rhizobium, Sinorhizobium, Mesorhizobium, Phylorhizobium, Azorhizobium* and *Bradyrhizobium.* They have the capacity to live either as free-living bacteria or as nitrogen-fixing bacteroids inside a specialized root organ of *Leguminosae* called nodules to establish a reciprocal profitable metabolic exchange with its host plant (Brewin, 2004; Peter et al., 1996). Whereas bacteroids convert atmospheric dinitrogen (N_2) into ammonia for the benefit of the host, leguminous plants in turn supply microsymbionts with photosynthate (Cooper, 2007; Prell and Poole, 2006). *Bradyrhizobium diazoefficiens* USDA110 and *Bradyrhizobium* sp. SUTN9-2 is a symbiotic nitrogen-fixing soil bacteria that induce root nodules formation in legume soybean (*Glycine max*) (Vauclare et al., 2013) and mung bean (*Vigna radiata*) (Piromyou et al., 2017), respectively.

Plant growth-promoting rhizobacteria (PGPR) are naturally occurring soil bacteria that aggressively colonize plant roots and benefit plants by providing growth promotion (Saharan and Nehra, 2011). Some PGPR strains, from a range of genera, enhance legume growth, nodulation and nitrogen fixation when coinoculated with rhizobia. Examples of these are *Azospirillum* (Remans et al., 2008), *Azotobacter* (Wu et al., 2012), *Bacillus*,

Pseudomonas (Atieno et al., 2012; Zahir et al., 2011), *Serratia* (Pan et al., 2002; Zahir et al., 2011) and *Streptomyces* (Tokala et al., 2002). As they share common microhabitats in the root–soil interface, rhizobia and PGPR must interact during their processes of root colonization. The effect of *Rhizobium*–PGPR coinoculation has been observed in different symbiotic and plant growth parameters. In the last few years, Aung *et al.* (2013) found that PGPR co-inoculation with either *B. japonicum* CB1809 or *B. diazoefficiens* USDA110 gave high nodule number, increased nodule dry weight and significantly increased seed yield over single inoculation. On the other hand, PGPR co-inoculation with *B. japonicum* CB1809 in nutrient-poor soil condition showed low nodule dry weight and biomass dry weight per plant when compared with PGPR co-inoculation with *B. diazoefficiens* USDA110 at 70 days after inoculation. Moreover, Prakamhang (2013) suggested the first observations that demonstrate the mechanism of PGPR strains *Bacillus subtilis* and *Staphylococcus* sp. on *Bradyrhizobium*-soybean symbiosis. The co-inoculation with *B. diazoefficiens* USDA110 and PGPR leads to an increased number of the most active nodules and plant yield, therefore, to a greater nitrogen fixation. Inoculation modes of PGPR and rhizobia may result in variable effects on legume growth, nodule morphology and this may depend on the phase of the process modified by PGPR. However, the effect of biological molecules produced via co-inoculation process on soybean and mung bean-bradyrhizobial symbiosis are needed to analyze in more detail.

1.2 Hypothesis

The overall nitrogen fixing efficiency and efficacy of the soybean and mung bean-bradyrhizobial symbiosis could be enhanced via co-inoculation with supernatant of PGPR strain. The biological molecules elicited the interactions between PGPR and

Bradyrhizobium co-inoculated in soybean and mung bean-bradyrhizobial symbiosis, resulting in better nodulation, nitrogen fixation, nodule morphology, growth and yield. Therefore, the aim of this study was to examine some determinants derived from PGPR affect on *Bradyrhizobium* co-inoculation that can enhance symbiosis.

1.3 Research objectives

1.3.1 General objectives

The general objectives of this research were to evaluate the biological molecules secreted from PGPR. The biological molecules that had the interactions between PGPR and *Bradyrhizobium* co-inoculated in soybean and mung bean-bradyrhizobial symbiosis, resulting in better nodulation, nitrogen fixation, nodule morphology, growth and yield.

1.3.2 Specific objectives

1. To evaluate the *Bacillus* sp. S141 and its supernatant influence on promoting nodulation and N_2-fixing efficiency of soybean (*Glycine max*) and mung bean (*Vigna radiata*) by co-inoculation with *Bradyrhizobium diazoefficiens* USDA110 and *Bradyrhizobium* sp. SUTN9-2, respectively
2. To reveal the whole genome of PGPR for better understanding the complexity of *Bradyrhizobium*-PGPR-plant interactions using Illumina MiSeq platform
3. To demonstrate the effect of biological molecules secreted from *Bacillus* sp. S141 on soybean and mung bean-bradyrhizobial symbioses and the possible combination of biological molecules between PGPR, *Bradyrhizobium* and plants

4. To determine the genes involved in biological determinant biosynthesis of PGPR

1.4 Scope and limitation of the study

The biological molecules and interaction mechanisms of biological molecules between PGPR and *Bradyrhizobium* co-inoculation in soybean and mungbean that could enhance nodulation, nitrogen fixation, nodule morphology, growth and yield were determined. The whole genome of PGPR was revealed by Illumina MiSeq platform. In addition, some genes involved in biological determinant biosynthesis of PGPR were investigated.

CHAPTER II

LITERATURE REVIEW

2.1 Soybean

Soybean [*Glycine max* (L.) Merrill] belongs to the family Leguminosae, subfamily Papilionideae. Soybean is a leguminous vegetable of the pea family that grows in tropical, subtropical, and temperate climates, the optimal temperature range for soybean growth is 25 - 30°C (Zhang et al., 1996). Soybean is relatively resistant to low and very high temperatures but growth rates decrease above 35°C and below 18°C (FAO, 2019a). Soybean is one of the world's most economically important legume crops (Prakamhang, 2013), with important roles in human and animal nutrition, besides broad industrial applications. The high protein content of its grains about 40% represents a nutritionally important part of the diets of humans and domestic animals. In addition, other seed products have broad industrial and pharmaceutical applications.

The United States and South American countries account to most of the world grain production (Hungria and Mendes, 2015). In 2017, the total area cropped to soybean was estimated at 130 million hectares, with a total production of 366 million metric tons, the three major producers are the United States, Brazil and Argentina. Soybean production in Thailand is reduced mostly due to the not cost effective (more expensive than import) and seed production problems. In 1982-1985 the area for soybean production between 800,000-1,000,000 rai that why USDA support loan fund for producing large scale inoculant production at DOA. The total area cropped to soybean in 2017 was account 3,100 hectares, with a total production of 54,000 metric tons (MT) (FAO, 2019b). The United States

Department of Agriculture (USDA) (2019) reported that soybean production in Thailand is forecast to decline slightly to 52,000 MT in Marketing Year (MY) 2019/20 due to no inoculant available for farmers area, low yields and unattractive returns when compared to competing crops like corn and off-season rice. The government still bans domestic cultivation of all transgenic or biotech plants, including soybeans. Due to low domestic production, Thailand depends almost entirely on imported soybeans to meet domestic demand for animal feed, vegetable oil, and food. Soybean imports are forecast to increase by 2 percent in MY2019/20 in line with anticipated total domestic consumption growth. Imports in MY2018/19 are anticipated to grow sharply by 27 percent from MY2017/18 due to a need to replenish depleted stocks and increased domestic consumption. Nearly all domestic soybean production is used to produce food products such as soybean milk, tofu, soybean sauces, and other soy food products. As a result, food-grade soybean imports are currently estimated at 200,000 MT. Canada and the United States are the only two foreign suppliers for this market segment. The food industry prefers domestic soybeans over imported soybeans due to their freshness and biotech-free status. However, due to growing demand and decreased domestic production, processors are increasingly relying on imported soybeans to meet their needs.

Soybean cultivars are also known to influence nodulation competition among *Bradyrhizobium* strains (Triplett and Sadowsky, 1992). In Thailand, soybean is grown in a variety of locations, cropping patterns, land types, and seasons. Many soybean cultivars have been developed with characteristics appropriate for different geographical areas. The examples of recommended Thai soybean cultivars are SJ1, SJ2, SJ4, SJ5, Nakorn Sawan 1 (NW1) and Chaing Mai 60 (CM60) (Boonkerd, 2002). The crop can be grown on a wide range of soils except those which are very sandy. Optimum soil pH is 6 to 6.5. The fertilizer requirements are 15 to 30 kg per hectare P and 25 to 60 kg per hectare K (FAO, 2019a). Soybean plants are able to facilitate biological nitrogen fixation (BNF) through a

symbiosis with soil bacteria, commonly called “rhizobia.” This relationship results in the formation of novel root organs, called nodules, which are critical for establishing an environment suitable for symbiotic nitrogen fixation (Lin et al., 2012). Soybean plants are able to fix 100-200 kg per hectare per year of atmospheric nitrogen (Smith and Hume, 1987). However, a starter dose of 10 to 20 kg per hectare nitrogen is beneficial for good early growth. In a survey of quantifications of BNF by agricultural legumes, soybean was rated as the highest among the grain producers, with contributions of up to 450 kg nitrogen per hectare, surpassed only by the fodder crops bitter lupin (*Lupinus mutabilis* Sweet) and alfalfa (*Medicago sativa* L.), with up to 527 and 470 kg nitrogen per hectare, respectively (Ormeño-Orrillo et al., 2013). Numerous studies have investigated significant contributions of atmospheric N_2 fixation to soybean nutrition and growth (Bai et al., 2003; Morel et al., 2012). Most estimation show that soybean derives between 25 and 75% of its N from fixation (McNeil, 2010).

2.2 Mung bean

The mung bean [*Vigna radiata* (L.) Wilczek], alternatively known as the green gram, is a fast growing warm-season legume species belonging to the papilionoid subfamily of the Fabaceae (Kang et al., 2014). Mung bean is such a minor crop that dryland smallholder farmers can use to break the downward spiral, and increase the profitability and sustainability of their farms. It is a nutritious warm season legume crop. The grains are rich in protein, minerals, and vitamins (Pataczek et al., 2018). Mung bean is widely grown in Asia, but also in parts of Africa and Australia. The mung bean is mainly cultivated in East Asia, Southeast Asia and Indian subcontinent. Nowadays, almost 90% of the mung bean production is found in Asia, where India, China, Pakistan and Thailand are among the most important producers (Lambrides and Godwin, 2007). Integration of mung bean in cropping systems, in Thailand intercropping with cassava were shown good for weed control,

additional nitrogen and organic matter (OM) and addition of income, and particularly in Central and South Asia, may increase the sustainability of dryland production systems. Diversification of local production systems through inclusion of mung bean as a catch crop provides additional income to farmers and has the potential to improve soil fertility (Pataczek et al., 2018). The United States Department of Agriculture (USDA) (2014) reported that mung bean production will likely continue to increase mainly due to acreage expansion. Farmers will likely replace off-season rice cultivation with mung bean due to attractive farm-gate prices. Mung bean farm-gate prices have increased significantly from the same period in 2013. In addition, the Royal Irrigation Department have warned farmers to avoid cultivating off-season rice due to possible water shortages in Thailand's northern and central regions. Moreover, the suspension of the MY2014 Off-Season Rice Paddy Pledging Program makes the second off-season rice crop less attractive. Mung bean consumption is expected to trend upward in anticipation of a stronger economy in the latter half of 2014. The Thai food processing industries are also likely to expand their production capacity to meet growing demand for diversified products such as vermicelli, bean flour, bean sprouts, and various confectionary items. The use of mung bean in the feed industry remains marginal due to its uncompetitive prices compared to other grains. MY2014 mung bean exports declined to 25,755 metric tons, down 37 percent from the previous year due to limited exportable supplies caused by growing domestic demand from the food processing industry. Meanwhile, MY2014 mung bean imports increased to 27,912 metric tons, mainly from Myanmar, which accounts for approximately 90% of total imports. Mung bean exports are expected to decline significantly to around 20,000 metric tons. The increase in mung bean production is likely to be offset by domestic consumption. In 2017, the total area cropped to mung bean in Thailand was account 166,576 hectares, with a total production of 313,596 metric tons (MT) (FAO, 2019c).

Mung bean is one of the important crops with the ability to improve soil fertility through N_2 fixation by symbiotic association with rhizobia present in root nodules (Ali and Gupta, 2012). Like other legumes, mung bean also possesses the ability to establish a symbiotic relationship with the nitrogen-fixing soil bacteria generally known as rhizobia, which is a term used for a broad range of microsymbionts from *α*- and *β-Proteobacteria* that establish symbiosis with leguminous plants for BNF. Mung bean is a promiscuous host that can be nodulated with a broad range of rhizobial species belonging to four different bacterial genera: *Bradyrhizobium*, *Ensifer* (formerly known as *Sinorhizobium*), *Mesorhizobium* and *Rhizobium* (Lu et al., 2009; Risal et al., 2012; Yang et al., 2008). Nevertheless, the successful nodulation of mung bean by the isolates showed that strains of both the genera *Bradyrhizobium* and *Ensifer* (*Sinorhizobium*) can be used for production of inoculum (Hakim et al., 2018). Mung bean can fix approximately 37–83 kg per hectare of nitrogen through nodule symbiotic association (Mohammad et al., 2010).

2.3 Biological nitrogen fixation; BNF

BNF accounts for roughly two-thirds of the nitrogen fixed globally, whereas the remaining portion is mostly contributed by the industrial Haber-Bosch process. BNF was firstly discovered by the German agronomist Hermann Hellriegel and Dutch microbiologist Martinus Beijerinck (Paracer and Ahmadjian, 2000). BNF is performed, generally at mild temperatures, by diazotrophic microorganisms, which are widely distributed in nature (Raymond et al., 2004). Most BNF is carried out by the activity of the molybdenum nitrogenase, which is found in all diazotrophs. In addition to the molybdenum nitrogenase, some diazotrophic microorganisms carry alternative vanadium and/or iron-only nitrogenases (Masepohl et al., 2002). BNF occurs when atmospheric nitrogen is converted to ammonia by nitrogenase enzyme (Postgate, 1998). Nitrogen fixation is catalyzed by the complex enzyme nitrogenase. The molybdenum nitrogenase enzyme complex has two

component proteins (Bulen and LeComte, 1966) including component I (also called dinitrogenase, or Fe-Mo protein), an alpha2beta2 tetramer encoded by the *nifD* and *nifK* genes, and component II (dinitrogenase reductase, or Fe protein) a homodimer encoded by the *nifH* gene (Rubio and Ludden, 2008; Zehr et al., 2003). Both component proteins are very oxygen-labile. Until now three different forms of nitrogenase have been reported (Rangaraj et al., 2000). All three different nitrogenases share similar structural properties but differ in the heterometal present in the active site of the dinitrogenase unit. The most widespread nitrogenase contains iron molybdenum cofactors at the active site and is encoded by the *nif* gene family. Vanadium containing and *vnf* encoded (vanadium-dependent nitrogen fixation) nitrogenase exhibits an iron vanadium cofactor. A third so-called "alternative" nitrogenase contains an iron-only cofactor (FeFe-co) and is *anf*-encoded (alternative nitrogen fixation). The reaction catalyzed by nitrogenase involves the Mg·ATP-dependent reduction of nitrogen gas to yield two molecules of ammonia. Many legumes (alfalfa, clover, lupins, peas, beans, lentils, soybeans, peanuts and others) contain symbiotic bacteria called rhizobia within nodules of their root systems. These bacteria have the special ability of fixing nitrogen from atmospheric, molecular nitrogen (N_2) into ammonia (NH_3). The chemical reaction is:

$$N_2 + 8H^+ + 16MgATP + 8e^- \rightarrow 2NH_3 + H_2 + 16MgADP + 16\ Pi$$

The ratio of hydrogen to ammonia produced can vary and is increased when conditions are not optimal for enzyme reaction and when the enzyme is inhibited. Some diazotrophs possess a so called uptake hydrogenase to regain energy lost by hydrogen evolution by the reduction of hydrogen and the generation of ATP and H_2O (Stacey et al., 1992). Ammonia is then converted to another form, ammonium (NH_4^+), usable by (some) plants by the following reaction:

$$NH_3 + H^+ \rightarrow NH_4^+$$

This arrangement means that the root nodules are sources of nitrogen for legumes, making them relatively rich in plant proteins.

2.4 *Bradyrhizobium*

Bradyrhizobium species are Gram-negative bacilli (rod shaped) with a single subpolar or polar flagellum. They are a common soil dwelling microorganism that can form symbiotic relationships with leguminous plant species where they fix nitrogen in exchange for carbohydrates from the plant. Like other rhizobia, they have the ability to fix atmospheric nitrogen into forms readily available for other organisms to use. They are slow growing in contrast to *Rhizobium* species, which are considered fast growing rhizobia. In a liquid media broth, it takes *Bradyrhizobium* species 3-5 days to create a moderate turbidity and 5-7 hours to double in population size. They tend to grow best with pentoses as a carbon source (Somasegaran and Hoben, 1994). The genus of bacteria can form either specific or general symbioses (Somasegaran and Hoben, 1994). This means that one species of *Bradyrhizobium* may only be able to nodulate one legume species, whereas other *Bradyrhizobium* species may be able to nodulate several legume species. *Bradyrhizobium* species as *B. betae* was isolated from tumor like root deformations on sugar beets, *B. elkanii*, *B. liaonigense* establish symbiosis with soybeans, *B. japonicum* nodulates soybeans, cowpeas, mung beans, and siratro, *B. yuanmingense* nodulates *Lespedeza*, *B. canariense* nodulates Genistoid legumes, Lupin and Serradella nodule. Some strains (for example, USDA 6 and CPP) are capable of oxidizing carbon monoxide aerobically. Ribosomal RNA is highly conserved in this group of microbes, making *Bradyrhizobium* extremely difficult to use as an indicator of species diversity. DNA-DNA hybridizations have been used instead and show more diversity (Rivas et al., 2009). Among rhizobia, *B.*

japonicum or *B. diazoefficiens* is the most agriculturally important species because it has the ability to form root nodules on soybeans.

B. diazoefficiens USDA110 (TAL 102, BCRC 13528, JCM 10833, 3I1B110, ACCC 15034), was isolated in 1959 from a nodule of a soybean grown in Florida, USA (Keyser and Griffin, 1987) and broadly used in commercial inoculants. The strain has been used since then in several commercial inoculants in the USA, Europe, Asia and Africa. (Hungria and Mendes, 2015). Symbiotic tests under both field (Caldwell, 1969) and greenhouse conditions (Israel, 1981) have demonstrated that strain USDA110 is superior at symbiotic nitrogen fixation compared with other strains. Strain USDA110, has been widely used for the purpose of molecular genetics, physiology, and ecology, because of its superior characteristics regarding symbiotic nitrogen fixation (Mathis et al., 1997). Kaneko et al., (2002) was determined the complete nucleotide sequence of the genome of a symbiotic bacterium *B. diazoefficiens* USDA110. The genome of *B. diazoefficiens* was a single circular chromosome 9,105,828 bp in length (Figure 1.) with an average GC content of 64.1%. No plasmid was detected. The chromosome comprises 8317 potential protein-coding genes, one set of rRNA genes and 50 tRNA genes. Fifty-two percent of the potential protein genes showed sequence similarity to genes of known function and 30% to hypothetical genes. The remaining 18% had no apparent similarity to reported genes. Thirty-four percentage of the *B. diazoefficiens* genes showed significant sequence similarity to those of both *Mesorhizobium loti* and *Sinorhizobium meliloti*, while 23% were unique to this species. A presumptive symbiosis island 681 kb in length, which includes a 410-kb symbiotic region. Six hundred fifty-five putative protein-coding genes were assigned in this region, and the functions of 301 genes, including those related to symbiotic nitrogen fixation and DNA transmission, were deduced. A total of 167 genes for transposases/10^4 copies of insertion sequences were identified in the genome. It was remarkable that 100 out of 167 transposase genes are located in the presumptive symbiotic

island. DNA segments of 4 to 97 kb inserted into tRNA genes were found at 14 locations in the genome, which generates partial duplication of the target tRNA genes. Recently, strains belonging to *B. japonicum* have been split into two species, *B. japonicum* and *B. diazoefficiens*. USDA110 now belongs to *B. diazoefficiens*, and the type strain for this new species is USDA 110T (Delamuta et al., 2013).

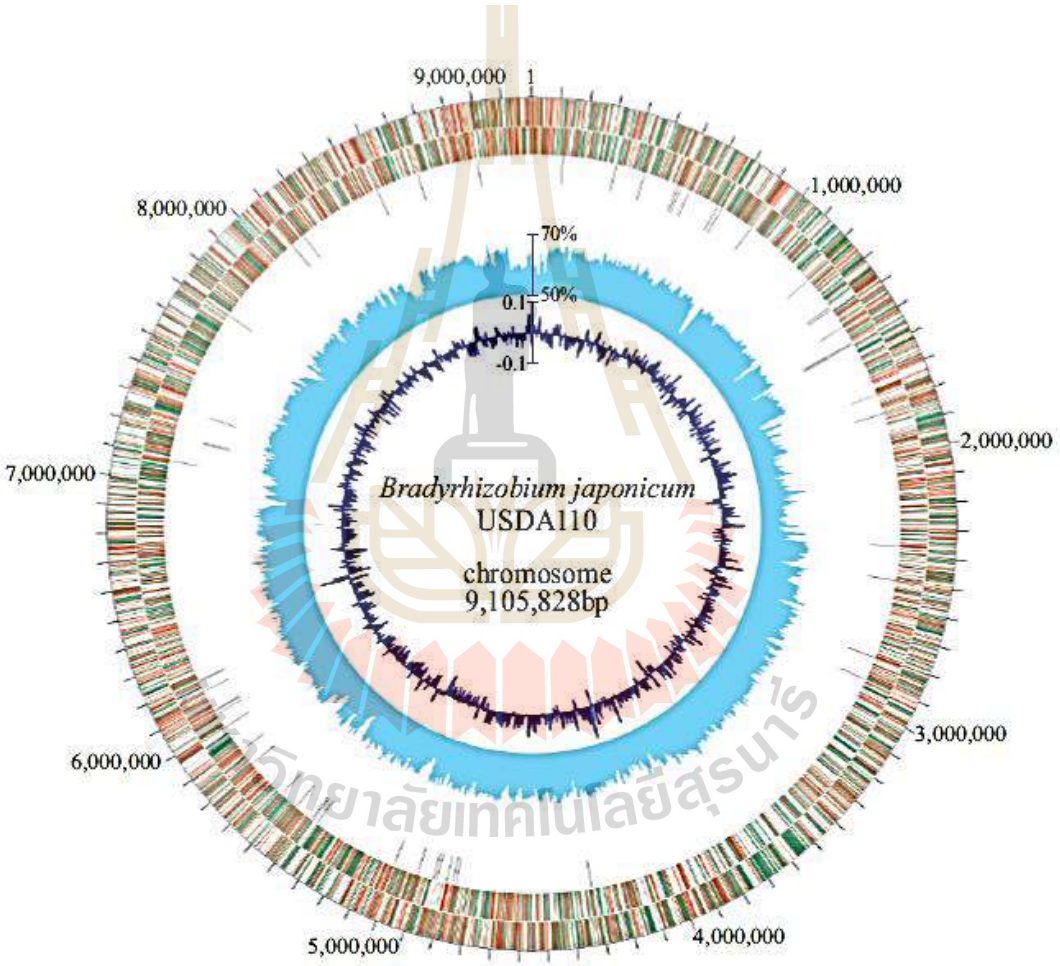

Figure 1. Circular representation of the chromosome of *B. japonicum* USDA110 (Kaneko et al., 2002).

B. japonicum CB1809 (USDA 136, 3I1b136, TAL 379, SEMIA 585, serogroup 122) is now reclassified as *B. diazoefficiens* (Delamuta et al., 2013). CB1809 was isolated in USA and sent to Australia, where it was characterized as exceptionally efficient, and thus

commercially recommended by the Commonwealth Scientific and Industrial Research Organization (CSIRO) since 1968. The strain is a subculture of USDA 136, which was derived from USDA 122 (Dr. Peter van Berkum, personal communication). Dr. Don Norris sent the strain from Australia to Dr. Johanna Döbereiner in Brazil in 1966, where its good symbiotic performance was confirmed (Hungria and Mendes, 2015), and it was then recommended for uing in commercial inoculants in 1977. However, poor nodulation with cultivar IAC-2, which was broadly used at that time, led to withdrawal of this recommendation in the following year (Hungria et al., 2006). CB1809 come from NifTAL (Nitrogen fixation for Tropical Agricultural Legumes) are used in rhizobial inoculant production for soybean-bradyrhizobial symbiosis (Aung, 2012). The great variability in nodule efficiency among strains of *B. japonicum* CB1809 and *B. elkanii*, CB1809 is one of the most efficient of them (Neves et al., 1985). In addition, Neves et al. (1985) distinguished two groups of soybean rhizobial strains for N_2 fixation, those with higher efficiency (CB1809, DF383 and 965) and those with lower efficiency (29W, DF395 and SM1b). Plants inoculated with the more efficient strains had higher ureide content of xylem sap, and higher relative efficiency (less H_2 evolved per N_2 fixed) than the others. The best strains increased yield by up to 30%. Strain CB1809 normally produces few nodules, has high nodule efficiency and high N accumulation averaged over cultivars, while 29W produces a large number of nodules, and has low nodule efficiency and low nitrogen accumulation in the plant. The superiority of CB1809 has also been confirmed in field experiments. Four decades of soybean cultivation in South Africa has resulted in the establishment of populations of bradyrhizobia against which the recently introduced inoculants strain CB1809 must compete (Botha et al., 2004).

Bradyrhizobium sp. SUTN9-2 was isolated in 2012 from a nodule of a *Aeschynomene americana* L. which is a leguminous weed found in rice fields of Thailand (Noisangiam et al., 2012). SUTN9-2 can form symbiotic relationships (nodule) with mung bean where they

fix atmospheric nitrogen in exchange for carbohydrates from the mung bean. Moreover, SUTN9-2 can establish themselves in rice tissues act as rice endophytic bradyrhizobia (is colonized in intercellular spaces), it can produce IAA, ACC deaminase and have a nitrogen fixation ability during symbiosis inside rice tissues (Piromyou et al., 2017).

2.5 Molecular basis of rhizobium-legume symbiosis

2.5.1 Rhizobial colonization

The establishment of symbiosis between legumes and rhizobia involves the activation of genes in both the host and the symbiont (Geurts and Bisseling, 2002) and an elaborated exchange of signals. The formation of a root nodule, the specialized organ from a plant host that contains the symbiotic nitrogen-fixing rhizobia, involves two simultaneous processes: infection and nodule organogenesis. The initiation of a root nodule is shown in Figure 2.

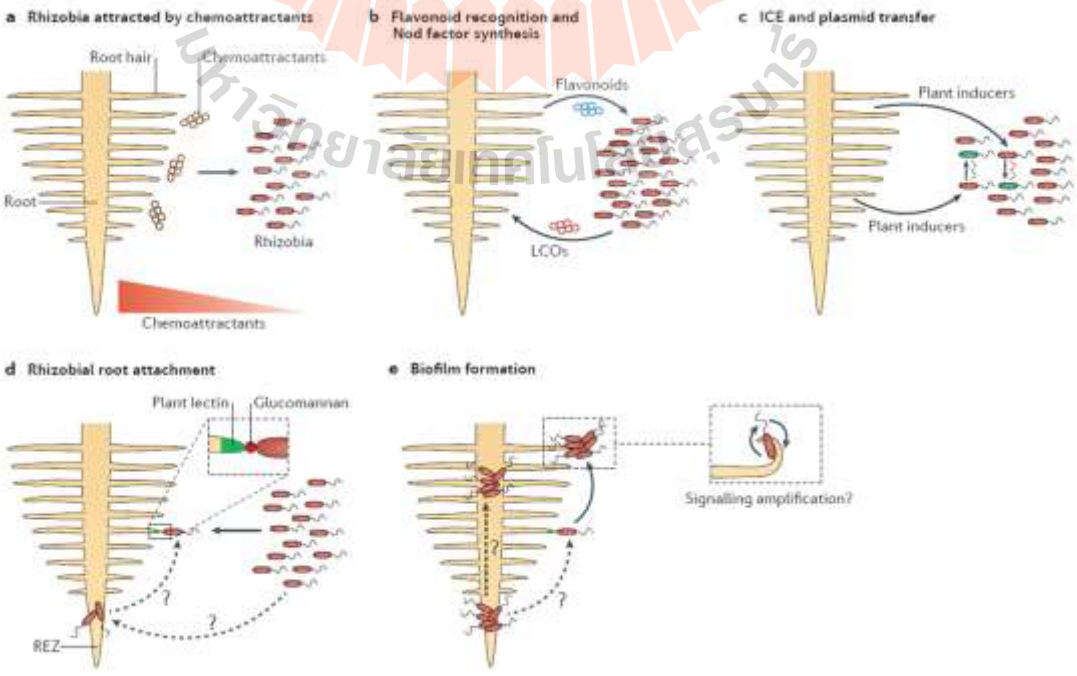

Figure 2. Rhizobial attachment and colonization of legume roots (Poole et al., 2018).

Colonization of roots likely requires bacterial motility and chemotaxis. Rhizobia are attracted towards legume roots by chemoattractants in root exudates. Once rhizobia are in close proximity to root hairs, flavonoids from the root hair induce bacterial nodulation (*Nod*) genes. This leads to the production of lipochitooligosaccharides (LCOs; also known as Nod factors). The transcriptional regulator nodulation protein (*NodD*) detects plant-derived flavonoids, inducing nod expression and the synthesis of LCOs. The pathway for the synthesis of the common core chitooligosaccharide is encoded by *nodABC*; *nodIJ* encodes an ABC export system for LCOs, and other *nod* genes are responsible for decorating the LCO core. (Poole et al., 2018). Production by the bacteria, Nod factors behave in many regards like plant hormones: They are diffusible signals that activate diverse developmental processes in the plant (Oldroyd and Downie, 2008). Nod factors that can recognized by the plant, binding of LCOs to lysine motif (LysM) receptors on root hairs initiates early signalling events, leading to calcium oscillations, initially in epidermal cells but later also in cortical cells preceding their colonization. Plant inducers in root exudates trigger the transfer of integrative and conjugative elements (ICEs) or plasmids to compatible rhizobia. It is unclear whether rhizobia first attach to the root elongation zone (REZ) or root epidermis and then to root hairs or whether they attatch directly to root hairs. At acidic pH, rhizobial polar glucomannan binds to plant lectin on the root hair tip. Rhizobia on the root surface and REZ or on the root hair form a biofilm with Rhizobium-adhering proteins (Raps), extracellular polysaccharides (EPSs) and cellulose fibrils. The biofilm structure on the root hair is called a root hair cap. There is probably strong induction of flavonoid and LCO signalling when rhizobia are attached to root hairs, which leads to root hair curling and entrapment of the rhizobia (Poole et al., 2018).

Rhizobia gain entry into the plant root by root hair cells that grow around the bacteria attached at the root surface, trapping the bacteria inside a root hair curl. Infection threads are invasive invaginations of the plant cell that are initiated at the site of root hair

curls and allow invasion of the rhizobia into the root tissue. The nucleus relocates to the site of infection, and an alignment of ER and cytoskeleton, known as the pre-infection thread, predicts the path of the infection thread. Nodules initiate below the site of bacterial infection and formed by de novo initiation of a nodule meristem in the root cortex. The infection threads grow towards the emergent nodules and ramify within the nodule tissue. In some cases, the rhizobia remain inside the infection threads, but more often, the bacteria are released into membrane-bound compartments inside the cells of the nodule, where the bacteria can differentiate into a nitrogen-fixing state (Oldroyd, 2013).

2.5.2 Symbiotic signaling

Arbuscular mycorrhiza (AM) fungi produce lipochitooligosaccharides structurally closely related to rhizobial Nod factors that induce lateral root formation in plants. It is thus conceivable that production of LCOs by rhizobia was a key step during the evolution of the root nodule symbiosis. This infection is preceded by signal transduction through a so-called ‘common symbiosis pathway’ (Kistner and Parniske, 2002) shared by Root Nodule Symbiosis (RNS) and AM (Figure 3).

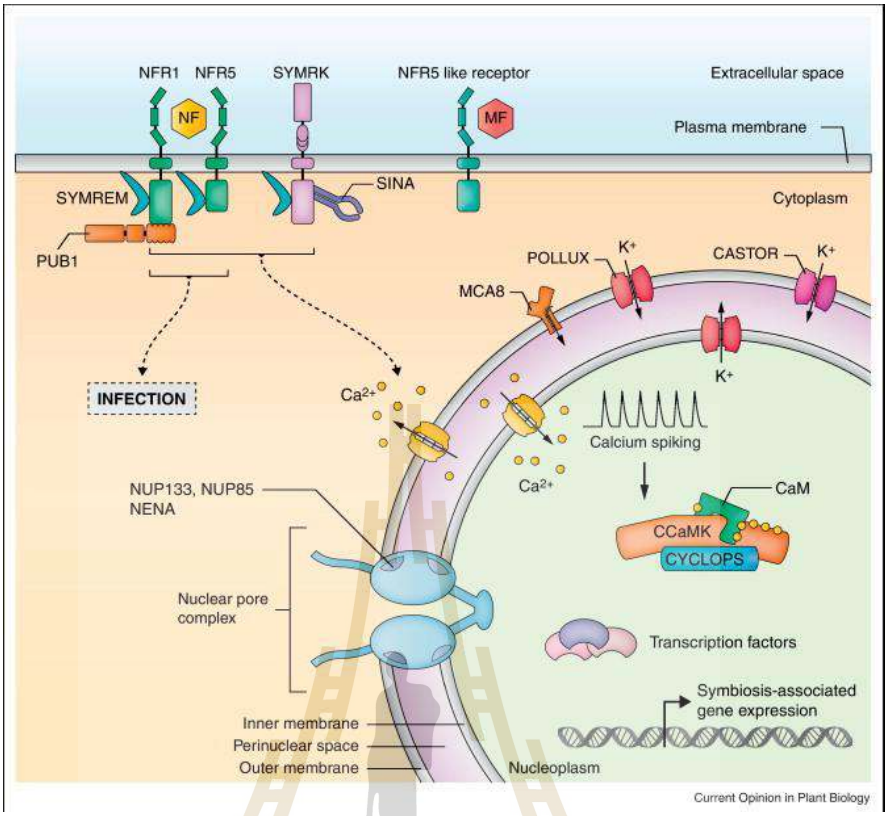

Figure 3. Symbiotic signal transduction in plant root cells (Singh and Parniske, 2012).

Symbiotic signal transduction starts with the percepting of rhizobial Nod factors (NFs) at the plasma membrane (PM) (Haney and Long, 2010). Plants detect LCOs with a heteroduplex of lysine motif (LysM) receptorsis; for example, LYK3–NFP (LysM domain receptor-like kinase 3–serine/threonine receptor-like kinase NFP) in *Medicago truncatula* and Nod-factor receptor 1 (NFR1)–NFR5 in *Lotus japonicus* (Oldroyd et al., 2011). Mycorrhizal signalling and plant immunity probably use similar LysM receptors and may share some receptors with the Nod pathway (Poole et al., 2018). An NFR5-like receptor may mediate perception of an AM fungus-derived ‘Myc factor’ (MF) (Parniske, 2008). PUB1: plant U-box protein 1 of *M. truncatula*, is an E3 ubiquitin ligase interacting with the kinase domain of LYK3, and exerts a negative regulatory role on nodulation signaling (Mbengue et al., 2010). SINA: the SEVEN IN ABSENTIA homolog SINA4

interacts with the kinase domain of SYMRK and mediates its relocalization and degradation (Den Herder et al., 2012). The symbiotic receptors at the PM interact with SYMREM1, a remorin protein specifically upregulated during nodulation and required for infection thread (IT) formation (Lefebvre et al., 2010; Tóth et al., 2012). Within minutes, LCOs perception at the PM, the LysM receptors transmit the signal through the common symbiosis (SYM) pathway. This leads to calcium oscillations in the nucleus, which are decoded by a calcium and calcium/calmodulin-dependent serine/threonine protein kinase (CCaMK). This steps bring about to a sustained nuclear Ca_2^+-spiking response. Downstream of this, the signals diverge between mycorrhizal and nodulation pathways; the generation, decoding and transduction of which is mediated by components common to both types of symbioses (Oldroyd, 2013). These are genetically positioned upstream (SYMRK/DMI2, CASTOR/POLLUX/DMI1, NUP85, NUP133, NENA) or downstream (CCaMK/DMI3, CYCLOPS/IPD3) of the Ca_2^+-spiking response. Several transcription factors including NSP1/2, NIN and others have been implicated in symbiosis-related gene expression (Kouchi et al., 2010). The observation that autoactive CCaMK does not restore epidermal IT formation in *nfr* mutants suggests the existence of a common *SYM* gene-independent pathway (Hayashi et al., 2010). MCA8: nuclear-membrane localized calcium ATPase pump (Capoen et al., 2011).

2.5.3 Legume symbiosis and root nodulation

For successful nodulation, plants must also initiate a nodule meristem, which contains dividing cells. As the nodule develops, the infection threads branch and carry rhizobia into the developing nodule. Eventually, bacteria are released and engulfed by plant cells in a process that requires vesicle SNAREs (v-SNAREs) (Ivanov et al., 2012). At this stage, bacteria differentiate into N_2-fixing bacteroids that are surrounded by symbiosome membranes. The symbiosome is the engine of fixation; the bacteroids are supplied with

dicarboxylates as carbon sources and they secrete ammonium to the plant (Udvardi and Poole, 2013). The legume symbiosis and root nodulation is shown in Figure 4.

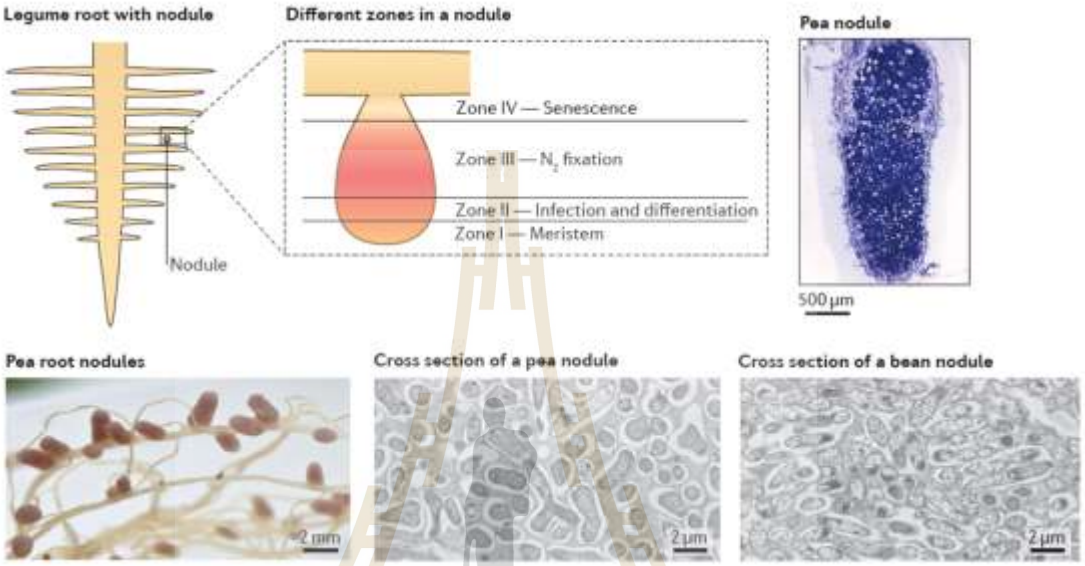

Figure 4. Legume symbiosis and root nodulation (Poole et al., 2018).

Nodules can be either determinate, as in beans, soybeans and *Lotus japonicus*, or indeterminate, as in alfalfa, peas and clover. In indeterminate nodules, the meristem persists, which leads to development zones in nodules. The distal zone I contains the nodule meristem, in which new plant cells are produced; zone II contains infection threads full of bacteria; in the interzone between zone II and III, bacteria are released from infection threads and engulfed by plant cells; zone III contains the mature, N_2-fixing bacteroids; and in zone IV, bacteroids senesce (Poole et al., 2018). Several nodulation-specific transcription factors (TFs), such as two GRAS-type TFs Nodulation Signaling Pathway 1 (NSP1), NSP2 and an AP2 family TF Ethylene Response Factor Required for Nodulation1 (ERN1) (Hirsch et al., 2009; Cerri et al., 2012), function downstream of the nodulation signaling pathway to stimulate cortical cell division and formation of the nodule

primordium development into mature nodules. Two types of nodules, indeterminate and determinate nodules, form in legume plants. Indeterminate nodules, which have a sustained nodule meristem, first mitotically activate inner cortical and pericycle cells, whereas nodule meristems originate from the third cortical layer, or the inner cortical layers in *M. truncatula* (Xiao et al., 2014), alfalfa and pea. Determinate nodules initiate from the outermost one or two layers of cortical cells and do not maintain a nodule meristem (Calvert et al., 1984), such as *L. japonicus* and soybean. Both types of nodule formation are tightly regulated by plant hormones (Oldroyd, 2013).

2.5.4 Hormone modulation of legume-rhizobial symbiosis

Auxin was the first identified phytohormone that plays essential roles to regulate diverse aspects of cell proliferation and differentiation (Chapman and Estelle 2009). In recent decades, more studies have focused on the role of auxin in regulating legume-rhizobium interactions. Libbenga et al. (1973) reported that when treated with both auxin and cytokinin, the cell proliferation pattern in explants from root-cortex tissue from pea was similar to the initial proliferative stage in nodule formation, which suggests both auxin and cytokinin are required for nodule organogenesis. The level of auxin is regulated spatio-temporally in the infective roots during nodulation (Liu et al., 2018). Inoculation of white clover with rhizobia induced a rapid, transient and local downregulated expression of *GH3*::*β*-glucuronidase (GUS), which is an auxin responsive reporter construct, during nodule initiation followed by upregulated reporter gene expression at the site of nodule initiation (Mathesius et al., 1998). The highly active synthetic auxin-responsive element *DR5* has been used in combination with a nuclear-localized green fluorescent protein (GFP) as a reporter to examine auxin response patterns during *L. japonicus* nodule development (Suzaki et al. 2012). It seems that decreasing the auxin levels locally, at the time of nodulation, is important to allow initiation to proceed in indeterminate nodules. However,

auxin is needed for nodule development just shortly after initiation. The *MtLAX* genes for *Medicago truncatula*-like *AUX1* genes of *Arabidopsis thaliana* encode auxin import carriers and play pleiotropic roles related to auxin uptake. In primary roots, the *MtLAX* genes are expressed preferentially in the root tips, particularly in the provascular bundles and root caps. During lateral root and nodule development, the genes are expressed in the primordia, particularly in cells that were probably derived from the pericycle. At slightly later stages, the genes are expressed in the regions of the developing organs where the vasculature arises (central position for lateral roots and peripheral region for nodules). These results are consistent with *MtLAX* being involved in local auxin transport and suggest that auxin is required at two common stages of lateral root and nodule development: development of the primordia and differentiation of the vasculature (de Billy et al., 2001). *LjTAR1* encodes an enzyme to produce indole-3-pyruvic acid (IPA), an IAA precursor. *LjTAR1* is transiently increased after inoculation with rhizobia (Suzaki et al. 2012). Recent studies indicated the critical role of auxin signaling pathways in root nodule symbiosis. *GmARF8a/b*-RNAi plants exhibited less nodule development in soybean (Wang et al. 2015). The miR167-*GmARF8* module also positively regulated nodulation efficiency under low microsymbiont density, a condition often associated with environmental stress. The regulatory role of miR167 on nodule initiation was dependent on the Nod factor receptor GmNFR1α, and it acts upstream of the nodulation-associated genes. miR167 also promoted lateral root numbers. Collectively, this findings establish a key role for the miR167-*GmARF8* module in auxin mediated nodule and lateral root formation in soybean (Wang et al. 2015). The overexpressing auxin receptor *GmTIR1*/*GmAFB3* genes, in soybean, significantly increased infection foci and eventual nodule number (Cai et al. 2017). Taken together, these findings indicated that the auxin signaling pathway is required for both infection thread formation and nodule development (Liu et al., 2018).

New molecular, genetic, biochemical and genomic approaches, scientists have uncovered the critical role of cytokinin signaling in root nodule symbiosis. It has been shown that rhizobia can produce cytokinin, which was proposed to be important for nodule formation (Sturtevant and Taller, 1989). Cooper and Long (1994) showed that rhizobium strain expressing the *tzs* gene, which encodes an isopentenyl transferase that catalyzes the rate-limiting step in the biosynthesis of two naturally occurring cytokinins, isopentenyl-adenine (iPA) and zeatin (ZT), could stimulate development of nodule-like structures, inducing normal periclinal mitoses in the innermost cortical cell layers and *MsENOD2* expression in the roots of alfalfa (Cooper and Long, 1994). Interestingly, cytokinin treatment can elicit expression of some nodulin genes, such as *Early nodulin 40* (*ENOD40*), *NIN*, *NSP1* and *NSP2* in alfalfa, *M. truncatula* and *L. japonicus* (Heckmann et al., 2011; Lee et al., 2007; Plet et al., 2011), which behaves as a NFs treatment (van Zeijl et al., 2015). In *L. japonicus*, NFs and *Mesorhizobium loti* induce a cytokinin oxidase/dehydrogenase CKX3 during the early stage of nodule initiation. *ckx3* knockout mutants show reduced nodule number and infection thread formation (Reid et al., 2016). However, ectopic expression of *AtCKX3* or *ZmCKX1* in *L. japonicus* significantly reduced nodule numbers (Lohar et al., 2004). Consistent with the role of cytokinin in nodulation, *MtLOG1* and *MtLOG2*, which encode cytokinin riboside 50-monophosphate phosphoribohydrolase to activate cytokinin, were also upregulated during nodulation. Both *MtLOG1* overexpressing and *MtLOG1*-RNAi lines show significantly reduced nodule numbers (Mortier et al., 2014), suggesting that an appropriate level of cytokinin is required for nodule development (Liu et al., 2018). Hormones regulate nodulation at different stages as shown in Figure 5.

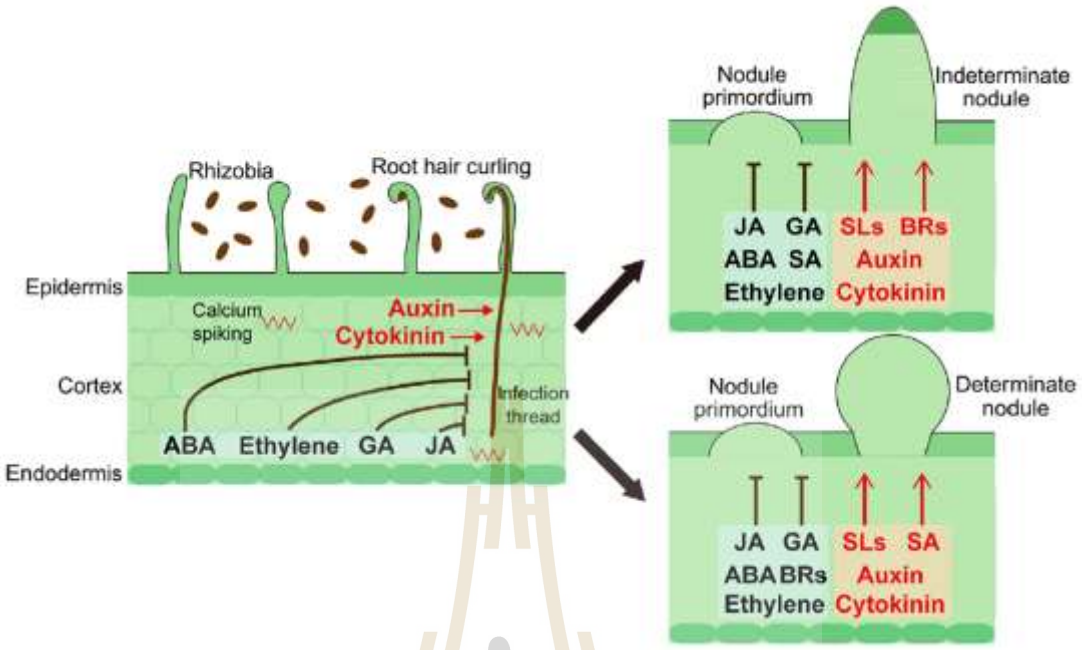

Figure 5. Hormones regulate nodulation at different stages (Liu et al., 2018).

Legumes release flavonoids to trigger rhizobia to produce Nod factors (NFs). NFs are detected by two LysM domain receptor-like kinases and initiate the symbiosis signaling pathway in plants. Calcium spiking and root hair deformation are early responses in host plants to NFs. Curled root hair entraps the rhizobia, which enters the root tissue of host plants through a specific path, called an “infection thread”. Auxin signaling initiates this infection thread and cytokinin signaling is required for infection thread elongation. Abscisic acid (ABA), ethylene, gibberellic acids (GA) and jasmonic acid (JA) all negatively regulate calcium spiking and infection thread formation. This symbiotic signal induces cortex cell division and initiates nodule organogenesis. Auxin and cytokinin are key regulators of cortical cell proliferation and differentiation and are essential for further nodule development. However, ethylene, GA, JA, ABA and brassinosteroids (BRs) suppress nodule formation and development. Interestingly, salicylic acid (SA) and BRs have opposite effects on the formation of determinate and indeterminate nodules (Liu et al., 2018).

2.5.5 Nodule organogenesis to senescence

Nodules can be either determinate, as in mung beans and soybeans, or indeterminate, as in alfalfa, peas and clover. In determinate nodules, the meristem dies, with all cells at the same development stage, and nodules grow larger by cell expansion, usually with several bacteroids enclosed by a single symbiosome membrane. In determinate nodules, the succeeding development stages are demonstrated in Figure 6.

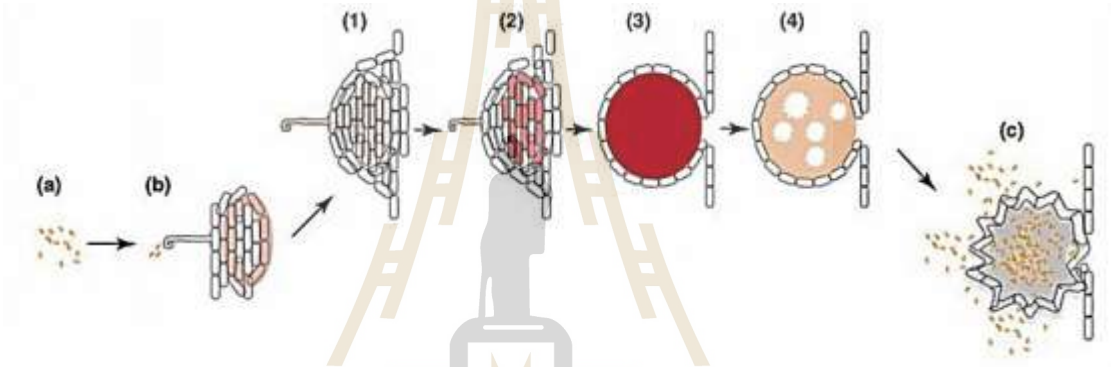

Figure 6. Nodule organogenesis to senescence (Prakamhang, 2013; Modified from Schumpp and Deakin (2010)).

Rhizobia can exist as saprophytes that feed on organic debris and compounds released from roots (Morgan et al., 2005).

(a) Rhizobia also react specifically to flavonoids released by legumes, resulting in NF synthesis and release, which triggers nodule development in the macro-symbiont (Broughton et al., 2000)

(b) Rhizobia attach to root hairs, NF simultaneously induce root-hair curling, allow the bacteria to enter the plants within the curls and provoke differentiation of cortical cells into meristematic primordial. Then, the plasma membranes of root hairs invaginate, forming the infection thread, dedicated to the transport of rhizobia to the developing

primordia (stages 1). When it reaches the center of the future nodule, the infection thread ramifies and rhizobia are enveloped by a plant derived membrane (symbiosome) and released into the cytosol of plant cells (stages 2). Alternatively, but not shown, rhizobia can also penetrate legume roots through disruptions in the epidermal layer. Rhizobial cells then multiply to form infection pockets and proceed intercellularly before infecting root cells. Rhizobia then enlarge, and differentiate into bacteroids that fix nitrogen. In parallel, infected plant cells undergo several cycles of endo-reduplication (Mergaert et al., 2006), resulting in large polyploid cells hosting thousands of symbiosomes (stages 3). After a period of active nitrogen fixation whose duration depends on the developmental stage of the plant and environmental conditions, the nodules senesce (stages 4).

(c) Decay of nodules releases bacteroids which dedifferentiate into free-living rhizobia and return to a saprophytic lifestyle (Müller et al., 2001). However a fraction of undifferentiated rhizobia from within the nodule remains, is able to divide and thus resume the free living life-style in the rhizosphere (Ratcliff et al., 2008).

2.5.6 Bacteroid development and root nodule metabolism

The nodules are colonized by the bacteria which then differentiate into a bacteroid that is capable of fixing nitrogen. This nitrogen, now in usable form, is provided to the plant in exchange for nutrients. The transformation of Rhizobium cells from vegetative bacteria into nitrogen-fixing bacteroids involves an alteration of cell fate, presumably with an underlying developmental pathway. The differentiation into bacteroids is accompanied by large changes in the transcriptome and proteome: N_2-fixation genes are induced, but most genes required for growth, including those encoding ribosomal proteins and involved in DNA replication and amino acid biosynthesis, are repressed (Karunakaran et al., 2009; Pessi et al., 2007). Nodule cysteine-rich (NCR) peptides expression is highly spatially resolved from proximal to distal parts of *M. truncatula* nodules (Guefrachi et al.,

2014). They induce bacterial cell swelling and genome endoreduplication and increase the permeability of the cell membrane, which causes terminal differentiation (Mergaert et al., 2006). Peptide NCR247 upregulates the master two-component sensor-regulator systems that control exopolysaccharide and cyclic glucan synthesis. It perturbs the cell cycle of *S. meliloti* and inhibits the master cell cycle transcriptional regulator CtrA and cell cycle components, including cell division protein FtsZ (Penterman et al., 2014). Inhibition of CtrA causes cell cycle arrest, swelling and branching in cultured bacteria (Pini et al., 2015). NCR247 occurs in three different oxidized regioisomers and a reduced form, which differ in their effects. Blocking secretion of NCR peptides prevents bacteroid formation in *M. truncatula* (Wang et al., 2010). NCR247 localizes to punctate regions in the cytoplasm and interacts with several proteins in *S. meliloti*: the main GroEL chaperonin, 60 kDa chaperonin 1 (Cpn60; also known as GroEL1), which is essential for bacteroid development (Bittner et al., 2007), FtsZ, pyruvate dehydrogenase (PDH) and nitrogenase (Farkas et al., 2014). The morphological changes characteristic for bacteroids in the legumes involve elongation of the bacteria from the free-living size of 1–2 μm to 5–10 μm and often the formation of Y-shaped branched cells that are packaged individually into peribacteroid membranes (PBMs) (Ivanov et al., 2010). These bacteroids have a highly amplified genome content that is condensed into multiple nucleoids of variable size (Mergaert et al., 2006). The polyploidy of these bacteroids and the induction of bacteroid-like cells by genetic or physiological interference with the rhizobial cell cycle (Van de Velde et al., 2010) suggested that the bacterial cell cycle is modified when the rhizobia differentiate into bacteroids, resulting in multiple rounds of DNA replication without cytokinesis. Bacteroids isolated from the nodules do not form colonies, as they have lost their reproductive capacity. Therefore, bacteroid differentiation is irreversible and terminal. The morphological and cytological changes are independent of the process of nitrogen fixation itself since mutants in the *fixLJ* regulatory genes or in the nitrogenase encoding

genes also exhibit terminal bacteroid differentiation (Maunoury et al., 2010). Bacteroids are fully differentiated when they commence the reduction of N_2 to ammonia through the nitrogenase enzyme complex. The main factor controlling N_2 fixation in bacteroids is O_2 tension as shown in Figure 7

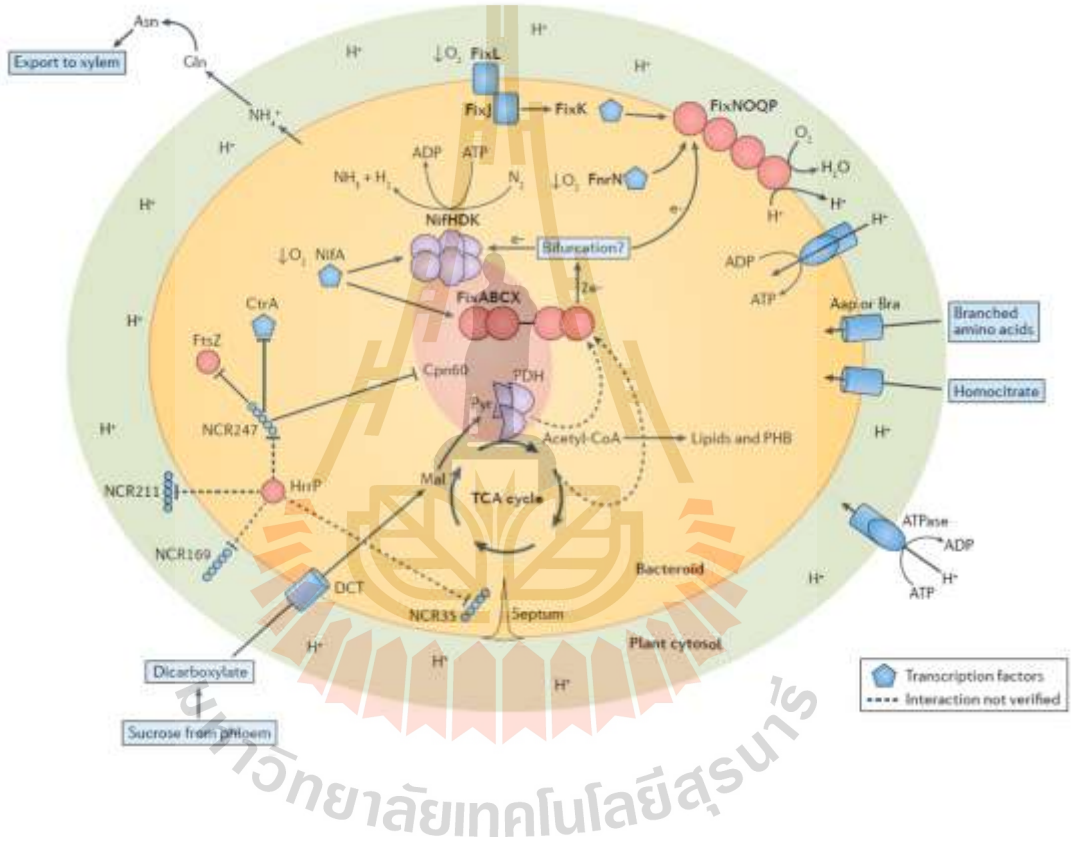

Figure 7. Nutrient exchange and regulation of bacteroid development (Poole et al., 2018).

O_2 is the main signal that regulates bacteroid development through two signalling circuits. The first involves FixLJK in *S. meliloti* and probable transcriptional activator (FnrN) in *R. leguminosarum*, which induce the expression of *fixNOQP*, and the second is Nif-specific regulatory protein (NifA), which induces *nifHDK* (encoding nitrogenase) and autoinduces *fixABCX–nifAB*. These circuits seem to be completely separate in *R. leguminosarum* but partly overlap in *S. meliloti* with weak induction of *fixABCX–nifAB* by N_2 fixation regulation protein FixK. FixABCX is an electron bifurcating

complex, probably donating electrons to either ferredoxin or to flavodoxin and CoQ. Pyruvate dehydrogenase (PDH), the tricarboxylic acid (TCA) cycle and 2-oxoglutarate dehydrogenase might form a complex with FixABCX to achieve this, or, possibly, NAD(P)H provides electrons directly. FixNOQP (also known as CBB3) is a high-affinity terminal electron acceptor needed in O_2-limited cells. Low O_2 limits the TCA cycle, causing acetyl-CoA to be used to produce lipids and polyhydroxybutyrate (PHB), this polyester storage reserve of carbon and reductant is considered to be an important source of oxidizable substrates to help maintain the respiratory demand of bacteroids and support nitrogen fixation when the supply of photosynthate from the host is reduced, as may occur during extended periods of low light intensity or pod filling (Bergersen et al., 1991). The peptidase HrrP is speculated to change host specificity by degrading nodule cysteine-rich (NCR) peptides. The HrrP–NCR peptide interactions in the figure are hypothetical and whether the shown peptides are important targets or in the correct compartment (for example, cytoplasm, symbiosome space or division septum) is unclear. The major chaperonin in bacteroids is 60 kDa chaperonin 1 (Cpn60). It can interact with multiple proteins, including PDH.

2.6 Plant growth-promoting rhizobacteria; PGPR

Plant growth-promoting rhizobacteria (PGPR) was firstly defined by Kloepper and Schroth (Kloepper and Schroth, 1978) to describe soil bacteria that colonize the roots of plants following inoculation onto seed and that enhance plant growth. The following are implicit in the colonization process: ability to survive inoculation onto seed, to multiply in the spermosphere (region surrounding the seed) in response to seed exudates, to attach to the root surface and to colonize the developing root system (Kloepper and Metting Jr, 1992). PGPR are naturally occurring soil bacteria that aggressively colonize plant roots and benefit plants by providing growth promotion (Saharan and Nehra, 2011). In the last few

years, the number of PGPR that have been identified has seen a great increase, mainly because the role of the rhizosphere as an ecosystem has gained importance in the functioning of the biosphere. Various genera of bacteria like *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Bacillus* and *Serratia* have been reported to enhance the plant growth (Joseph et al., 2007). Examples of the commercialized PGPB strains include; *Agrobacterium radiobacter*, *Azospirillum brasilense, A. lipoferum, Azotobacter chroococcum, Bacillus* spp., *B. fimus*, *B. licheniformis*, *B. megaterium*, *B. mucilaginous*, *B. pumilus*, *B. subtilis*, *B. subtilis* var. *amyloliquefaciens*, *Burkholderia cepacia, Delfitia acidovorans, Paenobacillus macerans, Pantoea agglomerans*, *Pseudomonas aureofaciens, P. chlororaphis*, *P. fluorescens*, *P. solanacearum, Pseudomonas* spp., *P. syringae, Serratia entomophilia*, *Streptomyces* spp., *S. griseoviridis*, *S. lydicus.* There are several PGPR inoculants currently used that seem to promote growth through at least one mechanism; suppression of plant disease (termed Bioprotectants), improved nutrient acquisition (Biofertilizers), or phytohormone production (Biostimulants) (Saharan and Nehra, 2011). However, PGPR inoculated crops represent only a small fraction of current worldwide agricultural practice (Glick, 2012). Generally, PGPR promote plant growth by exploiting either of direct or indirect mechanism. The direct mechanisms of plant growth promotion by PGPR include production of metabolites that is phytohormones or enhanced availability of nutrients, facilitating resource acquisition or modulating plant hormone levels (Glick, 2012). Briefly, the direct mechanism of plant growth promotion involves the production of substances by bacteria and its transport to the developing plants or facilitates the uptake of nutrients from the recipient environment. The direct growth promoting activity of PGPR includes N_2 fixation (Wani et al., 2007), solubilization of insoluble phosphorus (Khan et al., 2007), sequestering of iron by production of siderophores (Wani et al., 2007), production of phytohormones such as IAA, auxins, cytokinins, gibberellins and lowering of ethylene concentration by ACC deaminase

activity (Glick et al., 2007). On the contrary, the indirect mechanism of plant growth promotion by PGPR includes antibiotic production, depletion of iron from the rhizosphere, synthesis of antifungal metabolites and production of fungal cell wall lysing enzymes, competition for sites on roots and induced systemic resistance (Glick, 2012). The indirect promotion of plant growth takes place when PGPR lessen or prevent the injurious effects of plant pathogens by synthesizing inhibitory substances or by increasing the natural resistance of the host to the pathogens. A briefly schematic illustration of the plant growth-promoting mechanism from rhizobacteria is presented in Figure 8.

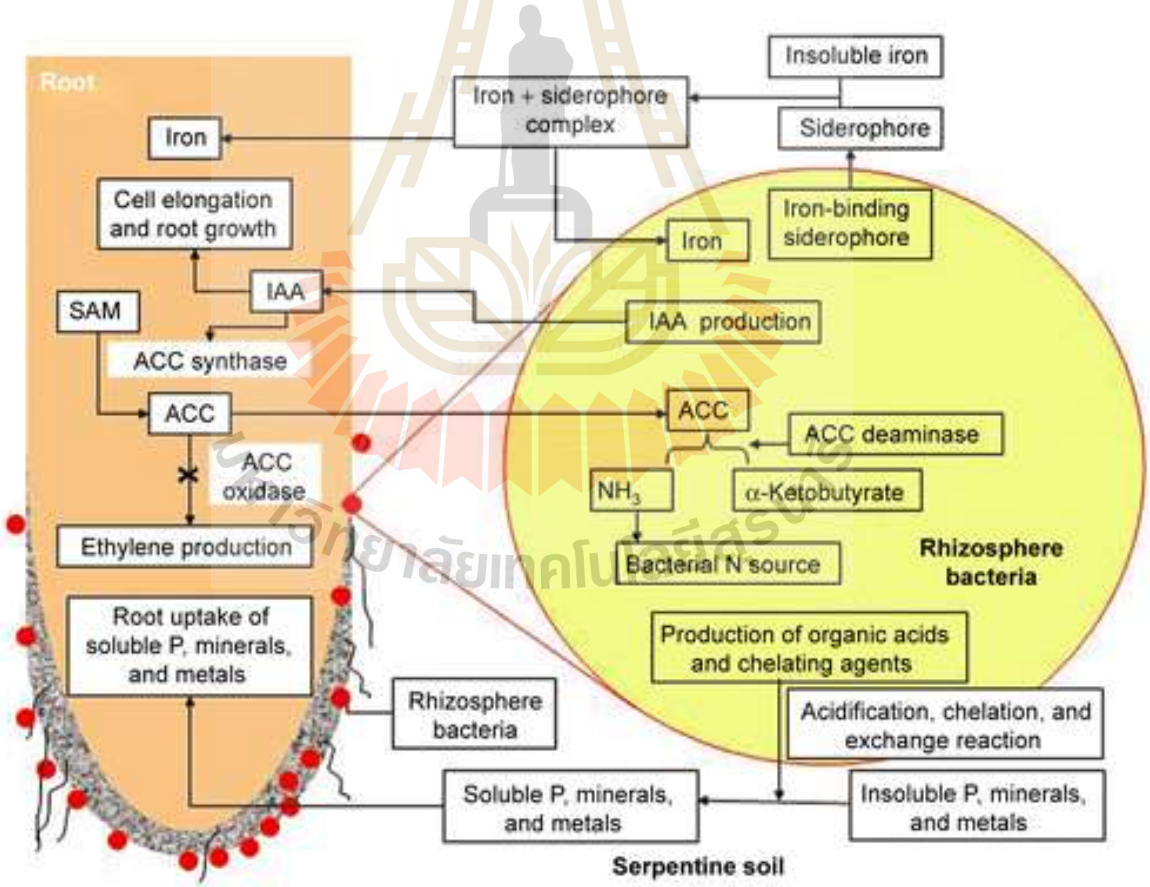

Figure 8. Plant growth-promoting mechanisms from rhizobacteria (Rajkumar et al., 2009).

2.6.1 Augmentation of nutrients supplied

Rhizobacteria have the ability to enhance plant growth in the absence of potentially pathogenic microorganisms. One way in which they can enhance plant growth is by solubilizing normally poorly soluble nutrients with either bacteria siderophores or lowering the pH by secreting acidic organic compounds (Van Loon, 2007). Plants acquire phosphorus from soil as phosphate anion. It is the least mobile element in plant in contrary to other macronutrients (Bhattacharyya et al., 2016). Phosphorous is a major macronutrient needed for plants, but is not easily up taken due to its reactive nature with iron, aluminum, and calcium; these common reactions result in the precipitation of phosphorous, thus making it unavailable to plants (Yang et al., 2009). Phosphorus solubilizing microorganisms (PSMs) play an essential role in phosphorus nutrition by enhancing its availability to plants through release of organic and inorganic soil phosphorus pools by solubilization and mineralization (Sharma et al., 2013; Walpola and MinHo, 2012). Lowering of soil pH by microbial production of organic acids and mineralization of organic phosphorus by acid phosphatase are the essential mechanisms usually involved during phosphorous solubilization (Bhattacharyya et al., 2016). *Alcaligenes*, *Acinetobacter*, *Arthrobacter*, *Azospirillum*, *Burkholderia*, *Bacillus*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Pseudomonas*, *Rhizobium* and *Serratia*, these bacteria solubilize phosphate through the production of acids, and by some other mechanism and are termed as phosphate solubilizing bacteria (PSB) (Chen et al. 2006). Some PGPR can convert phosphorous into a more plant attainable form, such as to orthophosphate (Rai, 2006; Vessey, 2003). Iron is also another essential nutrient, but it is scarce in soil. PGPR, can produce compounds called siderophores, which acquire ferric iron (Fe^{3+}), root cells can then take this up by active transport mechanisms (Ashraf et al., 2013).

2.6.2 Phytohormones

A wide range of microorganisms found in the rhizosphere are able to produce substances that regulate plant growth and development. PGPR produce phytohormones such as auxins, cytokinins, gibberellins and ethylene can affect cell proliferation in the root architecture by overproduction of lateral roots and root hairs with a subsequent increase of nutrient and water uptake (Arora et al., 2013).

2.6.2.1 Auxin

Microbial synthesis of the phytohormone auxin (indole-3-acetic Acid; IAA) has been known for a long time (Ahemad and Kibret, 2014). It is reported that 80% of microorganisms isolated from the rhizosphere of various crops possess the ability to synthesize and release auxins as secondary metabolites (Patten and Glick, 1996). Generally, IAA secreted by rhizobacteria interferes with the many plant developmental processes because the endogenous pool of plant IAA may be altered by the acquisition of IAA that has been secreted by soil bacteria (Glick, 2012; Spaepen et al., 2007). Evidently, IAA also acts as a reciprocal signaling molecule affecting gene expression in several microorganisms. Consequently, IAA plays a very important role in rhizobacteria-plant interactions (Spaepen and Vanderleyden, 2011). Most IAA is synthesized from the amino acid tryptophan present in plant root exudates at varying low concentrations based on the plant's genotype (Olanrewaju et al., 2017). IAA appears to be synthesized by at least three different biosynthetic pathways with each pathway being named for a key intermediate within the pathway. These pathways include: the indole pyruvic acid (IPyA) pathway, the indole acetamide (IAM) pathway, the indole acetaldoxime (IAOx)/indole acetonitrile (IAN) pathway (Duca et al., 2014), the indole acetaldehyde (IAH) pathway, and the tryptamine pathway. It should be noted that various PGPB can have one, two or even three functional IAA biosynthesis pathways suggesting that the synthesis of IAA is clearly very important

in the life and functioning of the bacterium. A schematic representation of bacterial IAA biosynthesis from tryptophan is shown in Figure 9.

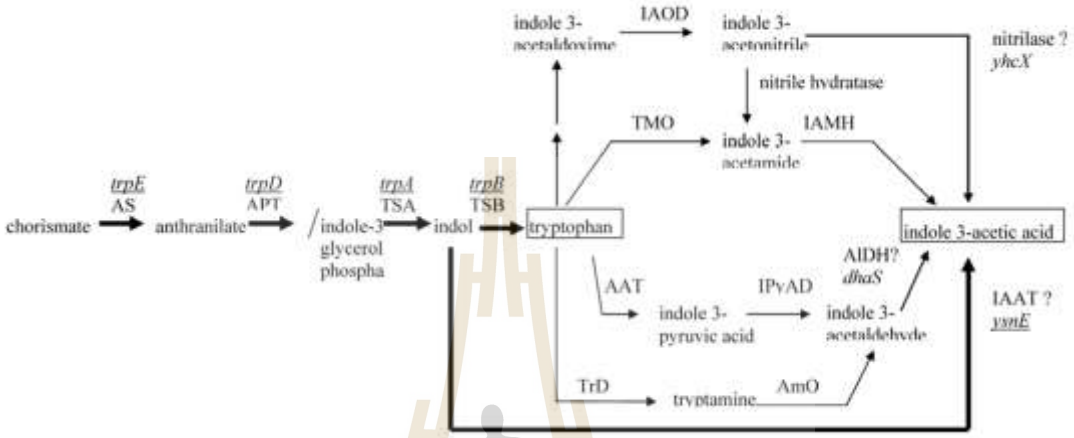

Figure 9. Tryptophan-dependent pathways of bacterial indole-3-acetic acid (IAA) synthesis (Modified from Idris et al. (2007)).

The chromosome or the plasmid of the auxin biosynthetic genes and this difference in hosts affects the IAA level with plasmid found in many copies. A case study is that observed between *P. savastanoi* pv. *savastanoi* and *P. syringae* pv. *syringae* where the genes are located on the plasmid and chromosomal DNA, respectively (Aragón et al., 2014). The IAM pathway is majorly attributed to phytopathology, while the IPA pathway is connected to epiphytic and rhizosphere fitness. The ipdC gene which is one of the genes involved in IAA synthesis in PGPB are regulated by the amount of IAA produced which serve as a positive feedback in up-regulating the expression of the gene (Spaepen and Vanderleyden 2011). This positive feed-back was reported in *A. brasilience* Sp245 as the first PGPB to show this observation (Broek et al. 2005). Idris et al. (2007) presented additional evidence for the existence of a tryptophan-dependent pathway as the main route of IAA biosynthesis in *B. amyloliquefaciens* FZB42. However, minor tryptophan-

independent pathways for IAA biosynthesis may exist in FZB42, because IAA biosynthesis was not abolished completely in the *trp*, *ysnE*, and *yhcX* mutant strains. Double mutants might be useful to prove the presence of alternative Trp-independent pathways. IAA is actively involved in its biosynthetic genes as it determines the activation, inactivation, over expression or under expression of these genes. Other regulatory mechanisms of IAA synthesis are reported in the work of Glick (2015).

The phytohormone auxin is a major regulator of plant growth and development. Many aspects of these processes depend on the multiple controls exerted by auxin on cell division and cell expansion. The specific roles of auxin at the various phases of the cell cycle in dividing cell suspension cultures has been hampered by the fact that auxin is required at the initial step of cell-cycle entry and thus its effects cannot easily be dissociated from this initial and critical step. The mechanisms by which auxin affects the cell cycle machinery are still far from being elucidated but recent data suggest that auxin acts on multiple targets and affects transcriptional regulation as well as protein turnover of core cell-cycle regulators. The understanding of auxin action on the cell cycle is still extremely fragmentary; primary evidence indicates that auxin acts on multiple targets, influencing directly or indirectly both transcriptional and posttranscriptional regulation (Perrot-Rechenmann, 2010) as shown in Figure 10.

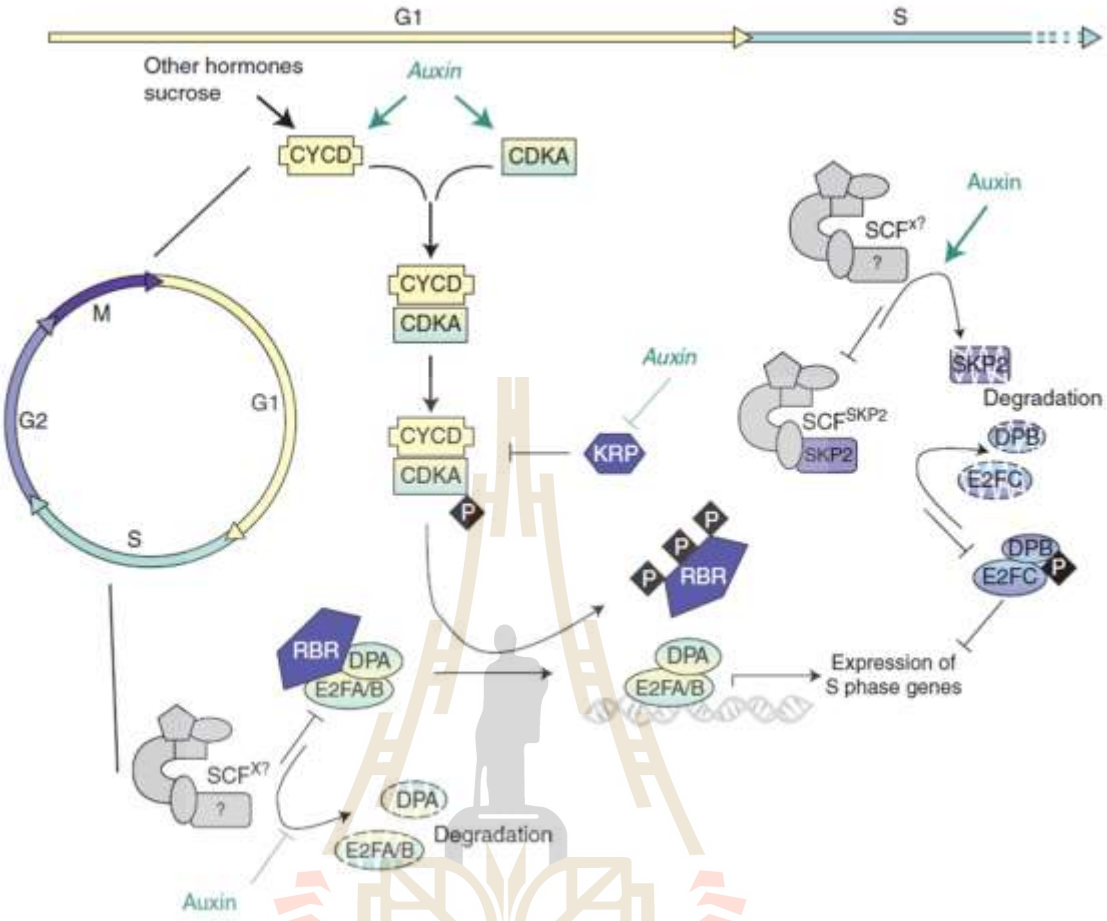

Figure 10. Auxin action on cell cycle (Perrot-Rechenmann, 2010).

Auxin and the G1/S transition, the cell cycle is divided into four phases: DNA replication (S), mitosis (M), and two Gap phases (G1 and G2, between M/S, and S/M, respectively). The cycle starts in G1. During this phase, expression of D-type cyclins and cyclin-dependent kinase (CDKA) is induced by various signals including auxin (Doerner et al., 2000). In *Arabidopsis*, CYCD3;1 was reported to be a rate-limiting factor for G1/S transition (Menges et al., 2006). Expression of CYCD3;1 is regulated by the availability of nutrients but also by cytokinin, brassinosteroids, and auxin (Dewitte and Murray 2003). The CDKA/CYCD complex is activated by phosphorylation but can still be blocked by CDK inhibitors (KRP). Auxin was reported to reduce the expression of some KRPs. Expression of KRP1 and KRP2, encoding two of the CDK inhibitors, was reported

to be down-regulated after auxin treatment at least in specific root cells (Himanen et al., 2002; Richard et al., 2001). The active CDK/CYCD complex provokes phosphorylation of the transcriptional repressor retinoblastoma-related protein (RBR) thus promoting expression of genes essential for the beginning of the S phase under the control of the E2FA/B and DPA complex (Jurado et al., 2008). Auxin was shown to stabilize these transcriptional regulators. Later in S phase, E2FC and DPB repress expression of S phase genes. Degradation of these proteins is under the control of the E3 ubiquitine ligase SCFSKP2 and auxin was shown to increase the degradation of the F-box SKP2, thus indirectly stabilizing E2FC and DPB (Perrot-Rechenmann, 2010).

Cell expansion is an increase in cell size accompanying the process of plant growth. Cells leaving meristematic zones often enlarge to involve or thousands of times their original size. This increase in size usually results from the combination of two processes: The increase in cell ploidy level by endoreplication (successive rounds of DNA replication with no mitosis), and the complex process of cell expansion, which is driven by internal turgor pressure and restricted by the ability of cell walls to extend. Endoreplication occurs when the cell cycle is truncated such that either mitosis or cytokinesis is skipped between rounds of DNA replication. The result is the formation of cells with increasing ploidy. In most *Arabidopsis* shoot tissues, a positive correlation was reported between the degree of ploidy and cell size. Conversely in roots, such correlation has not been observed however the switch from mitotic cycles to endocycles is coupled with the transition between the root meristem and cell differentiation (Beemster et al., 2002; Ishida et al., 2009). By modulating the levels of cell-cycle regulators that are involved in both the mitotic cycle and the endocycle, auxin is likely to play amajor role in the regulation of endoreplication. In roots, the mitotic to endocycle transition was recently proposed to be regulated by auxin and cytokinin, the combination of low auxin and increased cytokinin facilitating the switch from mitotic cells to endocycle and differentiation (Ioio et al., 2008).

Plant cell expansion requires uptake of water, which is then stored in vacuoles, and irreversible extension of the cell wall, which includes wall loosening and deposition of new wall material. Auxin is one of the major stimuli affecting these mechanisms but it is essential to keep in mind that cell expansion is also under the control of many other stimuli, such as blue light and most of the other phytohormones. Auxin-dependent cell expansion follows a dose-response curve in which high concentrations are inhibitory (Barbier-Brygoo et al., 1991; Evans et al., 1994). Auxin regulation of cell expansion and elongation is presented in Figure 11.

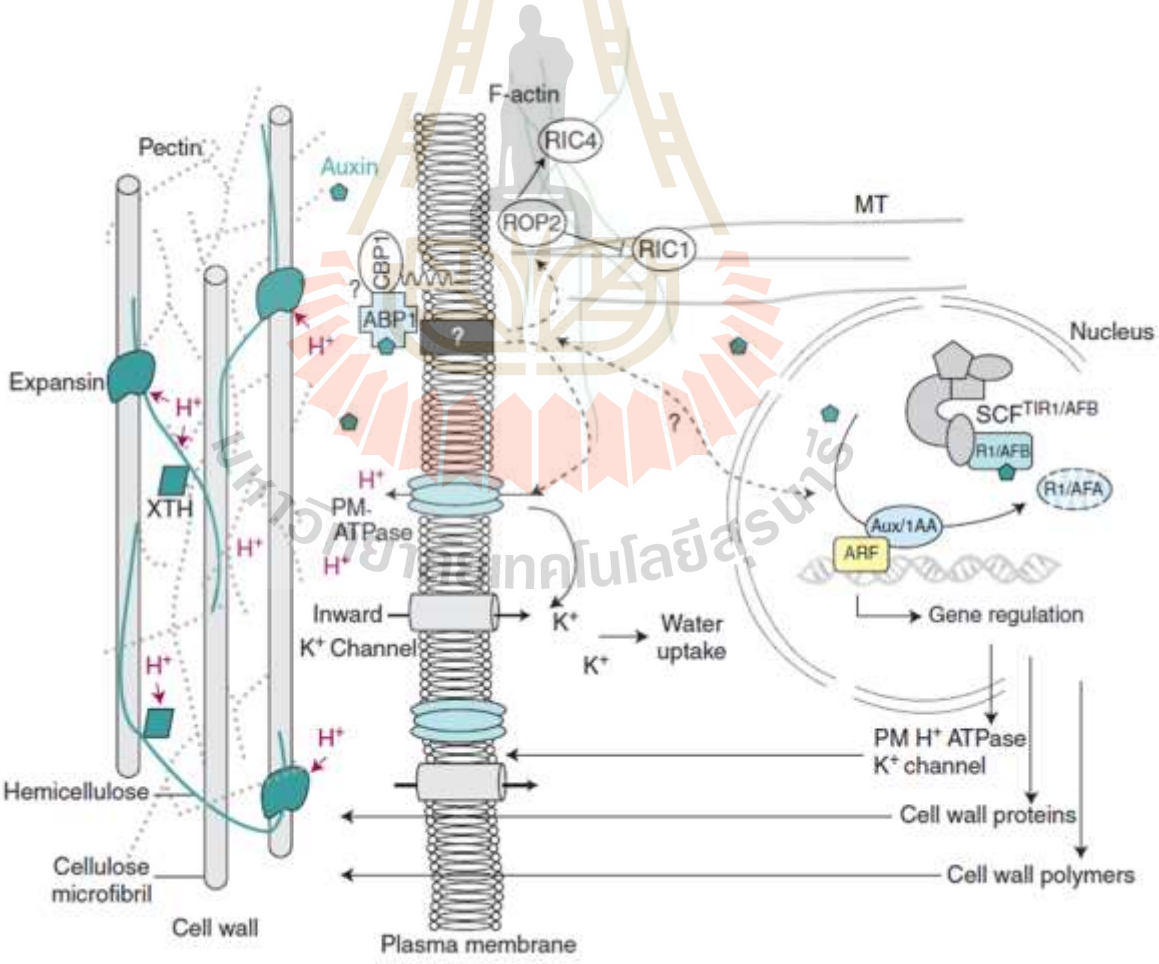

Figure 11. Auxin regulation of cell expansion and elongation (Perrot-Rechenmann, 2010).

Auxin-induced cell wall loosening and expansion. The scheme represents the cell wall, plasma membrane and cytoskeleton continuum with the consequences of auxin action. Auxin is perceived by the auxin receptor ABP1, which interacts with unknown membrane-associated proteins at the plasma membrane (such as the putative candidate GPI-anchored protein CBP1) (Shimomura, 2006). This activates the proton pump ATPase, provoking the acidification (H^+) of the extracellular space, the activation of cell wall proteins such as expansins and xyloglucan endotransglycosylase/hydrolases (XTH), which mediate cell wall loosening by acting on the cell wall polysaccharide network. Polysaccharides forming the cell wall are cellulose microfibrils, cross-linked hemicelluloses, and pectins. Activation of the H^+ ATPase also induces hyperpolarization of the plasma membrane and activation of K^+ inward rectifying channels, essential for the uptake of water sustaining cell expansion. Auxin also enhances these effects by inducing the expression of genes encoding plasma membrane ATPase, K^+ channels, expansins, and cell wall remodelling enzymes and promotes exportation of new cell wall material. Auxin is likely to act on actin microfilaments and microtubules via the modulation of ROP GTPases. Auxin produced by PGPR's is a class of plant hormones important in the promotion of lateral root formation increase root growth and length, modifying the plant (morphological functions), increased lateral root formation leads to an enhanced ability to take up nutrients for the plant (Saharan and Nehra, 2011) and uptake more nutrients from the soil (Das et al., 2013).

2.6.2.2 Cytokinin

Cytokinins are widely distributed in algae, bacteria, and higher plants; however, relatively little information is available on the roles of bacteria-produced cytokinins (Olanrewaju et al., 2017). Phytohormones including cytokinins were detected in the culture medium of several bacteria including *Halomonas desiderata*, *Proteus mirabilis*, *P. vulgaris*, *Klebsiella pneumoniae*, *Bacillus megaterium*, *B. cereus*, *B. subtilis* and

Escherichia coli (Arkhipova et al., 2005; Karadeniz et al., 2006). Different cytokinins are detected not only in the biomass of microorganisms (in free state or bound to certain tRNAs) but also in the culture medium in the form of either adenine derivatives, isoprenylated at N^6 position or their ribosides, such as 6-benzyladenine, N^6-isopentenyladenosine, zeatinriboside (Serdyuk et al., 2003). Transzeatine has also been found in the culture of *Agrobacterium tumefaciens* (Krall et al., 2002). Not surprisingly therefore, the *ipt*-gene coding for isopentenyltransferase (an enzyme controlling synthesis of cytokinins from adenine derivatives) was initially discovered in *A. tumefaciens* (tumour stimulating bacteria) (Nester et al., 1984) Not only bacteria, this gene also detected in plant genome (Kakimoto, 2001). Ryu et al. (2003) reported that cytokinin from bacterial origin improve growth in *Arabidopsis*. Inoculation of plant with bacteria producing cytokinin has been shown to stimulate shoot growth and reduce root/shoot ratio in droughted plants (Arkipova et al., 2007).

Cytokinins are important regulators of cell proliferation and differentiation in plant development (Mens et al., 2018). Cytokinin hormones are a class of structurally similar N^6-substituted adenine derivatives with a central role in plant growth and development. Since their discovery in cell proliferation and differentiation, several other functions have been attributed to this class of phytohormones, including maintenance of the shoot apical meristem, branching, organogenesis, delay of senescence, long-distance communication of nutritional status and the plant's response to biotrophic pathogens (Kamada-Nobusada and Sakakibara, 2009). Like auxins, they can act both locally and as long-distance messengers. Natural cytokinins are classified into aromatic and isoprenoid cytokinins, the latter are most prevalent in higher plants and include trans-zeatin (tZ), cis-zeatin (cZ), dihydrozeatin (DZ) and N^6-(Δ^2-isopentenyl)-adenine (2-iP). Cytokinins show further variation through the addition of side chains, which seem to confer receptor specificity.

Isopentenyl transferases (*IPTs*) carry out the first and rate limiting step in cytokinin biosynthesis, where an isopentenyl group is transferred to either AMP, ADP or ATP (Kakimoto, 2001). Recently, *IPT3* was shown to be required for nodule development in the model legume *L. japonicus* (Chen et al., 2013; Reid et al., 2017). It has also been proposed that *LjIPT3* could have a role in the synthesis of cytokinin molecules that act as the shoot-derived inhibitory factor in autoregulation of nodulation (AON) (Sasaki et al., 2014). *LjIPT2* is thought to be responsible for the initial cytokinin build-up required for nodulation initiation, alongside *LjLOG4* and independent of the *LHK1* receptor (Reid et al., 2017). Interestingly, *IPT* encoding genes are also differentially expressed following treatment with certain nitrogen sources (Miyawaki et al., 2004), with nitrogen promoting plant development, but also inhibiting nodule organogenesis. In addition, 17 *IPT* genes were identified in the soybean genome and genetically characterized. Of these genes, the tandem duplicates *GmIPT3* and *GmIPT15* were upregulated in the root upon nitrate treatment, indicating a role for these genes in nitrate response. Upon rhizobia inoculation, *GmIPT5* (a soybean ortholog of *LjIPT3*) was significantly upregulated in the shoot following rhizobia inoculation of the root. Upregulation of *GmIPT5* occurred in both the root and shoot in wild-type and GmNARK mutant plants (*nts382*), indicating that it functions in the leaf and root in response to nodulation, but likely does not have a role in the AON pathway (Mens et al., 2018). However, it is important to remember that in addition to synthesis, posttranscriptional processing and degradation are also important to maintaining homeostasis of biologically active cytokinins.

2.6.2.3 Gibberellins

There is little information regarding microorganisms that produce gibberellins, although it is known that symbiotic bacteria existing within nodules in leguminous plants to fix nitrogen (rhizobia) are able to produce gibberellins, auxins and cytokinins in very low concentrations when the plant is forming the nodule and there is a

high cell duplication rate (Atzorn et al., 1988). However, the production of gibberellins by PGPR is rare, with only two species being documented that produce gibberellins, *B. pumilus* and *B. licheniformis*. These bacteria were isolated from the rhizosphere of *Alnus glutinosa* and have shown a capacity to produce large quantities of gibberellins GA_1, GA_3, GA_4 and GA_{20} in vitro. These types of hormones are the largest group of plant regulators, including more than 100 different molecules with various degrees of biological activity. The common structure of these diterpenic growth regulators is a skeleton of 19–20 carbon atoms, and there is a clear relationship between structure and biological effect. The reason for the pronounced effect of gibberellins is that these hormones can be translocated from the roots to the aerial parts of the plant. The effects in the aerial part are notable and more so when the bacteria also produce auxins that stimulate the root system, enhancing the nutrient supply to the sink generated in the aerial part. The first report of gibberellin characterization in bacteria using physico-chemical methods was by Atzorn et al. (1988), who demonstrated the presence of GA1, GA3, GA4 and GA20 in gnotobiotic cultures of *R. meliloti*. Apart from *Azospirillum* sp. and *Rhizobium* sp., production of gibberellin-like substances has also been claimed in numerous bacterial genera, production of gibberellins has been confirmed in *Acetobacter diazotrophicus, Herbaspirillum seropedicae* and *Bacillus* sp. (Jha and Saraf, 2015).

2.6.2.4 Ethylene

Ethylene is a significant phytohormone, ethylene in low levels has been observed to promote plant growth, but excess secretion of ethylene will lead to root curling and shortening, even it can result in plant death under extreme conditions (Das et al., 2013). In plants, 1-aminocyclopropane-1-carboxylate (ACC) and 5'-deoxy 5'methylthioadenosine (MTA) is converted to ACC by ACC synthase (Glick et al., 2007). A number of plant growth-promoting bacteria have been found to contain the enzyme ACC deaminase, ACC deaminase activity of PGPR helps plant combat abiotic stress by hydrolyzing ACC, the

precursor of ethylene, to alpha-ketobutyrate and ammonia, and encourages plant growth under stress environment (Das et al., 2013; Saharan and Nehra, 2011). Thus lowers the level of ethylene in a developing or stressed plant (Glick et al., 2007). The presence of PGPR thereby moderates concentration of ACC so that it does not reach a level where it begins to impair root growth.

2.6.3 Nitrogen fixation

Nitrogen is one of the important nutrients essential for the growth of all living organisms including plants and bacteria. The observation of nitrogen deficiency in soil has led to the use of large amounts of nitrogenous fertilizers in order to make up for the necessary plant requirements to achieve maximum plant yield in most soils (Zhang et al., 2015). Nitrogen availability has become one of the yield-limiting factors in plant growth due to rainfall and mineral leaching into ground water (Mantelin and Touraine, 2004). Although the nitrogen presents 78 % of the atmosphere, this form of gaseous nitrogen [N_2 (g)] remains unavailable to the plants (Gupta et al., 2015), it is not suitable for plant assimilation until it is first converted to ammonia (Baas et al. 2014). This conversion to ammonia requires a significant amount of energy because of the stability of the triple bond in N_2 (g). This energy may be provided at the expense of fossil fuels, through biological nitrogen fixation at the expense of ATP, and through other mechanisms of nitrogen input in terrestrial systems (Olanrewaju et al., 2017). Regrettably no plant species is capable for fixing atmospheric dinitrogen into ammonia and expend it directly for its growth. Thus the atmospheric nitrogen is converted into plant utilizable forms by biological nitrogen fixation (BNF) which changes nitrogen to ammonia by nitrogen fixing microorganisms using a complex enzyme system known as nitrogenase (Gaby and Buckley, 2012). PGPR have the ability to fix atmospheric nitrogen and provide it to plants by two mechanisms: symbiotic and non-symbiotic. Symbiotic nitrogen fixation is a mutualistic relationship between a

microbe and the plant. The microbe first enters the root and later on form nodules in which nitrogen fixation occurs (Ahemad and Kibret, 2014). There are a number of PGPR, which are able to fix atmosphere nitrogen (N_2) and make it more accessible to plants. Broad range of nitrogen fixing bacteria have been identified including a number of organisms that fix nitrogen symbiotically with specific plants (mostly legumes). Examples of symbiotic nitrogen fixers are *Rhizobium*, *Sinorhizobium*, *Azorhizobium*, *Allorhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Frankia*, *Azoarcus*, *Achromobacter*, *Burkholderia*, and *Herbaspirillum* (Babalola 2010; Pérez-Montaño et al. 2014; Turan et al. 2016). On the other hand, non-symbiotic nitrogen fixation is carried out by free living diazotrophs and this can stimulate non-legume plants growth such as radish and rice. Non-symbiotic Nitrogen fixing rhizospheric bacteria belonging to genera including: *Burkholderia*, *Enterobacter*, *Gluconacetobacter*, *Pseudomonas*, *Azospirillum*, Cyanobacteria, *Azoarcus*, *Azotobacter* and *Acetobacter*, these bacteria are free living nitrogen fixation that is capable for fixing atmospheric dinitrogen into ammonia (Bhardwaj et al., 2014; Das et al., 2013; Vessey, 2003). Although PGPR have the ability to fix nitrogen, they are not able to provide a sufficient amount to sustain the plants. Due to their effect on shoot elongation and stimulation of nitrate (NO_3^-) transport systems, they are able to greatly increase the intake of nitrogen by the plants, despite not fixing enough nitrogen on its own for sustenance (Mantelin and Touraine, 2004). Inoculation by biological nitrogen fixing plant growth promoting rhizobacteria on crop provide an integrated approach for maintain the nitogen level in agricultural soil.

2.6.4 Induced systemic resistance

Induced resistance may be defined as a physiological state of enhanced defensive capacity elicited in response to specific environmental stimuli and consequently the plant's innate defenses are potentiated against subsequent biotic challenges (Avis et al.,

2008). Biopriming plants with some plant growth promoting rhizobacteria can also provide systemic resistance against a broad spectrum of plant pathogens. Diseases of fungal, bacterial, and viral origin and in some instances even damage caused by insects and nematodes can be reduced after application of PGPR (Naznin et al., 2013). PGPR are able to control the number of pathogenic bacteria through microbial antagonism, which is achieved by competing with the pathogens for nutrients, producing antibiotics, and the production of anti-fungal metabolites (Bloemberg and Lugtenberg, 2001). Besides antagonism, certain bacteria-plant interactions can induce mechanisms in which the plant can better defend itself against pathogenic bacteria, fungi and viruses (Compant et al., 2005). This is known as induced systemic resistance (ISR) and was firstly discovered by Van Peer et al. (1991). The inducing rhizobacteria triggers a reaction in the roots that creates a signal that spreads throughout the plant which results in the activation of defense mechanisms, such as, reinforcement of plant cell wall, production of antimicrobial phytoalexins and the synthesis of pathogen related proteins (Van Loon, 2007). Components of bacteria that can activate ISR includes lipopolysaccharides (LPS), flagella, salicylic acid, sideophores (Lugtenberg and Kamilova, 2009), cyclic lipopeptides, homoserine lactones, 2, 4-diacetylphloroglucinol and volatiles like, acetoin and 2, 3-butanediol (Doornbos et al., 2012). Moreover, induced systemic resistance involves jasmonate and ethylene signaling within the plant and these hormones stimulate the host plant's defense responses against a variety of plant pathogens (Glick, 2012). During root colonization, fluorescent pseudomonads produce antifungal antibiotics, elicit induced systemic resistance in the host plant or interfere specifically with fungal pathogenicity factors as present Figure 12.

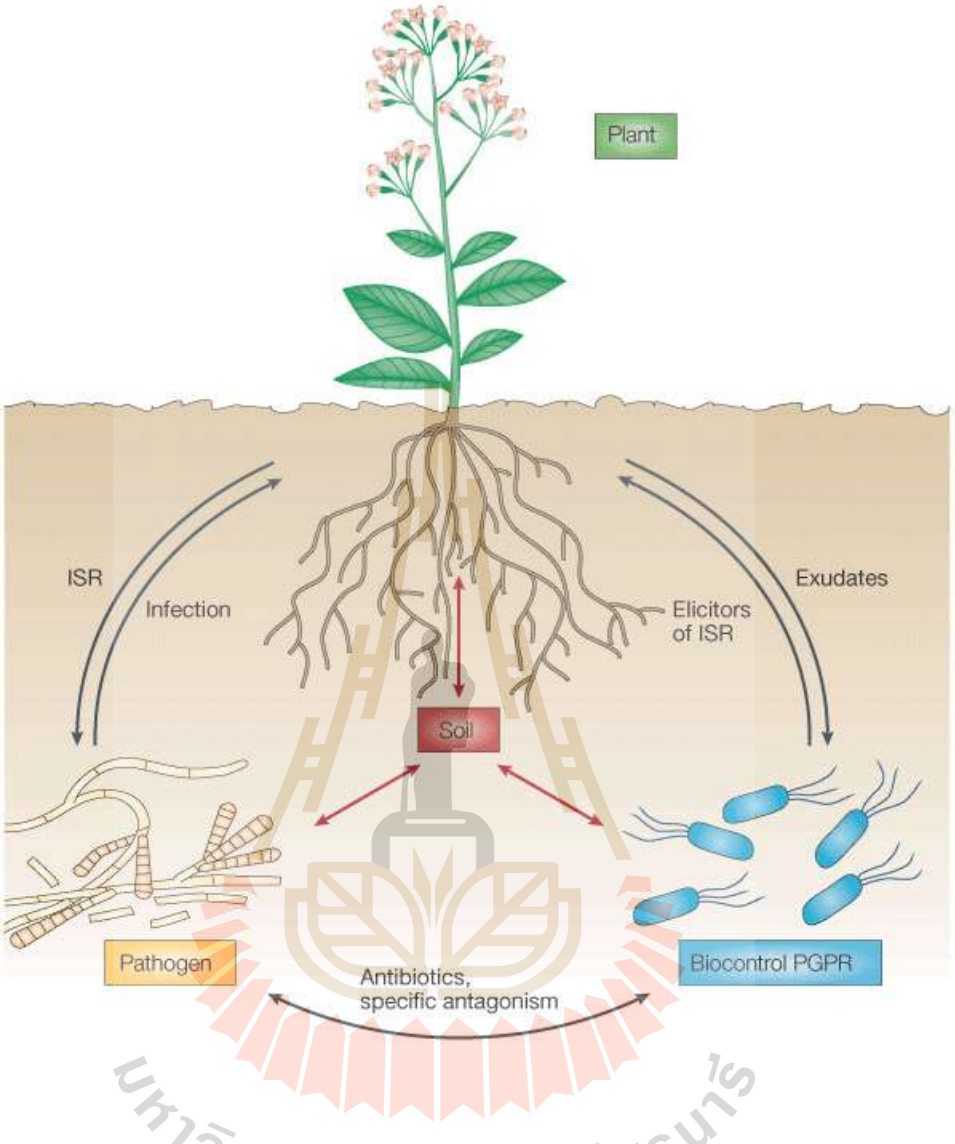

Figure 12. Interactions between biocontrol plant growth-promoting rhizobacteria (PGPR), plants, pathogens and soil (Haas and Défago, 2005).

2.6.5 Application of PGPR as biofertilizer

Agriculturally, the beneficial bacteria can be used as inoculants for crops and plants (Vessey, 2003). Microbial biofertilizers are the best modern tools in agriculture (Bhardwaj et al., 2014). The additional advantages of biofertilizers include longer shelf life causing no adverse effects to ecosystem (Sahoo et al., 2014). Biofertilizers keep the soil environment rich in all kinds of micro- and macro-nutrients via nitrogen fixation, phosphate and potassium solubalisation or mineralization, release of plant growth

regulating substances, production of antibiotics and biodegradation of organic matter in the soil (Sinha et al., 2010). In term of biofertilizer, which is a substance that contains living microorganisms and when applied to seeds, plant surfaces or soil inoculants, it promotes growth by increasing the supply or availability of primary nutrients to the host plant (Bhattacharyya and Jha, 2012; Vessey, 2003) and/or they multiply and participate in nutrient cycling and benefit crop productivity (Singh et al., 2011). In general, the total applied fertilizer is lost and the remaining 10% to 40% is taken up by plants. In this regard, microbial inoculants have paramount significance in integrated nutrient management systems to sustain agricultural productivity and healthy environment (Adesemoye and Kloepper, 2009). Biofertilizer is different from organic fertilizers, which contains organic compounds that increase soil fertility either directly or as a result of their decay. Not all PGPR are considered as a biofertilizer; if they control plant growth by control of deleterious organisms, they are instead regarded as biopesticides. Biofertilizers must contain living microorganisms that promote plant growth by improving the nutrient status of the plant.

PGPR has become increasingly important in the agricultural production of certain crops. However the commercialization and utilization of PGPR has been currently limited due to the fact that there have not been consistent responses in different host cultivars and at different field sites (Vessey, 2003). Additionally, their effects have been used in environmental application, such as promoting re-vegetation in eroded deserts (Bashan et al., 2008; Compant et al., 2005). Although the use of PGPR in agriculture and solving environmental problems seems promising, there is not enough knowledge about these bacteria for them to be put into use. A lot more research needs to be investigated before they can be proven as useful to mankind. However, scientific preparation and application of microbial formulation is important while developing the agriculture in a sustainable way.

2.7 Co-inoculation of PGPR and *Rhizobium* for legume production

A recent response by Kiers et al. (2013) highlights the potential problems of single-strain inoculation experiments, and argues that variation in nodulation speed confounds symbiotic competitive ability and host fitness. Most studies of beneficial plant-microbe interactions focus on a single plant-microbe partnership at a time (Benito et al., 2017). Inoculating with a single strain, in keeping with the long tradition of reductionism, is a straight forward way to conclusively ascribe an effect on the host to the action of that strain. Single-strain inoculation provides information about the potential benefit conferred by that strain, without the complications of non additive interactions between strains (Friesen and Heath, 2013). In some cases, multiple strains are synergistic for plants (Egamberdieva et al., 2016; İçgen et al., 2002), while in others, mixed inoculations result in plants that perform worse than predicted (Heath and Tiffin, 2007). Studying ways to augment plant productivity through the use of beneficial microbes will increase our knowledge of plant-microbe interactions, which has deep implications for agriculture and biotechnology (Benito et al., 2017). As the moving from the monoculture-based approach of traditional agriculture into the realism of complex communities of plants and microbes, the urgently need to integrate ecologically grounded experiments with mathematical frameworks provided by statistical genetics and community ecology (Friesen, 2012; Friesen and Heath, 2013).

Enhancement of legume nitrogen fixation by co-inoculation of rhizobia with some PGPR is a way to improve nitrogen availability in sustainable agriculture production systems. Moreover, simultaneous infection with rhizobia and a number of other bacteria also present in nodules enhances nodulation and plant growth in a wide variety of legumes. Some PGPR strains, from a range of genera, enhance legume growth, nodulation and nitrogen fixation when co-inoculated with rhizobia. Examples of these are *Azospirillum* (Askary et al., 2009; Aung et al., 2013; Cassán et al., 2011; Chibeba et al., 2015; de Souza

et al., 2017; Ferri et al., 2017; Groppa et al., 1998; Hungria et al., 2013; Hungria et al., 2015; Puente et al., 2018; Remans et al., 2008; Rodrigues et al., 2016; Vicario et al., 2016), *Azotobacter* (Hadi et al., 2010; Wu et al., 2012), *Bacillus* (Atieno et al., 2012; Bai et al., 2003; Li and Alexander, 1988; Masciarelli et al., 2014; Mishra et al., 2009a; Mishra et al., 2009b; Qureshi et al., 2011; Santos et al., 2018a; Santos et al., 2018b; Shehata et al., 2012; Subramanian et al., 2015; Xue et al., 2011; Yuttavanichakul et al., 2012), *Pseudomonas* (Afzal et al., 2010; Argaw, 2011; Li and Alexander, 1988; Kaur et al., 2015), *Serratia* (Pan et al., 2002; Zahir et al., 2011) and *Streptomyces* (Gregor et al., 2003; Htwe et al., 2018; Htwe and Yamakawa 2015; Santos et al., 2018a; Soe and Yamakawa 2013; Tokala et al., 2002). As they share common microhabitats in the root soil interface, rhizobia and PGPR must interact during their processes of root colonization. The effect of *Rhizobium*–PGPR co-inoculation has been observed in different symbiotic and plant growth parameters. Compared to single *Rhizobium* inoculation, co-inoculation of *Rhizobium* spp. and *Azospirillum* spp. can enhance the number of root hairs, the amount of flavonoids exuded by the roots and the number of nodules formed (Remans et al., 2008). The beneficial influence of PGPR on nodulation of legumes by *Rhizobium* has been variously attributed to their ability to produce phytohormones (Schmidt, 2008), toxins (Zaidi et al., 2012), antibiotics (Yuttavanichakul et al., 2012), or antioxidant response (Santos et al., 2018a), as well as by other unidentified mechanisms (Paulucci et al., 2012). For example, Argaw (2011) demonstrated that some *Pseudomonas* strains colonized the root and increased nodule number and acetylene reduction in soybean plants inoculated with *B. japonicum*. Current data suggest that although *Micromonospora* species do not induce nodules or fix nitrogen in association with a host plant, they provide many benefits to the plant by increasing the number of nodules, enhancing aerial growth and nutrient uptake (Martínez-Hidalgo et al., 2014; Solans et al., 2009; Trujillo et al., 2014). Moreover, complex interactions and competition between the three microorganisms (*B. japonicum*, *A.*

canadense and *Glomus irregular*) were also induced differential growth and nodulation responses, which can be linked to metabolic changes (Juge et al., 2012). Considering the co-inoculation of symbiotic nitrogen fixers and PGPR on legumes response was summarized in Table 1.

Table 1. Co-inoculation of symbiotic nitrogen fixers and PGPR on legumes response.

Symbiotic nitrogen fixers	Co-inoculating PGPR	leguminous plants	Legumes response to the co-inoculation	References
Bradyrhizobium spp.	*Bacillus subtilis*	Soybean	Increasing the nodulation, shoot dry matter and N-content.	Shehata et al. (2012)
	P.fluorescens	Soybean	Salient superiority in suppressive disease.	
Bradyrhizobium spp.	*A. brasilense*	Soybean	Promotion of early nodulation and increase in nodule biomass.	Chibeba et al. (2015)
Bradyrhizobium spp.	*A. brasilense*	Soybean	Co-inoculation improves soybean yield.	Hungria et al. (2015)
Bradyrhizobium sp.	*Bacillus* sp. *Phyllobacterium* sp.	Soybean	Increasing the number of nodules, nodule dry weight, plantdry weight and total N and P contents.	Xue et al. (2011)
Bradyrhizobium sp.	*Pseudomonas* sp.	Soybean	Increasing grain yield in pot and field experiment and increasing survival efficiency of *Bradyrhizobium*.	Afzal et al. (2010)
B. japonicum	*Azomonas agilis* *A. lipoferum* *P. fluorescens*	Soybean	Enhancing nodulation and ARA but *P. fluorescens* decreased the nodule number and ARA.	Chebotar et al. (2001)
B. japonicum	*Azospirillum* sp.	Soybean	Co-inoculation effects on competitive nodulation and rhizosphere eubacterial community structures of soybean under rhizobia-established soil conditions.	Aung et al. (2013)
B. japonicum	*A. brasilense*	Soybean	Increasing number of the most active nodules, therefore, to a greater nitrogen fixation and assimilation.	Groppa et al. (1998)
B. japonicum	*A. brasilense*	Soybean Common bean	Increasing soybean yield and improved nodulation under field experiment.	Hungria et al. (2013)
B. japonicum	*A. chroococcum*	Soybean	Increasing the number and fresh weight of nodules and seedling dry weight under drought stress.	Hadi et al. (2010)

Table 1. Co-inoculation of symbiotic nitrogen fixers and PGPR on legumes response (continued).

Symbiotic nitrogen fixers	Co-inoculating PGPR	leguminous plants	Legumes response to the co-inoculation	References
B. japonicum	*B. subtilis*	Soybean	Increasing the yield and nodule occupancy using the liquid and granule-based formulation type.	Atieno et al. (2012)
B. japonicum	*B. subtilis* *B. thuringiensis*	Soybean	Enhanced soybean plant growth and soybean production systems in short growing season regions.	Bai et al. (2003)
B. japonicum	*B.thuringiensis*	Soybean	Provided the highest and most consistent increase in nodule number, shoot and root weight, root volume and total biomass.	Mishra et al. (2009b)
B. japonicum	*Bacillus* sp. *S. griseus*	Soybean	Enhancing nodule number, pod formation, yield and seed weight.	Li and Alexander (1988)
B. japonicum	*S. proteamaculans* *S.liquefaciens*	Soybean	There are no additional improvements in nodulation and nitrogen fixation by using PGPR pre-incubated with genistein.	Pan et al. (2002)
B. japonicum	*S. kanamyceticus* *S. coeruleoprunus* *S. rimosus* *Streptomyces* sp.	Soybean	Increasing in nodule occupancy varied from 0% to 18.3% and shoot nitrogen composition.	Gregor et al. (2003)
B. japonicum	*B. megaterium* *M. oryzae*	Soybean	Increasing in nodule number, nodule activity which was measured in terms of nodule leghemoglobin content, nodulated root ARA and total plant nitrogen content.	Subramanian et al. (2015)
B. japonicum	*P. putida*	Soybean	Improvement of soybean salt tolerance through altering root system architecture facilitating nitrogen and phosphorus acquisition and nodule formation.	Egamberdieva et al. (2017a)

Table 1. Co-inoculation of symbiotic nitrogen fixers and PGPR on legumes response (continued).

Symbiotic nitrogen fixers	Co-inoculating PGPR	leguminous plants	Legumes response to the co-inoculation	References
B. japonicum	*A. brasilense*	Soybean	Increasing the nodule number, nodule dry weight, shoot dry biomass and shoot N content.	Ferri et al. (2017)
B. japonicum	*B. amyloliquefaciens*	Soybean	Enhancing the capacity of the latter to colonize plant roots and increase the number of nodules.	Masciarelli et al. (2014)
B. japonicum	*Stenotrophomonas rhizophila*	Soybean	Promoting plant growth, nutrient uptake and fitness of hydroponically grown soybean under salt stress condition.	Egamberdieva et al. (2016)
B. japonicum	*A. brasilense*	Soybean	Increasing nodule weight, total plant and root length, aerial and root dry weight, number of nodules on the primary root and increased in the symbiosis with *B. japonicum.*	Puente et al. (2018)
B. elkanii	*S. griseoflavus*	Soybean	Increasing N_2 fixation, plant growth, nodulation, N uptake and shoot growth.	Htwe et al. (2018)
B. elkanii *B. japonicum*	*S. griseoflavus*	Soybean	Increasing nitrogenase activity, root dry weight and promoted plant growth.	Htwe and Yamakawa (2015)
B. yuanmingense	*S. griseoflavus*	Soybean	Increasing the nodule number, nodule dry weight and seed yield.	Soe and Yamakawa (2013)
Rhizobium sp.	*B. megaterium* *P. polymyxa*	Common bean	Increasing nodule weight and shoot dry weight.	Korir et al. (2017)
R. tropici	*A. brasiliense*	Common bean	Increasing of nodule number, nodule dry weight, root dry weight and shoot dry weight.	de Souza et al. (2017)
R. tropici	*Bradyrhizobium* sp.	Common bean	Plants produced more nodules, accumulated more shoot dry biomass and nitrogen.	da Conceição Jesus et al. (2018)

Table 1. Co-inoculation of symbiotic nitrogen fixers and PGPR on legumes response (continued).

Symbiotic nitrogen fixers	Co-inoculating PGPR	leguminous plants	Legumes response to the co-inoculation	References
R. tropici	*A. brasilense*	Common bean	Increasing nodule number.	Rodrigues et al. (2016)
Bradyrhizobium sp.	*Actinomadura* sp.	Cowpea	Lowering hydrogenperoxide content, increasing superoxide dismutase, catalase and phenol peroxidase activities in nodulesexposed to salt stress.	Santos et al. (2018a)
	Paenibacillus graminis		Lowering hydrogenperoxide content, increasing superoxide dismutase, catalase and phenol peroxidase activities in nodulesexposed to salt stress.	
	Streptomyces sp.		Increasing catalase activity in plants.	
	Bacillus sp.		Indution of positive responses for coping with salt-induced oxidative stress in cowpea nodules.	
Bradyrhizobium sp.	*Bacillus* sp.	Cowpea	Providing better symbiotic performance, mitigating the deleterious effects of salt stress.	Santos et al. (2018b)
Mesorhizobium sp.	*Pseudomonas* sp.	Chickpea	Improvement in symbiotic parameters and yield.	Kaur et al. (2015)
M. ciceri	*B. subtilis*	Chickpea	Decrease H_2O_2 concentrations and increased proline contents indicating an alleviation of adverse effects of salt stress and reduced the infection rate of rootrot in chickpea caused by *F. solani*	Egamberdieva et al. (2017b)
R. leguminosarum	*B. thuringeinsis*	Pea Lentil	Providing the highest and most consistent increase in nodule number, shoot weight, root weight and total biomass.	Mishra et al. (2009a)

Table 1. Co-inoculation of symbiotic nitrogen fixers and PGPR on legumes response (continued).

Symbiotic nitrogen fixers	Co-inoculating PGPR	leguminous plants	Legumes response to the co-inoculation	References
Bradyrhizobium sp.	*A. brasilense*	Peanut	Increasing the nodule number, nodule dry weight, shoot dry weight and pod yield dry weight in field experiments	Vicario et al. (2016)
S. meliloti	*Delftia* sp.	Alfalfa	Production of some molecules positively affects the microbe-plant association, increasing alfalfa shoot and root matter,	Morel et al. (2015)
Rhizobium sp.	*P. polymyxa*	Mung bean	25 percent nitrogenous and phosphatic fertilizer of the recommended dose can be substituted by seed co-inoculation with phosphate solubilizing bacteria and rhizobia without affecting the yield compared to 100 per cent RDF.	Tarafder et al. (2016)
R. phaseoli	*P. syringae* *P. fluorescens*	Mung bean	PGPR containing ACC deaminase and rhizobia enhanced plant growth, reducing the inhibitory effect of salinity.	Ahmad et al. (2011)
R. phaseoli	*B. megaterium*	Mung bean	Enhancing the mungbean growth, nodulation and yield.	Qureshi et al. (2011)
Bradyrhizobium sp.	*B. subtilis*	Mung bean	Improve nodulation and grain yield of the mung bean.	Tariq et al. (2012)
Rhizobium sp.	*Pseudomonas* sp.	Mung bean	Promoting growth of green gram plants.	Kumar et al. (2015)
Rhizobium sp.	*Pseudomonas* sp. *Azospirillum* sp.	Mung bean	Increasing grain yield and its components in mungbean plant	Hosseini et al. (2014)
B. diazoefficiens *B. japonicum*	*B. subtilis* *Staphylococcus* sp.	Soybean	Promoting N_2-fixation, nodule number, nodule and plant dry weight with both of the commercial bradyrhizobial strains	Prakamhang et al. (2015)

Many PGPR secrete phytohormones, such as cytokinins, gibberellins, auxin and ACC deaminase and influence plant growth and functions (Vessey, 2003). They are also capable of alleviating drought stress by promoting root growth and hampering stomatal conductance (Vessey, 2003; Gray and Smith, 2005). It has been also shown that presence of ACC deaminase enzyme in PGPR enhanced the nodulation of mung bean up on co-inoculation with *Bradyrhizobium* (Ahmad et al., 2011). The possibility that metabolites other than phytohormones, such as siderophores, phytoalexins, and flavonoids which enhanced *nod* gene expression, might enhance nodule formation has also been proposed (Daayf et al., 2012; Piccoli and Bottini, 2013), but this hypothesis has not been verified. Phytomicrobiome members synthesize and excrete a range of inter-organismal signal compounds that defend their host plant against pathogens and abiotic stresses: broad-spectrum antibiotics, lytic enzymes, organic acids and other metabolites, proteinaceous exotoxins and antimicrobial peptides (bacteriocins) (Smith et al., 2015). In addition, an increase in soil enzymatic activities (phosphatase, β-glucosidase and dehydrogenase) and of auxin production around PGPR inoculated roots could also be involved in the PGPR effect on nodulation-dependent (Bargaz et al., 2013; Krey et al., 2011; Turan et al., 2012). Some of the most intimate beneficial interactions between plants and microbes, and between different microbes take place on the surface of the root (Bolaños et al., 2004; Chebotar et al., 2001). In 2015, Prakamhang et al. proposed model for the effect of single inoculation of *B. japonicum* and co-inoculation of *B. japonicum* and PGPR on soybean was demonstrated in Figure 13.

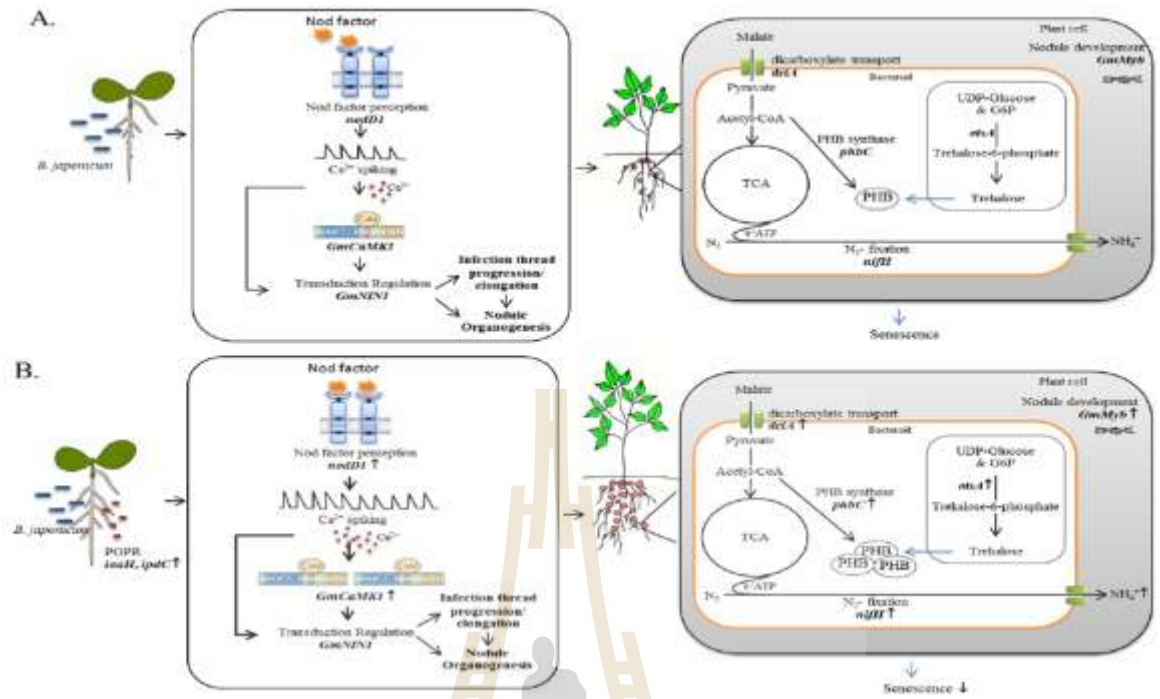

Figure 13. Proposed model for the effect of single inoculation of *B. japonicum* (A) and co-inoculation of *B. japonicum* and PGPR (B) on soybean (Prakamhang et al., 2015).

Co-inoculation of *B. japonicum* with PGPR, the up-regulation of PGPR mode of action related genes, *iaaH* and *ipdC* genes were up-regulated. Some genes involved in symbiosis were also performed up-regulated. Nod factor perception (*nodD1*) was induced and affected to the increasing of Ca_2^+ spiking and a set of transcriptional regulators and nodule inception (*GmNIN1*), resulting increased infection thread progression/elongation and nodule organogenesis. During nodule formation, a higher percentage of genes were related to primary metabolism, cell-wall modifications. The *GmMyb* gene expression was induced, this indicated that this gene was expressed specifically in developing nodules but not in the late mature nodules which might be involved in bacterial infection or in controlling the first steps of nodule development. The co-inoculation seems to influence the expression of *dctA* gene especially during the bacteroid differentiation into the fully

differentiated bacteroids. Moreover, the *dctA* gene could be consistent with an increased need for transport of C4-dicarboxylic acids by the nitrogen-fixing bacteroids. In trehalose biosynthetic pathways, trehalose 6-phosphate synthase encoded by *otsA* gene plays an important role as a protectant during periods of physiological stress. Gene expression analysis reported here demonstrated that the trehalose biosynthesis gene *otsA* was induced by co-inoculation with PGPR that caused an increase in trehalose accumulation levels. Furthermore, the accumulation of carbon source and PHB granules were also induced. The co-inoculation with *B. japonicum* and PGPR forced accumulation of PHB during symbiosis does not appear to have a negative effect on plant yield, PHB synthesis during symbiosis may not be the sole contributor to symbiotic performance. The accumulation of trehalose represented an increase in PHB accumulation and could be prolonged nodule senescence. Moreover, the expression level of *nifH* gene was strongly induced by the co-inoculation with *B. japonicum* and PGPR. Taken together indicated that the co-inoculation approachs are able to enhance the nodulation and nitogen fixation in soybean, resulting increased soybean yield (Prakamhang et al., 2015).

A variety of complex microbial interactions might be anticipated across the diversity of environments created in the plant rhizosphere. It is unlikely that any one rhizobacterium would be predominant and effective in all environments and hence mixtures of compatible strains might be more significant than a single bacterial species in promoting plant growth. Indeed, many of the positive effects of PGPR on plant growth seem to involve activation of plant response systems. Understanding the mechanisms and consequences of signal interactions occurring between the PGPR and host plants and development of methods to manipulate these interactions for increased plant growth, is an important challenge for this century.

CHAPTER III

METERIALS AND METHODS

3.1 Materials

3.1.1 Bacteria

3.1.1.1 *Bradyrhizobium diazoefficiens* strain USDA110 is commercially used in rhizobial inoculants production for soybean in Thailand was obtained from the Department of Agricultural (DOA), Bangkok, Thailand.

3.1.1.2 *B. diazoefficiens* strains CB1809 was supplied by Department of Agricultural Research (DAR), Myanmar.

3.1.1.3 *Bradyrhizobium* sp. SUTN9-2 was sourced from Applied Soil Microbiology Laboratory, School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

3.1.1.4 *Bacillus velezensis* S141 was kindly provided by Dr. Janpen Prakamhang, was sourced from Applied Soil Microbiology Laboratory, School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

Other bacterial strains used in this study are listed in Table 2.

Table 2. Bacterial strains used in this study.

Bacterial strains	Relevant genotype or description	Source or references
Bradyrhizobium diazoefficiens USDA110	Wild type	DOA
B. diazoefficiens CB1809	Wild type	DAR
Bradyrhizobium sp. SUTN9-2	Wild type	Noisangiam et al. (2012)
B. diazoefficiens GUS-tagged USDA110	marked with mTn*5SSgusA20* (pCAM120); Sm^r Sp^r	Aung et al. (2013)
Bradyrhizobium sp. dsRed-tagged SUTN9-2	marked with mTn*5SSgusA20* (pCAM120); Sm^r Sp^r	Piromyou et al. (2015)
Bacillus subtilis 168:ytsJ:gfp	*ytsJ::gfp::erm*^{r}	Kobe University
B. subtilis TSU077	*trpC2, epr::PrpsO-dam (Phle)*	Kobe University
B. subtilis TMO310	*trpC2 aprE::(spc lacI* P*spac-mazF)*	Morimoto et al. (2009)
B. subtilis TMO311	*trpC2 aprE::(kan lacI* P*spac-mazF)*	Morimoto et al. (2009)
B. subtilis YNB100	*trpc2 aprE::kan yhcT::(oriTLS20-F erm)* pLS20catΔ*oriT*	Miyano et al. (2018)
Bacillus velezensis S141	Wild type	Prakamhang et al. (2015)
B. velezensis S141:GFP	*tuf::gfp::phle*^{r}	This study
B. velezensis S141Δ*dhaS*	*dhaS* deletion, Δ*dhaS::erm*^{r}	This study

Table 2. Bacterial strains used in this study (continued).

Bacterial strains	Relevant genotype or description	Source or references
B. velezensis S141Δ*yhcX*	*yhcX* deletion, Δ*yhcX::kan*^{r}	This study
B. velezensis S141Δ*IPyAD*	*IPyAD* deletion, Δ*IPyAD::spm*^{r}	This study
B. velezensis S141Δ*ipt*	*IPT* deletion, Δ*ipt::phle*^{r}	This study
B. velezensis S141Δ*ipi*	*IPI* deletion, Δ*ipi::kan*^{r}	This study

3.1.2 Leguminous plants

3.1.2.1 Soybean [*Glycine max* (L.) Merrill] cultivar Chaing Mai 60 (CM60) is a recommended commercial line for use under Thai field condition.

3.1.2.2 Soybean [*Glycine max* (L.) Merrill] cultivar Enrei is a reference for Japanese domestic soybean cultivars.

3.1.2.3 Mung bean [*Vigna radiata* (L.) Wilczek] cultivar SUT4 was sourced from SUT farm, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

3.1.3 Primers

Primers or oligonucleotides used for combining the contigs in whole genome sequence of *B. velezensis* S141 were summarized in Appendix 6. Primers used for Sanger sequencing method to closed gaps between the contig in whole genome sequence were documented in Appendix 7. Primers used for confirm the transconjugants of *B. velezensis* S141 were documented in Appendix 8 and primer used for construction of bacterial strain in this study were summarized in Table 3.

3.1.4 Instruments

Autoclave:	Hiclave HA-3000MIV, Hirayama, Japan.
Automated electrophoresis:	Agilent 2100 Bioanalyzer, USA.
Balance:	Precisa 205A, Precisa Instruments, Switzerland.
	Precisa 3000C, Precisa Instruments, Switzerland.

Centrifuge machine:	Thermoscientific, Sorvall legend XTR centrifuge, USA.
	Eppendrof centrifuge 5810 R, Eppendrof, USA.
	Eppendrof minispin plus, wiswspin® feedback control digital timer function, Eppendrof, USA.
Deep freezer -80 ^{o}C:	Thermo Sciencetific, forma 900 series, USA.
Fluorometer:	Qubit 2.0 Fluorometer, Invitrogen, USA.
Freezer -20 ^{o}C:	Heto, HLLF 370, Denmark.
	Haier, China.
Gel Document set:	White/Ultraviolet Transilluminator GDS7500, UVP, USA.
	Digital Graphic Printer UP-D890, Sony, Japan.
Gel electrophoresis apparatus:	Mini Protean® 3 cell, BioRad, USA.
Heat Box:	HB1, Wealtee Corp., USA.
Next Generation Sequencer:	Illumina MiSeq, Illumina, Inc., San Diego, USA.
Incubator shaker:	Innova 4230 refrigerated incubator shaker, New Brunswick Scientific, USA.
	Innova® 42 incubator shaker series, USA.

Incubator:	Memmert, BE 500, WTB Binder BD115, Germany.
Laminar hood:	Thermo Scientific 1300 series A2, USA.
	BH 2000 Series ClassII Biological Safety Cabinets.
	BHA120 & BHA180, Clyde-Apac.
Microcentrifuge:	Mini spin plus, Eppendrof, USA.
	Eppendorf 54154, Eppendorf, Germany.
pH meter:	Ultra Basic pH meter, Denver Instruments, Germany.
PCR machine:	DNA Engine PTC 200 peltier Thermal cycler,
	MJ Research, USA.
Rotator:	Certomat TCC, B. Braun Biotech International, Germany.
Shaker:	Innova 2300 platform shaker, New Brunswick Scientific, UK.
	Certomat TC2, B. Braun Biotech International, Germany.
Sonicator:	Waken GE100 Ultrasonic processor, Japan.
Spectrophotometer:	Ultrospec 2000, Pharmacia biotech, UK.
Stirrer:	Variomag Electronicrührer Poly 15, Germany.
	Magnetic stirrer MSH300, USA.
Thermomixer:	Thermomixer compact, Eppendrof, USA.
Vortex:	Vortex-Genie 2 G506, Scientific Industries, USA.

3.2 Methods

3.2.1 Co-inoculation effect of S141 on soybean and mung bean-*Bradyrhizobium* symbiosis

3.2.1.1 Bacterial cells preparation

Bradyrhizobial strains (*B. diazoefficiens* USDA110, *B. diazoefficiens* CB1809 and *Bradyrhizobium* sp. SUTN9-2) were cultured in yeast extract mannitol (YEM) medium broth at 28°C for 6 days to obtain bacteria cell numbers of 10^6-10^8 CFU ml^{-1}. The early stationary phase of bradyrhizobial cultures were centrifuged at 4,000 rpm for 10 min and washed with sterilized 0.85% (w/v) NaCl to remove the excess media, and the cell pellet was resuspended in 0.85% (w/v) NaCl solution. Cultures were adjusted with 0.85% (w/v) NaCl solution to a final concentration of 10^6 CFU ml^{-1} prior to inoculate into plants**.**

Bacillus velezensis strain S141 was grown in Luria-Bertani (LB) medium broth for 48 h at 30°C to obtain bacteria cell numbers of 10^6-10^8 CFU ml^{-1}. The early stationary phase of S141 was centrifuged at 4,000 rpm for 10 min and washed with sterilized 0.85% (w/v) NaCl to remove the excess media, and the cell pellet was resuspended in 0.85% (w/v) NaCl solution. Culture was adjusted with 0.85% (w/v) NaCl solution to a final concentration of 10^6 CFU ml^{-1} prior to inoculate into plants.

3.2.1.2 Leonard's jar experiment

The soybean and mung bean cultivar tested in this experiments including: soybean cultivar Chiang Mai 60 is recommending lines for using under Thai field condition, Soybean cultivar Enrei is a reference for Japanese domestic soybean cultivars and mung bean cultivar SUT4.

Seeds of soybean and mung bean were cultivated in growth chambers using modified Leonard's jar assemblies (Blauenfeldt et al., 1994). Seeds of soybean and mung bean were surface sterilized by full immersion in 70% ethanol for 30 sec, followed

by rinsing five times with sterilized water. Unless otherwise, the seeds were sown in sterilized grade-2 vermiculite in modify Leonard's jars. The early stationary phase of S141 and *Bradyrhizobium* culture were centrifuged (4,000 rpm for 10 min) and washed with sterilized 0.85% (w/v) NaCl to remove the excess media, and the cell pellet were resuspended in 0.85% (w/v) NaCl solution. The Leonard's jar experiment was conducted to evaluate the co-inoculation effects of S141 with *B. diazoefficiens* on soybean and S141 with *Bradyrhizobium* sp. SUTN9-2 on mung bean. For the single inoculation, the seedlings were inoculated separately with 1 ml of 10^6 CFU ml^{-1} of S141 or *Bradyrhizobium* and mixed in a ratio of 1 : 1 for co-inoculation treatment. In the control treatment, the cell suspensions were replaced by distilled water. Plant growth conditions in laboratory were controlled using a 16-h-day/8-h-night cycle at 28°C/23°C, respectively. During the experiment, the plants were watered regularly with N-free nutrient solution (Zhang et al., 1996). The experiment was laid out with ten replicates for each treatment. Plants were grown for 45 days after inoculation (DAI), when they were then harvested for scoring the nodule number, nodule dry weight, shoot and root dry weight and detection of nitrogen-fixing activity by Acetylene Reduction Assay (ARA) were measured (Somasegaran and Hoben, 1994). Plant dry weights were recorded. The tissue were placed in an oven at 65°C for 4 days prior to weighing.

3.2.2 Co-inoculation effect of S141 and biological molecules secreted from S141 on soybean-*Bradyrhizobium* symbiosis

3.2.2.1 Bacterial cells and supernatant preparation

Bacterial cells preparation were followed as mention in 3.2.1.1. For supernatant preparation, culture medium for 48 h at 30°C were filtrated through 0.22 μm polytetrafluoroethylene (PTFE) membrane. The culture filtrate was used for single inoculation and co-inoculation treatments or stored at -80°C for further experimentation.

3.2.2.2 Leonard's jar experiment

The Leonard's jar experiment was followed as mention in 3.2.1.2. The experiment was conducted to evaluate the co-inoculation effects of *B. diazoefficiens* with cells or supernatant of S141 on soybean. For the single inoculation, the seedlings were inoculated separately with 1 ml of 10^6 CFU ml^{-1} of S141 or *B. diazoefficiens* or 1 ml of supernatant of S141 and mixed in a ratio of 1 : 1 for co-inoculation treatment. In the control treatment, the cell suspensions were replaced by distilled water. Plant growth conditions in laboratory were followed as mention in 3.2.1.2.

3.2.3 Effect of co-inoculation dose of *Bradyrhizobium* with biological molecules secreted from S141 on soybean-*Bradyrhizobium* symbiosis

Soybean cultivar CM60 (as recommended used for soybean cultivation in Thailand) seedlings co-inoculated with cells or supernatant of S141 at 10^6 cells/seed were mixed in a ratio of 1 : 1 with *B. diazoefficiens* at six inoculum doses; 10^3, 10^4, 10^5, 10^6, 10^7 and 10^8 CFU ml^{-1}. *B. diazoefficiens* cells or supernatant of S141 culture were used 1 ml $seed^{-1}$ in a full dose for single inoculation or in a half dose in co-inoculation treatment. In the control treatment, the cell suspensions were replaced by sterilized distilled water. The plants cultivated in growth chambers using modified Leonard's jar assemblies and harvested at 45 DAI. The nodule number, nodule dry weight, shoot and root dry weights and detection of nitrogen-fixing activity by Acetylene Reduction Assay (ARA) were measured (Somasegaran and Hoben, 1994).

3.2.4 Co-inoculation effect of S141 on *Bradyrhizobium* competition

The soybean and mung bean cultivars tested are recommended lines for using under Thai field condition. Seeds of soybean cultivar Chiang Mai 60 and mung bean cultivar SUT4 were cultivated in pot. Seeds were surface sterilized and gnotobiotically

germinated on wet tissue paper (Somasegaran and Hoben, 1994). Bradyrhizobial strains (*B. diazoefficiens* GUS-tagged USDA110, and *Bradyrhizobium* sp. dsRed-tagged SUTN9-2) were cultured in yeast extract mannitol (YEM) medium broth supplemented with antibiotics: 100 μg ml^{-1} streptomycin and 100 μg ml^{-1} spectinomycin at 28°C for 6 days to obtain bacteria cell numbers of 10^6-10^8 CFU ml^{-1}.

The early stationary phase of S141 and bradyrhizobial strains (*B. diazoefficiens* GUS-tagged USDA110, and *Bradyrhizobium* sp. dsRed-tagged SUTN9-2) cultures were centrifuged (4,000 rpm for 5 min) and washed with sterilized 0.85% (w/v) NaCl to remove the excess media, and the cell pellet was resuspended in 0.85% (w/v) NaCl solution. The pots experiment with local soil collected from fields on the basis of cropping history were conducted to evaluate the co-inoculation effects of S141 with *B. diazoefficiens* GUS-tagged USDA110, and *Bradyrhizobium* sp. dsRed-tagged SUTN9-2 on soybean and mung bean, respectively. The chemical characteristics of the soil are listed in Appendix 9. For the single inoculation, the seedlings were inoculated separately with 1 ml of 10^6 CFU ml^{-1} of S141 or *Bradyrhizobium* and mixed in a ratio of 1 : 1 for co-inoculation treatment. In the control treatment, the cell suspensions were replaced by distilled water. Plants were grown in greenhouse condition. During the experiment, the plants were watered regularly with tap water. The experiment was laid out with five replicates for each treatment. Plants were sampled at 45 DAI, the nodule formation of gus-marked *B. diazoefficiens* GUS-tagged USDA110 strains were checked on soybean hosts by gus-staining method. Staining for GUS activity, histochemical staining of plant material was performed as described (Jefferson, 1987) with the following modifications. Intact or hard-cut sections of nodules were incubated, without prior fixation, in 1 mM 5-bromo-4-cloro-3-indolyl-β-D-glucuronic acid (X-gluc), 50 mM Sodium phosphate buffer (pH 7.0), 0.2 % (w/v) sodium dodecyl sulfate and 20 % (v/v) methanol. The explants were subjected to vacuum infiltration for 10 min before incubation of them overnight at 37°C. The nodulation competitiveness of

inoculated *Bradyrhizobium* sp. dsRed-tagged SUTN9-2 strain was detected on mung bean host by fluorescence compound microscope and the nodule number, nodule and plant dry weight were recorded.

3.2.5 Extra/intracellular proteome of S141 or USDA110

3.2.5.1 Bacterial cells preparation

B. diazoefficiens strain USDA110 was grown in yeast extract mannitol broth at 28°C for 6 days. S141 was grown in Luria-Bertani (LB) broth for 48 h at 28°C. The early stationary phase of S141 was inoculated with 1 ml of 10x of supernatant of *B. diazoefficiens* USDA110 (cell free) and for the *B. diazoefficiens* USDA110 was inoculated with 1 ml of 10x of supernatant of S141 (cell free). In the control treatment, the inoculants were replaced by distilled water. Cells culture were grown for 24 and 48 h after inoculation, then harvested cells culture by centrifuge at 4,000 rpm at 4°C for 10-15 min and immediately frozen in liquid nitrogen and store at -80°C until extraction.

3.2.5.2 Whole-cell protein extraction

Three milliliter of bacterial culture were centrifuged at 4,000 rpm, at 4°C and cells were added with protein extraction buffer containing 20 mM Tris HCl pH 8.0, 1.0 % Triton X-100, 400 μg ml^{-1} lysozyme and 40 μg ml^{-1} DNase (Appendix 10), incubated at 37°C for 30 min. Bacterial cells were sonicated (100W, 40-60 second/time) on ice for breakdown cell several times until completed (avoid the bubble), and then centrifuged at 4,000 rpm at 4°C for 10-15 min to collect supernatant and stored in ultra-freezer (-80°C) until analyses.

For total protein extraction, the solubilized proteins were quantified using the Bradford protein assay (Bradford, 1976) with a bovine serum albumin (BSA) standard curve (Rodrigues et al., 2012).

3.2.5.3 One-dimensional gel electrophoresis and visualization

For separating the size of proteins, 35 μg of extracted protein were separated using sodium dodesyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The one dimension electrophoresis was performed in 5 and 12% polyacrylamide gel electrophoresis. The components of 5% polyacrylamide gel including: 166 μl ml^{-1} of 30% acrylamide solution (29:1; acrylamide:Bis-acrylamide); 250 μl ml^{-1} of 0.5 M Tris pH 6.8; 10 μl ml^{-1} of 10% SDS; 10 μl ml^{-1} of 10% APS and 1 μl ml^{-1} of TEMED (Appendix 11), and 12% polyacrylamide gel containing 400 μl ml^{-1} of 30% acrylamide solution (29:1; acrylamide:Bis-acrylamide); 250 μl ml^{-1} of 1.5 M Tris pH 8.6; 10 μl ml^{-1} of 10% sodium dodesyl sulfate (SDS); 10 μl ml^{-1} of 10% ammonium persulfate (APS) and 0.6 μl ml^{-1} of TEMED (Appendix 12). The electrophoresis was carried out for 90 minutes at 100V. Following electrophoresis, gels were stained with staining solution containing 1 g L^{-1} of Coomassie Brilliant Blue G-250, 400 ml L^{-1} of methanol and 100 ml L^{-1} of glacial acetic acid (Appendix 13), and then destained with destain solution containing 400 ml L^{-1} of methanol and 100 ml L^{-1} of glacial acetic acid (Appendix 14).

3.2.5.4 Protein identification

The protein of interested bands were extracted and digested according to Yuan et al. (2006). Nano-liquid chromatography electrospray ionization quadrupole-time of flight MS (nano-LC–ESI–MSMS) measurements were performed on a Bruker Ultraflex III TOF/TOF-MS (Bruker Daltonics, Bremen, Germany) equipped with a 337 nm wavelength nitrogen laser working in reflectron mode. Peptide mass fingerprinting (PMF) were performed using MASCOT v2.2.06 (Matrix Science, http://www.matrixscience.com). The MASCOT search engine used MS data to identify the proteins from primary sequence databases as described previously by Yuan et al. (2006). For protein identification by PMF, peptide masses were searched from the publicly available NCBInr protein databases. Among the proteins identified in the NCBInr database, proteins S141 were selected as the

best hits from the lists of homologue proteins. All MS/MS ion database searches were performed using MASCOT v2.2.06 on the freely accessible internet website (http://www.matrixscience.com) against protein databases of NCBInr (v20100430, 10 927 723 sequences; 372 0794 783 residues of all bacteria). The homologue proteins from databases of NCBInr were used to design primers.

3.2.6 Whole genome sequence of *B. velezensis* S141

Whole genome sequence of S141 was aimed to determine the genetic background of its soybean growth-promoting capacity involving symbiotic N_2 fixation with *B. diazoefficiens* USDA110. S141 genomic DNA was prepared using the Wizard Genomic DNA purification kit (Promega, USA), and a genomic DNA library was constructed using the NEBNext ultra DNA library prep kit for Illumina (New England BioLabs Inc., USA) according to the manufacturer's protocol. S141 genome sequencing was performed on an Illumina MiSeq platform. After quality filtering, high quality paired-end reads (2X300 bp) were assembled using CLC Genomics Workbench version 10.0.1 (Qiagen, USA). Total read coverage was 300-fold. After trimmed (paired) assembly using the CLC Genomics Workbench, a draft genome with number of scaffolds was obtained. Gaps between scaffolds were closed using PCR and Sanger sequencing to obtain the complete sequence of the circular chromosome. The genome sequence was automatically annotated using the Microbial Genome Annotation Pipeline (MiGAP) (Sugawara et al., 2009). Based on 16S rRNA gene analysis using BLAST searches, the highest sequence identities were identified. Average nucleotide identity analysis using the Map Reads to Reference tool were indicated the highest similarity species.

3.2.7 Colonization of S141 on soybean-*Bradyrhizobium* symbiosis

3.2.7.1 Construction of the green fluorescent protein (GFP)

The green fluorescent protein (GFP) and phleomycin resistance gene was activated in S141 by insertion to *tuf* promoter gene, as follows. Two DNA fragments, each of which corresponded to upstream and downstream regions of *tuf* promoter, were amplified by PCR using S141 chromosomal DNA as a template with primers tuf-uF/tuf-uR for the upstream fragment and tuf-dF/tuf-dR for the downstream fragment (Table 3). Another DNA fragment containing the phleomycin resistance gene of *B. subtilis* strain TSU077 (Table 2) was amplified using primers phl-F/phl-R (Table 3) and the green fluorescent protein of strain 168:ytsJ:gfp (Table 2) was amplified using primers gfp-F/gfp-R (Table 3). The four fragments were ligated by recombinant PCR using primers tuf-uF/tuf-dR to sandwich the green fluorescent protein and phleomycin resistance gene between the upstream and downstream regions of *tuf* promoter. The recombinant PCR fragment was transformed into S141 conferring phleomycin resistance and yielding the new strain, S141:GFP (*tuf::gfp::phle*^{r}), which was used as the GFP-tagged strain for the colonization on soybean-*Bradyrhizobium* symbiosis in this study.

3.2.7.2 Leonard's jar experiment

The Leonard's jar experiment was followed as mention in 3.2.1.2. The experiment was conducted to reveal the colonization of S141 on soybean-*Bradyrhizobium* symbiosis. S141:GFP was grown in Luria-Bertani (LB) medium broth supplemented with phleomycin 8 μg ml^{-1} at 30°C for 48 h. For the single inoculation, the seedlings were inoculated separately with 1 ml of 10^{6} CFU ml^{-1} of S141::GFP or *B. diazoefficiens* and mixed in a ratio of 1 : 1 for co-inoculation treatment. In the control treatment, the cell suspensions were replaced by distilled water. Plant growth conditions in laboratory were controlled using a 16-h-day/8-h-night cycle at 28°C/23°C, respectively. During the

experiment, the plants were watered regularly with N-free nutrient solution (Zhang et al., 1996). The experiment was laid out with five replicates for each treatment. Plants were grown for 15 DAI, when they were then harvested for checked the colonization on soybean hosts by fluorescence compound microscope.

Table 3. Primers used for construction of bacterial strain in this study.

Primer	**Sequences (5′→3′)**
tuf-uF	CGTTGTATTTGTCAACAAAATGGACAAAATC
tuf-uR	**GTACCCTCGACTCTAGATCACC**TTACTCAGTGATTGTAGAAACAACGCCTGAAC
tuf-dF	**GCAATCGCCCTAATATATG**TGTTTCTACAATCACTGAGTAA
tuf-dR	GCGCTTAAGAACATCACGAGGATCTTTC
gfp-F	GGTGATCTAGAGTCGAGGGTAC
gfp-R	**CCGAATAGCAAAAAACTGG**CGGGCTGCACTAGTGCTCG
phl-F	CCAGTTTTTTGCTATTCGG
phl-R	CATATATTAGGGCGATTGC
dhaS_uF	TACATCCTGAGAAATGCAGATGGTC
dhaS_uF_nest	CGCGTAAATTTTTCTTCTCAGCATC
dhaS_uR	**ATTTTAAGATACTGCACTATCAACACACTC**CCACCTTGAAGATACGGTGACAAAC
dhaS_dF	**GAAAGTTACACGTTACTAAAGGGAATGTAG**GCGTCATTTAACACGATCGTATGGT
dhaS_dR_nest	TAGGAAAACAGCTTGTCGATAAACG
dhaS_dR	CCCTTTTAAAAGGCGTGTTTTTTTC

cont.

Table 3. Primers used for construction of bacterial strain in this study (continued).

Primer	**Sequences (5′→3′)**
Erm_F	GAGTGTGTTGATAGTGCAGTATCTTAAAATTTTG
Erm_R	CTACATTCCCTTTAGTAACGTGTAACTTTCC
yhcX_uF	ATGATAGCCTTTCACTTTGTAATGG
yhcX_uF_nest	TCAGCATATGACTGTCCTCAAATAA
yhcX_uR	**TTTACTGGATGAATTGTTTTAGTACCTAGA**TTTCATTTCATATTGAATGACGCAG
yhcX_dF	**CCAATTCACTGTTCCTTGCATTCTAAAACC**AAGATTTACTCCTTCGAGGAATTTGC
yhcX_dR_nest	ATAGTTCGGTGTCTGAAGTCGTCAC
yhcX_dR	TAAGCGGTGTCAATCTTTTTTTGTC
Kan_F	TCTAGGTACTAAAACAATTCATCCAG
Kan_R	GGTTTTAGAATGCAAGGAACAGTGAATTGG
IPyAD_uF	ATTAAGAGATGAATCGGCTTGATGC
IPyAD_uF_nest	CTCTTTCTCCTATGCCGTTAATGGC
IPyAD_uR	**CTGTTCAATAAAGCTGACCGTTAGCGTTTA**GGAAAAATTATTGAAACGCAAATTCC
IPyAD_dF	**ACGCTTTATTACTTTAATTTAGTGAAGCTT**GATTTCAGCAGGATCAATATCGATATG

cont.

Table 3. Primers used for construction of bacterial strain in this study (continued).

Primer	**Sequences (5′→3′)**
IPyAD_dR_nest	TAATATGCCATTGTTGGATACACGG
IPyAD_dR	AACGAGTGGAAAGATATCACACAGAAC
Spm_F	TAAACGCTAACGGTCAGCTTTATTG
Spm_R	AAGCTTCACTAAATTAAAGTAATAAAGCGTTCTC
IPT_uF	CCCTTGGCAAAACATCACTT
IPT_uF_nest	TGATGAAATGCTGCTTGGAG
IPT_uR	**CCGAATAGCAAAAAACTGG**GATTTCCGCATTCAGCATTT
IPT_dF	**GCAATCGCCCTAATATATG**CAGGCGATCGGCTATAAAGA
IPT_dR_nest	AGCCGATTTCGTCTTCTTCA
IPT_dR	AACCAAGGTTGCAGGAAATG
Phl_F	CCAGTTTTTTGCTATTCGG
Phl_R	CATATATTAGGGCGATTGC
IPI_uF	ACTTGATGAAGCGCCTCTGT
IPI_uF_nest	TCAACAACCATCCCCATCTT

cont.

Table 3. Primers used for construction of bacterial strain in this study (continued).

Primer	**Sequences (5′→3′)**
IPI_uR	**CTGGATGAATTGTTTTAGTACCTAGA**CAAAAGACGCCTTTGGTCAT
IPI_dF	**CCAATTCACTGTTCCTTGCATTCTAAAACC**TAAAAATCGGCGAACTGCTT
IPI_dR_nest	TCCGGCAGTTCATGATACAA
IPI_dR	GGAAATGAAGCGGATTCAAA
Kan_F	TCTAGGTACTAAAACAATTCATCCAG
Kan_R	GGTTTTAGAATGCAAGGAACAGTGAATTGG

3.2.8 Co-inoculation effect of S141 and plant growth hormone on soybean-*Bradyrhizobium* symbiosis

Bacterial cells preparation was followed as mention in 3.2.1.1. The Leonard's jar experiments were followed as mention in 3.2.1.2. The experiment was conducted to evaluate the co-inoculation effects of *B. diazoefficiens* with S141 or plant growth hormone on soybean. For the single inoculation, the seedlings were inoculated separately with 1 ml of 10^6 CFU ml^{-1} of S141 or *B. diazoefficiens* and mixed in a ratio of 1 : 1 for co-inoculation treatment. In the control treatment, the cell suspensions were replaced by distilled water. During the experiment, the plants were watered regularly with N-free nutrient solution (Zhang et al., 1996). When necessary, the N-free nutrient solution was supplemented with appropriate plant growth hoemone: 1 μg ml^{-1} cytokinin; 6-Benzylaminopurine (Sigma-Aldrich) (Hussain and Hasnain, 2009) and 10 μg ml^{-1} auxin; Indole-3-Acetic Acid (Sigma-Aldrich) (Gadallah, 2000; Spaepen et al., 2007). Plant growth conditions in laboratory were followed as mention in 3.2.1.2.

3.2.9 Gene disruption related to plant growth homone biosynthesis pathway in S141

3.2.9.1 Construction of gene related IAA biosynthesis pathway

The putative gene related to IAA biosynthesis pathway including: *dhaS*, *yhcX* and *IPyAD* were disrupted. In order to replace the respective wild-type genes, appropriate gene cassettes consisting of the antibiotics resistance determinant flanked by the respective gene sequences were constructed.

The *dhaS*, *yhcX* and *IPyAD* genes of S141 were inactivated by replacement with an erythromycin, kanamycin and spectinomycin resistance genes, respectively. Two DNA fragments, each of which corresponded to upstream and downstream regions of *dhaS*, *yhcX* and *IPyAD* were amplified by PCR using S141

chromosomal DNA as a template with primers dhaS_uF/dhaS_uR, yhcX_uF/yhcX_uR and IPyAD_uF/IPyAD_uR, respectively for the upstream fragment and dhaS_dF/dhaS_dR, yhcX_dF/yhcX_dR and IPyAD_dF/IPyAD_dR, respectively for the downstream fragment (Table 3). Another DNA fragment containing the erythromycin resistance gene of *B. subtilis* strain YNB100, kanamycin resistance gene of *B. subtilis* strain TMO311 and spectinomycin resistance gene of *B. subtilis* strain TMO310 (Table 2) were amplified using primers Erm_F/Erm_R, Kan_F/Kan_R and Spm_F/Spm_R, respectively (Table 3). The three fragments (upstream, downstream and antibiotic resistance gene) of each gene: *dhaS*, *yhcX* and *IPyAD* were ligated by recombinant PCR using primers dhaS_uF_nest/dhaS_dR_nest, yhcX_uF_nest/yhcX_dR_nest and IPyAD_uF_nest/IPyAD _dR_nest, respectively to sandwich each antibiotic resistance gene between the upstream and downstream regions of *dhaS*, *yhcX* and *IPyAD*. The recombinant PCR fragment was transformed into S141 conferring erythromycin, kanamycin and spectinomycin resistance and yielding the new strain, S141Δ*dhaS* (*dhaS::erm*^{r}), S141Δ*yhcX* (*yhcX::kan*^{r}) and S141Δ*IPyAD* (*IPyAD::spm*^{r}), which were used as the mutant strains for the IAA biosynthesis pathway in this study.

3.2.9.2 IAA production Assay

The IAA production of S141 and S141 IAA related mutant strains were grown in LB medium broth supplement with 0.1% tryptophan for 48 h at 30°C. After incubation, the IAA production were colorimetrically determined as described by Costacurta et al., (1998). Pure indole-3-acetic acid (Sigma, USA) was used as standard.

3.2.9.3 Construction of gene related cytokinin biosynthesis pathway

In S141, the gene related to cytokinin biosynthesis pathway including: *IPT*-gene coding for isopentenyl transferase and *IPI*-gene coding for isopentenyl isomerase were disrupted. In order to replace the respective wild-type genes, appropriate gene

cassettes consisting of the antibiotics resistance determinant flanked by the respective gene sequences were constructed.

The *IPT* and *IPI* genes of S141 were inactivated by replacement with a phleomycin and kanamycin resistance genes, respectively. Two DNA fragments, each of which corresponded to upstream and downstream regions of *IPT* and *IPI* were amplified by PCR using S141 chromosomal DNA as a template with primers IPT_uF/IPT_uR and IPI_uF/IPI_uR, respectively for the upstream fragment and IPT_dF/IPT_dR and IPI_dF/IPI_dR, respectively for the downstream fragment (Table 3). Another DNA fragment containing the phleomycin resistance gene of *B. subtilis* strain TSU007 and kanamycin resistance gene of *B. subtilis* strain TMO311 (Table 2) were amplified using primers Phl_F/Phl_R and Kan_F/Kan_R, respectively (Table 3). The three fragments (upstream, downstream and antibiotic resistance gene) of each gene: *IPT* and *IPI* were ligated by recombinant PCR using primers IPT_uF_nest/ IPT_dR_nest and IPI_uF_nest/IPI_dR_nest, respectively to sandwich each antibiotic resistance gene between the upstream and downstream regions of *IPT* and *IPI*. The recombinant PCR fragment was transformed into S141 conferring phleomycin and kanamycin resistance and yielding the new strain, S141Δ*IPT* (*IPT::erm*^{r}) and S141Δ*IPI* (*IPI::kan*^{r}), which were used as the mutant strains for the cytokinin biosynthesis pathway in this study.

3.2.9.4 Leonard's jar experiment

The Leonard's jar experiments were followed as mention in 3.2.1.2. The experiment was conducted to evaluate the co-inoculation effect of S141 and its mutant strains on soybean-*Bradyrhizobium* symbiosis. S141 mutant strains were grown on LB medium borth at 30 °C. When necessary, the medium was supplemented with appropriate antibiotics: 1 μg ml^{-1} erythromycin, 10 μg ml^{-1} kanamycin, 100 μg ml^{-1} spectinomycin and 8 μg ml^{-1} phleomycin. For the single inoculation, the seedlings were inoculated separately with 1 ml of 10^6 CFU ml^{-1} of each strains and mixed in a ratio of 1 : 1 for co-inoculation

treatment. In the control treatment, the cell suspension were replaced by distilled water. Plant growth conditions in laboratory were controlled using a 16-h-day/8-h-night cycle at 28°C/23°C, respectively. During the experiment, the plants were watered regularly with N-free nutrient solution (Zhang et al., 1996). The experiment was laid out with five replicates for each treatment. Plants were grown for 30 DAI, when they were then harvested for scoring the nodule number, nodule dry weight, shoot and root dry weight and detection of nitrogen-fixing activity by Acetylene Reduction Assay (ARA) were measured (Somasegaran and Hoben, 1994). Plant dry weights were recorded. The tissue were placed in an oven at 65°C for 4 days prior to weighing.

3.2.10 The statistical analysis

Data from each experiment were first submitted to tests of normality and homogeneity of variances for each variable and then to analysis of variance (ANOVA). When confirming a statistically significant value in the F-test ($p \leq 0.05$), a post hoc test (Duncan's multiple-range test at $p \leq 0.05$) was used as a multiple comparison procedure (Duncan, 1955) by SPSS® software for WINDOWS™, Version 14.0 (SPSS, Chicago, IL).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 *Bacillus velezensis* strain S141 as a plant growth promoting rhizobacterium

S141 strain is a PGPR isolated from soybean rhizosphere soil in Thailand by Prakamhang et al. (2015), and is closely related to *B. subtilis* GB03 based on 16S rRNA gene sequencing. Strain S141 was isolated using LG N-free medium in order to obtain the most abundant root adhering bacteria. Enhancement of nitrogen fixation is the ultimate goal of any Rhizobium-legume symbiosis; therefore, all the experiments in this study were carried out in N-free conditions, making nitrogen the limiting nutrient for plant growth and PGPR might have more chances to persist and provide some nitrogen to plants than non-nitrogen-fixing bacteria (Piromyou et al., 2011). In 2015, Prakamhang et al. reported that S141 is capable of increasing soybean growth and yields via co-inoculation with *Bradyrhizobium diazoefficiens* strains USDA110 and THA6 in field experiments. We hypothesized that the increasing in nodulation will promotes better root growth, nodule occupancy by the rhizobial strain and subsequent nitrogen fixation resulted in the increased plant growth parameters. The overall nitrogen-fixing efficiency and efficacy of the soybean and mung bean-bradyrhizobial symbiosis could be enhanced via co-inoculation with cells or supernatant of S141 strain. The biological molecules harbouring the interactions between PGPR and *Bradyrhizobium* co-inoculated in soybean and mung bean-bradyrhizobial symbiosis, resulting in better nodulation, nitrogen fixation, nodule morphology, growth and

yield. Therefore, the aim of this study was to examine some determinant derived from PGPR affect on *Bradyrhizobium* co-inoculation that can enhance symbiosis.

4.2 Co-inoculation of S141 enhanced N_2 fixation efficiency in soybean and mung bean-*Bradyrhizobium* symbiosis

Bacillus velezensis S141 was tested for plant growth promotion using grown soybean and mung bean in Leonard's jar trails. S141 showed significantly capable of promoting one or more plant parameters including nitrogen-fixing efficiency, nodule dry weight, root, shoot and total plant dry weight when co-inoculated with strains of *B. diazoefficiens* USDA110 and *Bradyrhizobium* sp. SUTN9-2 over the single inoculation of *Bradyrhizobium* on soybean and mung bean at 45 and 30 DAI, respectively ($P<0.05$). Variations in plant growth parameters of soybean [*Glycine max* (L.) Merrill] cultivar Chaing Mai 60 (CM60) at 45 DAI as consequent of single inoculation and co-inoculation is presented in Figures 14 and 15. The co-inoculation of S141 with USDA110 showed the highest nitrogen-fixing efficiency, nodule dry weight, root dry weight and total plant dry weight, which higher than single inoculation for 55.75%, 29.41%, 40.52% and 22.88%, respectively.

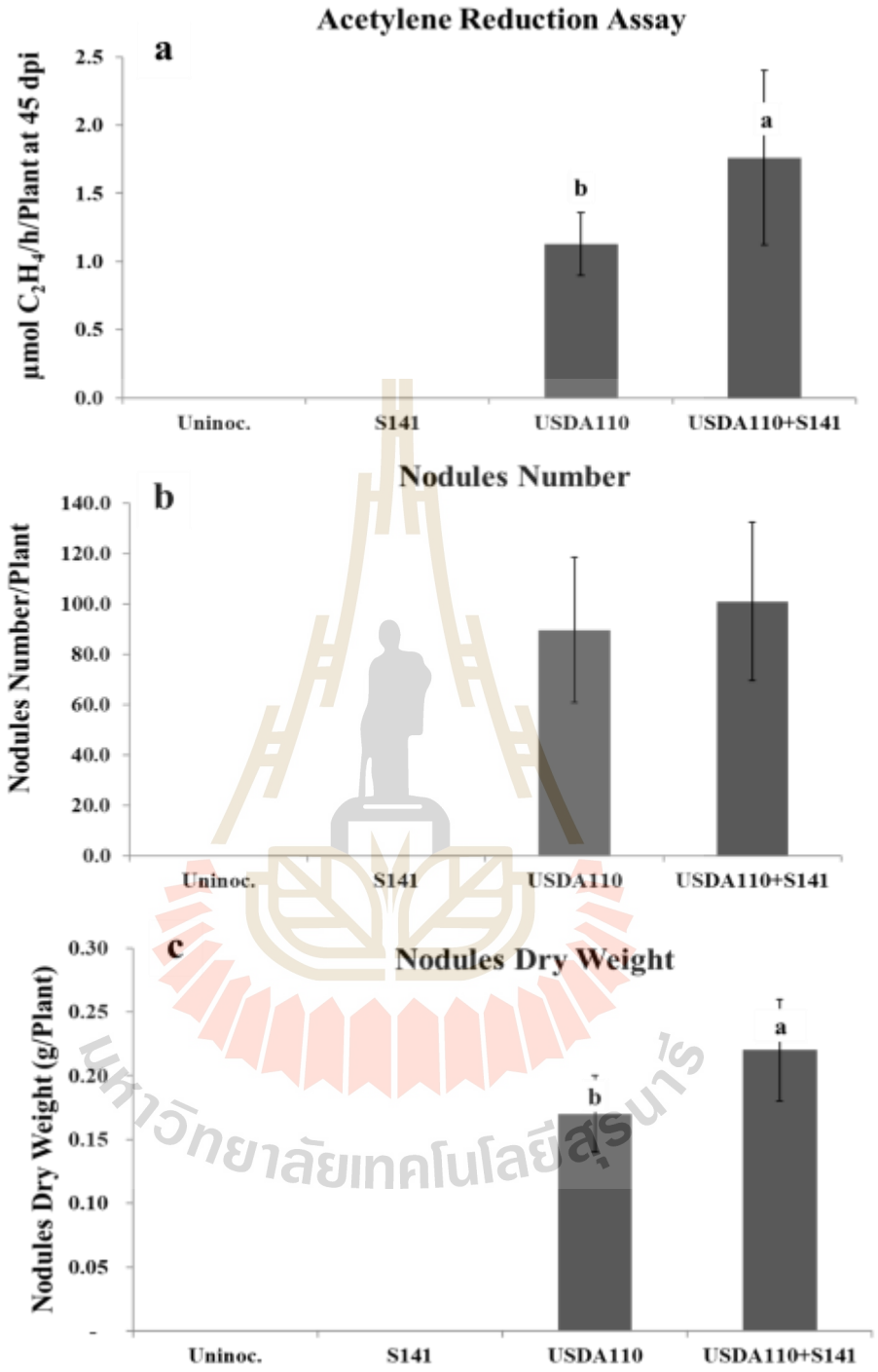

Figure 14. Plant growth and symbiotic parameters of soybean cultivar Chaing Mai 60 by co-inoculation with S141 and USDA110 at 45 DAI: (a) nitrogenase activity was determined using the acetylene reduction assay, (b) nodule numbers per plant and (c) nodule dry weight, (e) root dry weight. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=8).

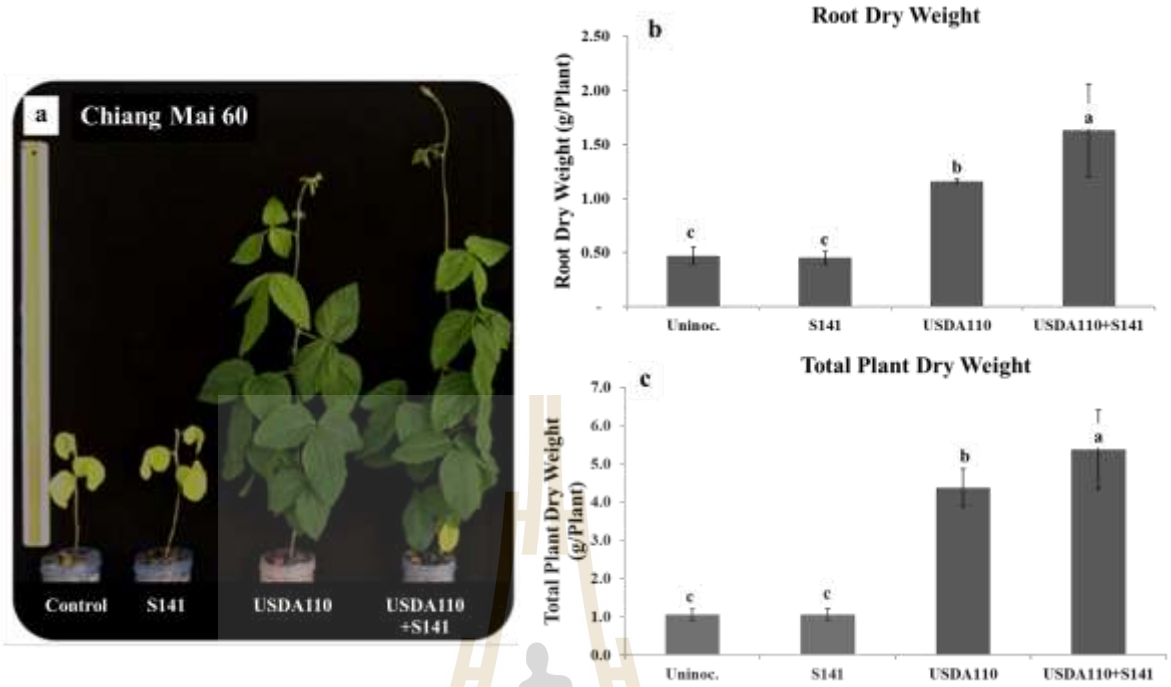

Figure 15. Plant growth and symbiotic parameters of soybean cultivar Chaing Mai 60 by co-inoculation with S141 and USDA110 at 45 DAI: (a) soybean cultivar Chaing Mai 60 growth under Leonard's jar experiments, (b) root dry weight and (c) total plant dry weight. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=8).

Interestingly, co-inoculation of S141 with USDA110 did not showed significantly different between single inoculation and co-inoculation of nodule number per plant, but nodule dry weight showed significantly different that sizes of the nodule were indicated the difference. The nodule could be classified into different sizes by diameter. There are 4 groups of nodule size as categorized (Table 4).

Table 4. Size of nodule

Size	Diameter of nodule (mm)
Very large (VL)	more than 4
Large (L)	3-4
Medium (M)	2-3
Small (S)	Less than 2

The nodule sizes separation demonstrated significantly different between single inoculation and co-inoculation (Figure 16). The co-inoculation of S141 with USDA110 showed the highest number of VL size nodule and L size nodule when compared with single inoculation treatment whereas, the single inoculation of USDA110 showed the highest number of S size nodule compared with co-inoculation treatment.

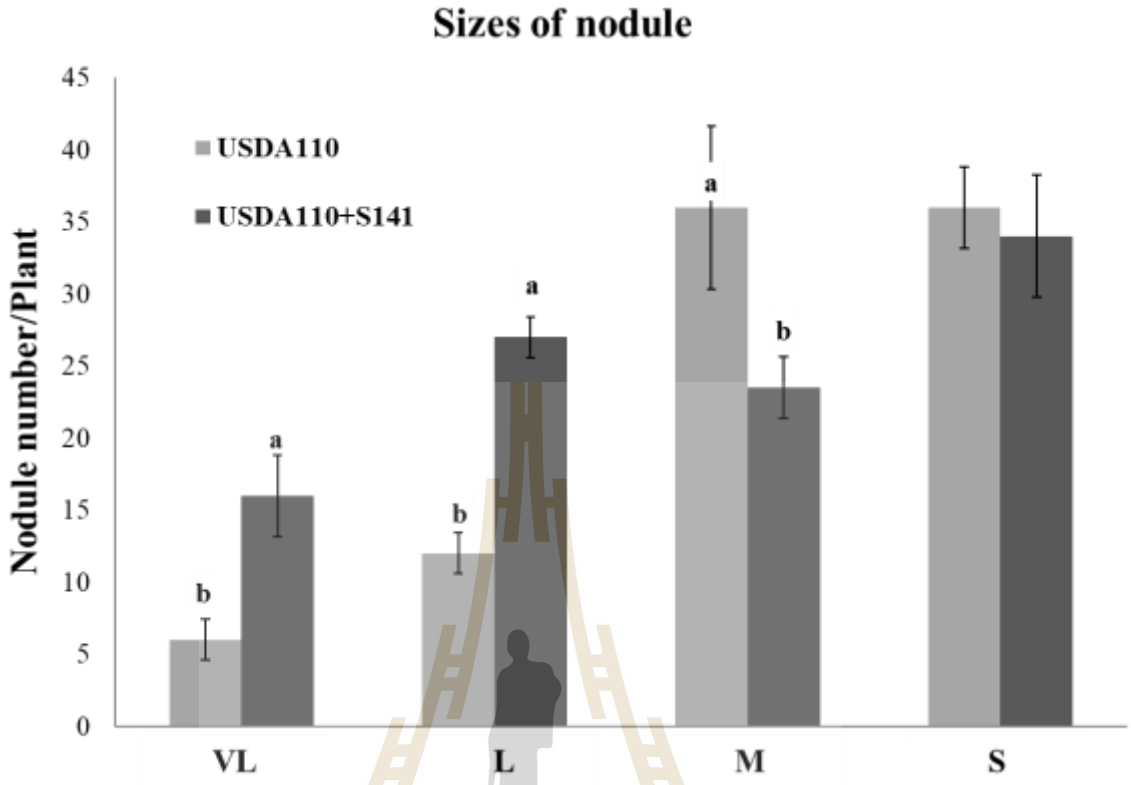

Figure 16. The nodule sizes separation of soybean cultivar Chaing Mai 60 by co-inoculation with S141 and USDA110 at 45 DAI. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=8).

The variations in plant growth and symbiotic parameters of soybean cultivar Enrei at 45 DAI as a consequent of single inoculation and co-inoculation was depicted in Figure 17. The nitrogen-fixing efficiency and nodule dry weight showed the highest when drew a comparison between co-inoculation of S141 with USDA110, single inoculation and control. In addition, co-inoculation of S141 with USDA110 also did not show significantly different between single inoculation and co-inoculation of nodule number per plant, but nodule dry weight showed significantly different, indicated that sizes of the nodule are the difference consistent with the result of CM60**.**

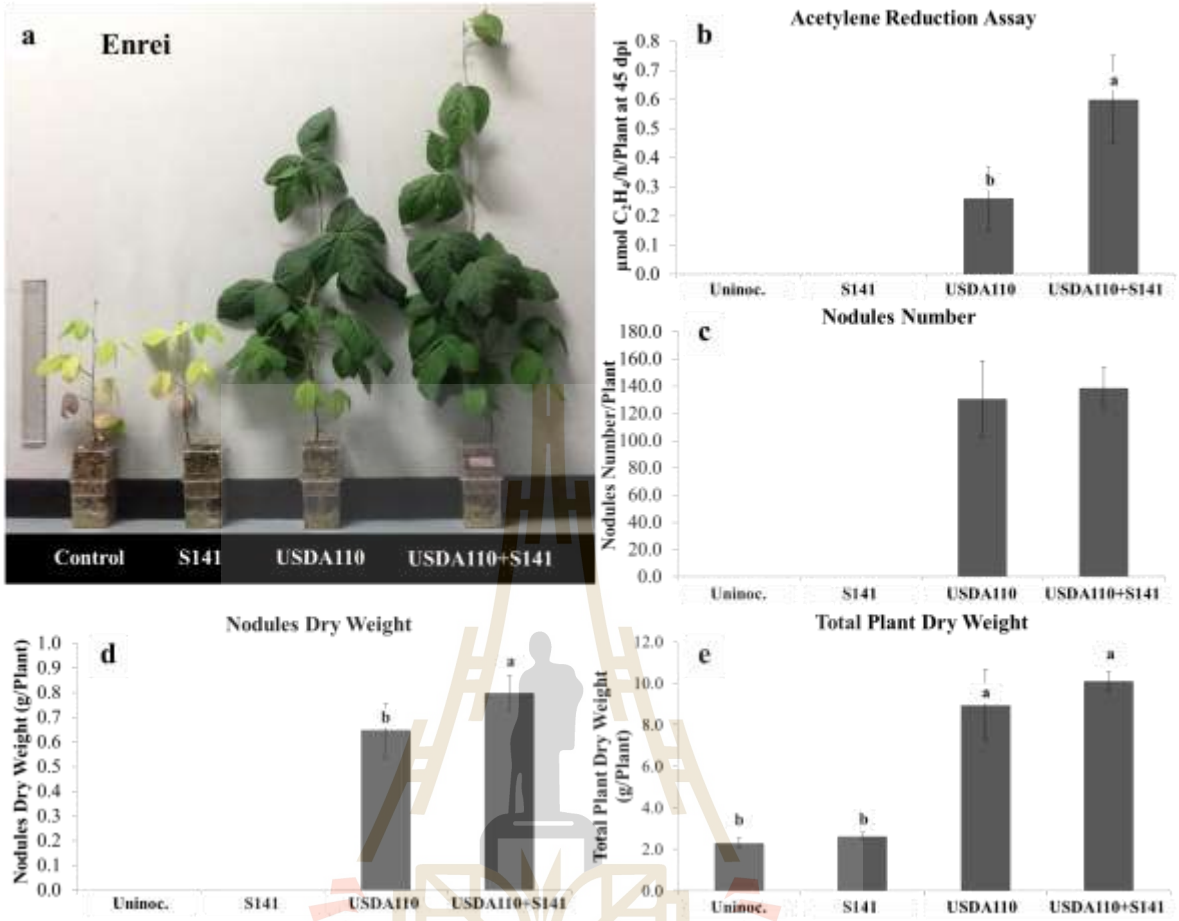

Figure 17. Plant growth and symbiotic parameters of soybean cultivar Enrei by co-inoculation with S141 and USDA110 at 45 DAI: (a) soybean cultivar Enrei growth under Leonard's jar experiments, (b) nitrogenase activity was determined using the acetylene reduction assay, (c) nodule numbers per plant, (d) nodule dry weight and (f) total plant dry weight. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=6).

The nodule sizes separation demonstrated significantly different between single inoculation and co-inoculation as indicated in Figure 18. The co-inoculation of S141 with USDA110 showed the highest number of VL size nodule, which increased to 45.67% when compared with single inoculation treatment.

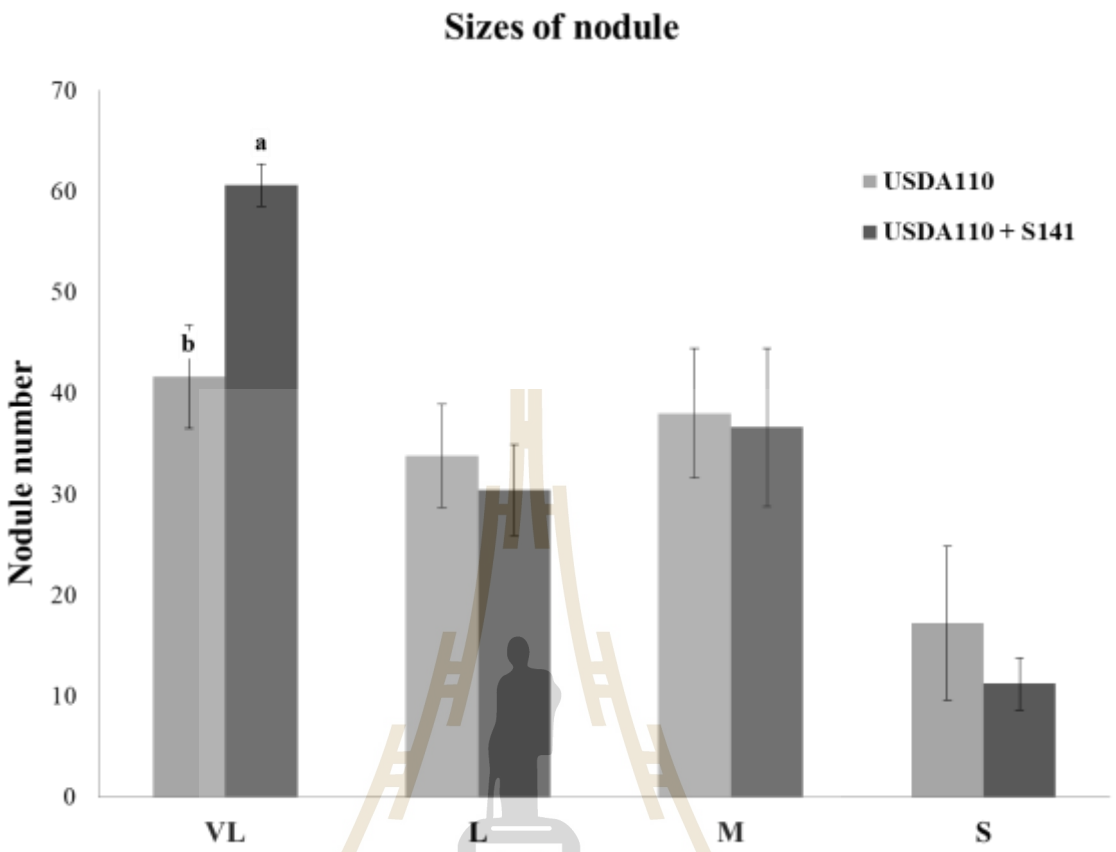

Figure 18. The nodule sizes separation of soybean cultivar Enrei by co-inoculation with S141 and USDA110 at 45 DAI. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=6).

The co-inoculation of *B. velezensis* S141 and *Bradyrhizobium* sp. SUTN9-2 was tested for their plant growth promotion using grown mung bean [*Vigna radiata* (L.) Wilczek] cultivar SUT4 in Leonard's jar experiments. S141 performed significantly capable of promoting plant growth and symbiotic parameters including nitrogen-fixing efficiency, nodule dry weight, root dry weight, shoot dry weight and total plant dry weight when co-inoculated with SUTN9-2 over the inoculation of SUTN9-2 alone on mung bean at 30 DAI ($P<0.05$), which higher nitrogen-fixing efficiency, nodule dry weight, root dry weight, shoot dry weight and total plant dry weight than single inoculation for 92.86%,

125.00%, 47.13%, 45.20% and 48.42%, respectively. The results of single inoculation and co-inoculation on plant growth and symbiotic parameters of mung bean at 30 DAI were showed in Figure 19. The co-inoculation of S141 with SUTN9-2 showed the highest nitrogen-fixing efficiency, nodule dry weight, root dry weight, shoot dry weight and total plant dry weight when compared with single inoculation treatment and control. Just in case of root dry weight, single inoculation of S141 demonstrated the highest result similarly with co-inoculation of S141 with SUTN9-2 when compared with SUTN9-2 single inoculation treatment and control. Nevertheless, co-inoculation of S141 with USDA110 did not showed significantly different between single inoculation and co-inoculation of nodule number per plant, but nodule dry weight displayed significantly different, this indicated that sizes of the nodule are the difference consistent with the results of soybean cultivars CM60 and Enrei. The nodule sizes separation also demonstrated significantly different between single inoculation and co-inoculation (Figure 20). The co-inoculation of S141 with SUTN9-2 showed the highest number of L size nodule when compared with single inoculation treatment. On the other hand, the single inoculation of SUTN9-2 showed the highest number of S size nodule when compared with co-inoculation treatment because of not significantly different in nodule number per plant.

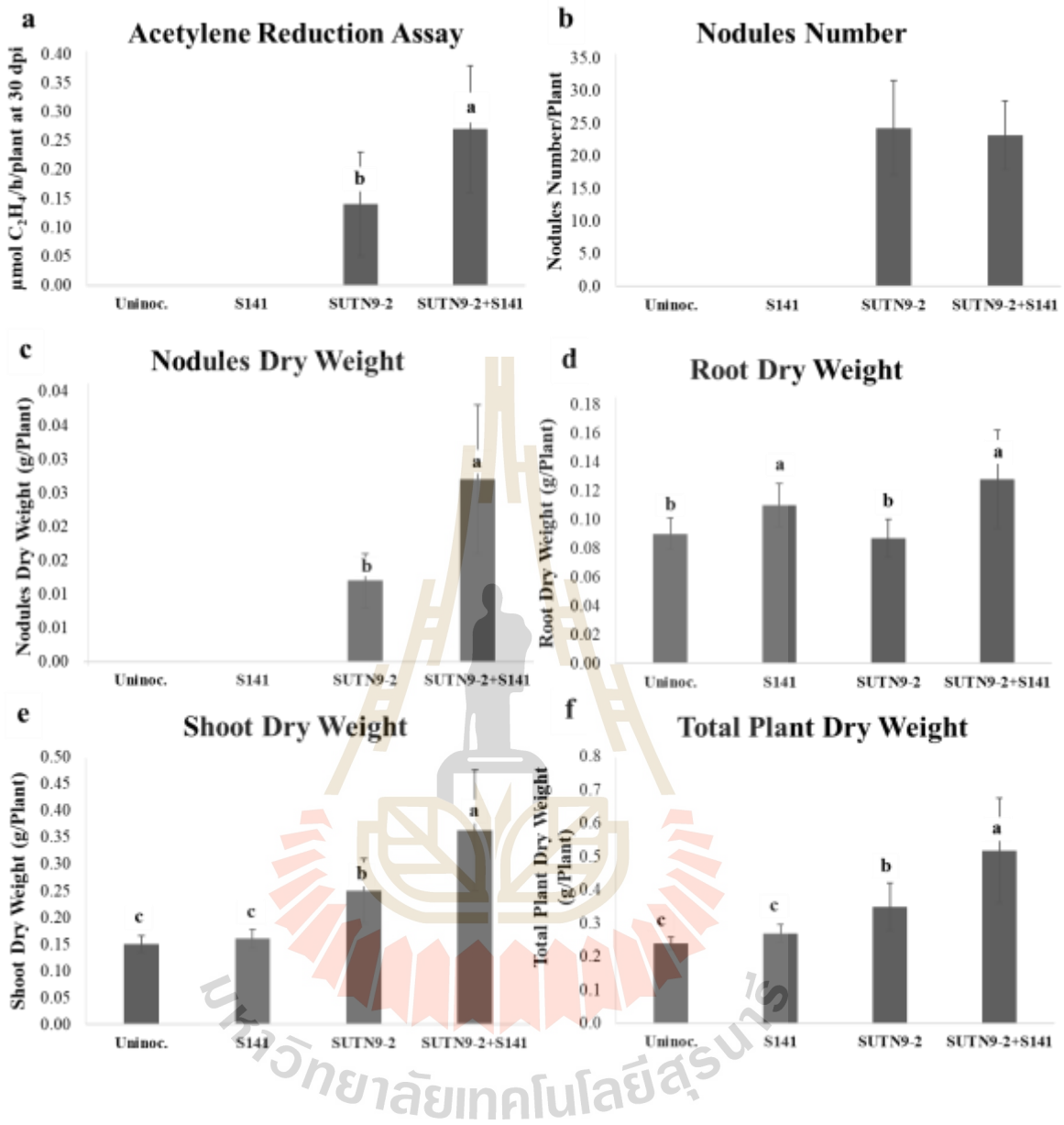

Figure 19. Plant growth and symbiotic parameters of mung bean cultivar SUT4 by co-inoculation with S141 and SUTN9-2 at 30 DAI: (a) nitrogenase activity was determined using the acetylene reduction assay, (b) nodule numbers per plant, (c) nodule dry weight, (d) root dry weight, (e) shoot dry weight and (f) total plant dry weight. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=5).

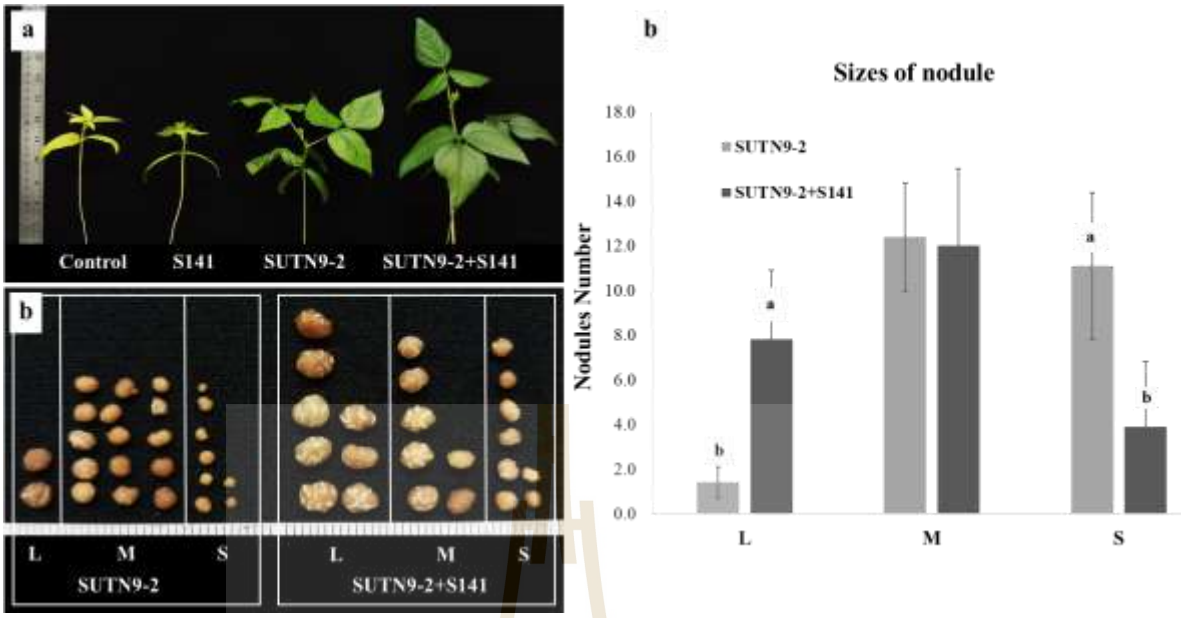

Figure 20. Mung bean plant growth and the nodule sizes separation of mung bean cultivar SUT4 by co-inoculation with S141 and SUTN9-2 at 30 DAI: (a) mung bean cultivar SUT4 growth under Leonard's jar experiments, (b) photograph of mung bean nodule and (c) nodule sizes separation of mung bean. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=5).

Taken together with the results of co-inoculation experiments between S141 with USDA110 in soybean-*Bradyrhizobium* symbiosis and S141 with SUTN9-2 in mung bean-*Bradyrhizobium* symbiosis, co-inoculation were significantly capable of promoting one or more plant growth and symbiotic parameters including nitrogen-fixing efficiency, nodule dry weight, root dry weight, shoot dry weight and total plant dry weight. In conclusion, all of the co-inoculation enhanced soybean and mung bean-*Bradyrhizobium* symbiosis rendering produced large size of nodules resulting in increasing nitrogen-fixing efficiency.

4.3 Influence of biological molecules secreted from S141 on soybean-*Bradyrhizobium*

The Leonard's jar experiment was conducted to evaluate the co-inoculation effects of *B. diazoefficiens* USDA110 with cells or supernatant of S141 on soybean cultivar Chaing Mai 60. The strain S141 and its supernatant showed the ability to promote one or more plant parameters including nitrogen-fixing efficiency, nodule dry weight, root, shoot and total plant dry weight when co-inoculated with USDA110 over the single inoculation of USDA110 on soybean at 45 DAI ($P<0.05$). Variations in plant growth parameters of soybean at 45 DAI based on single inoculation and co-inoculation was illustrated in Figures 21 and 22. The co-inoculation either with cells or supernatant of S141 with USDA110 showed the highest nitrogen-fixing efficiency, nodule dry weight, root dry weight and total plant dry weight when compared with single USDA110 inoculation treatments and control, which higher than single inoculation for 20.26-41.18%, 16.67-33.33%, 24.14-40.52% and 15.71-27.14%, respectively. Meanwhile, co-inoculation of USDA110 with supernatant of S141 showed not significantly different with single inoculation of USDA110 in terms of nitrogen-fixing efficiency, nodule dry weight, root dry weight and total plant dry weight on soybean-*Bradyrhizobium* symbiosis. The results revealed that co-inoculation of USDA110 with supernatant of S141 trend to increased nitrogen-fixing efficiency, nodule dry weight, root dry weight and total plant dry weight on soybean-*Bradyrhizobium* symbiosis at 45 DAI.

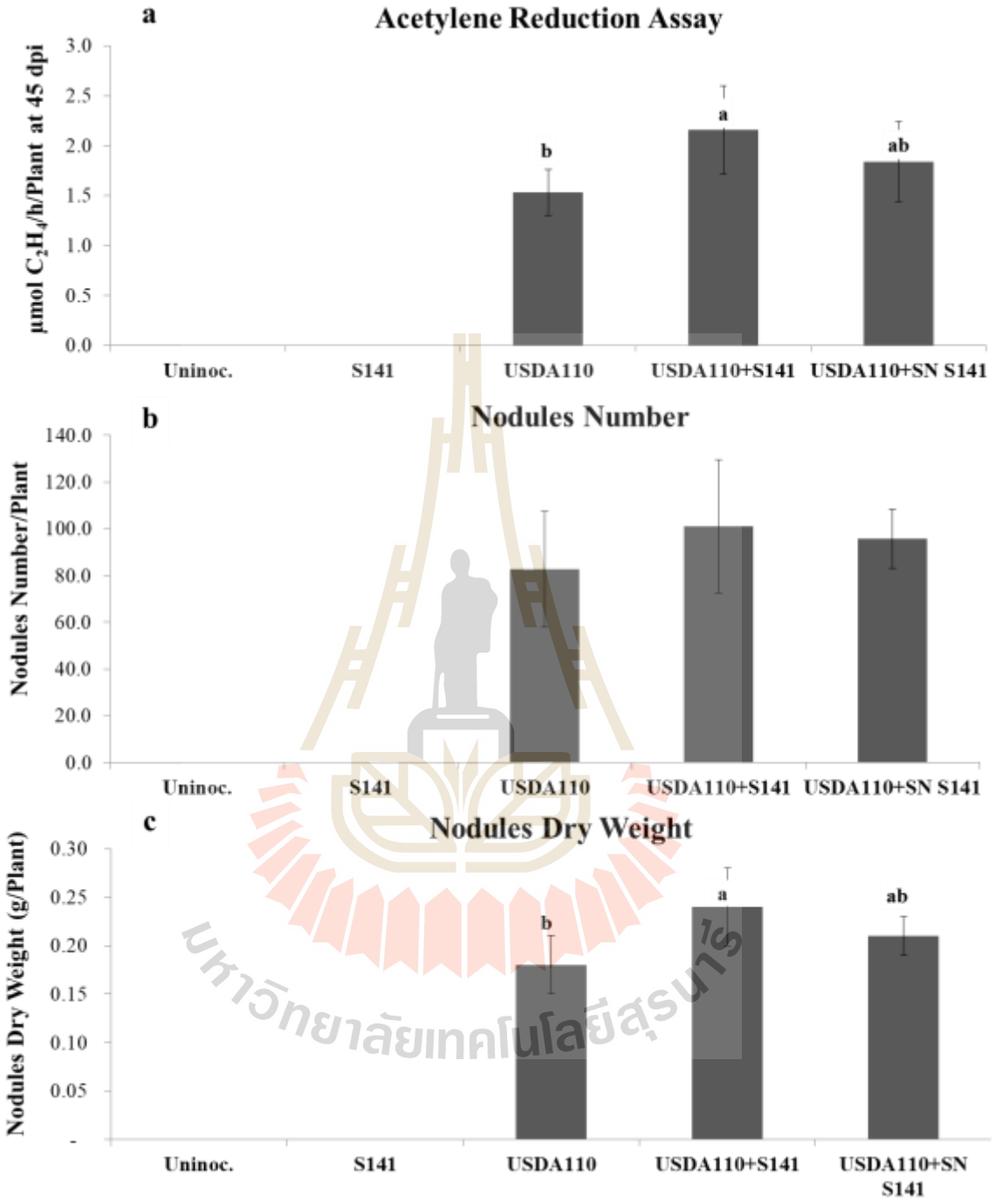

Figure 21. Plant growth and symbiotic parameters of soybean cultivar Chaing Mai 60 by co-inoculation with cells or supernatant of S141 and USDA110 at 45 DAI: (a) nitrogenase activity was determined by the acetylene reduction assay, (b) nodule numbers per plant and (c) nodule dry weight. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=10).

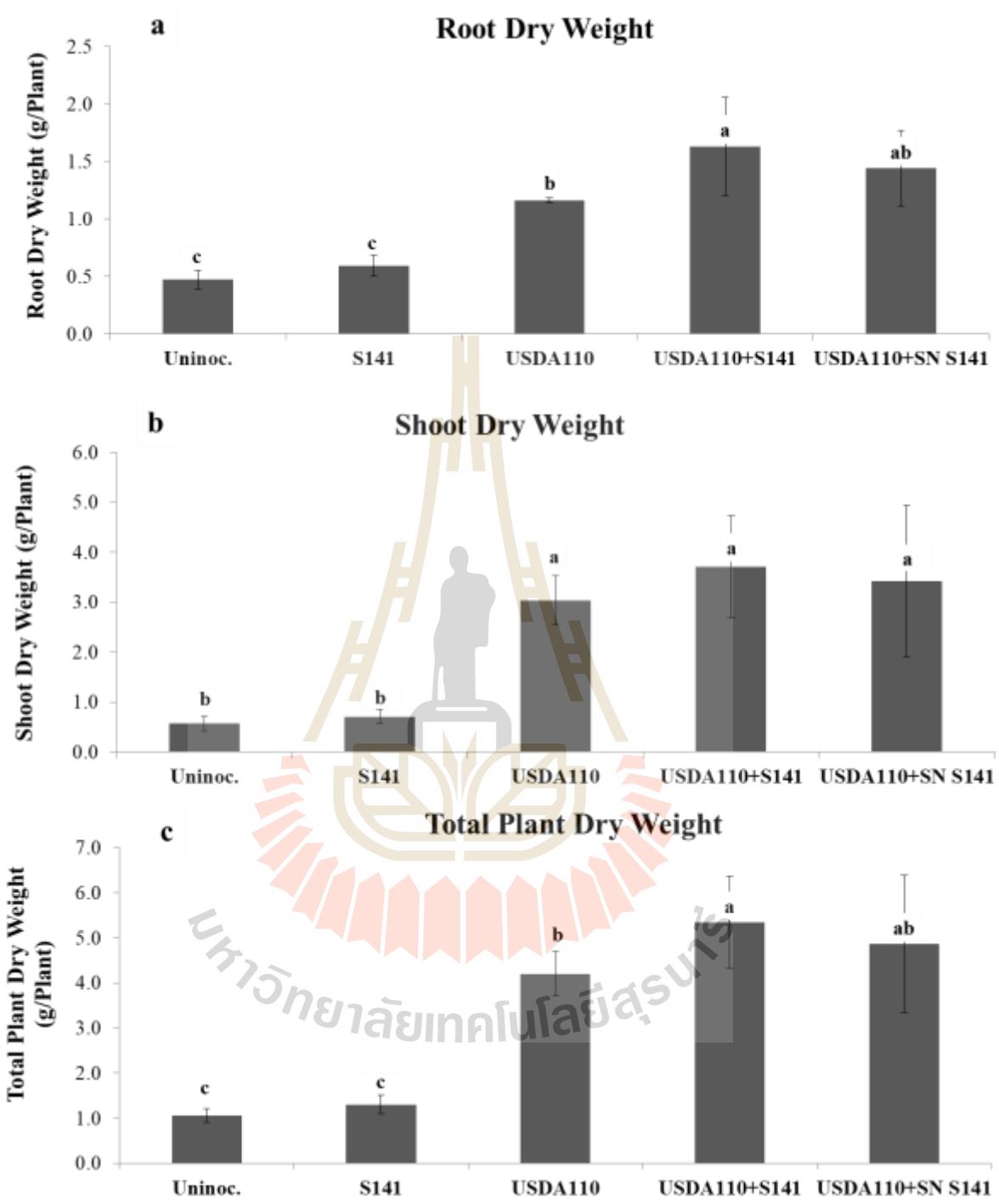

Figure 22. Plant growth parameters of soybean cultivar Chaing Mai 60 by co-inoculation with cells or supernatant of S141 and USDA110 at 45 DAI: (a) root dry weight, (b) shoot dry weight and (c) total plant dry weight. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=10).

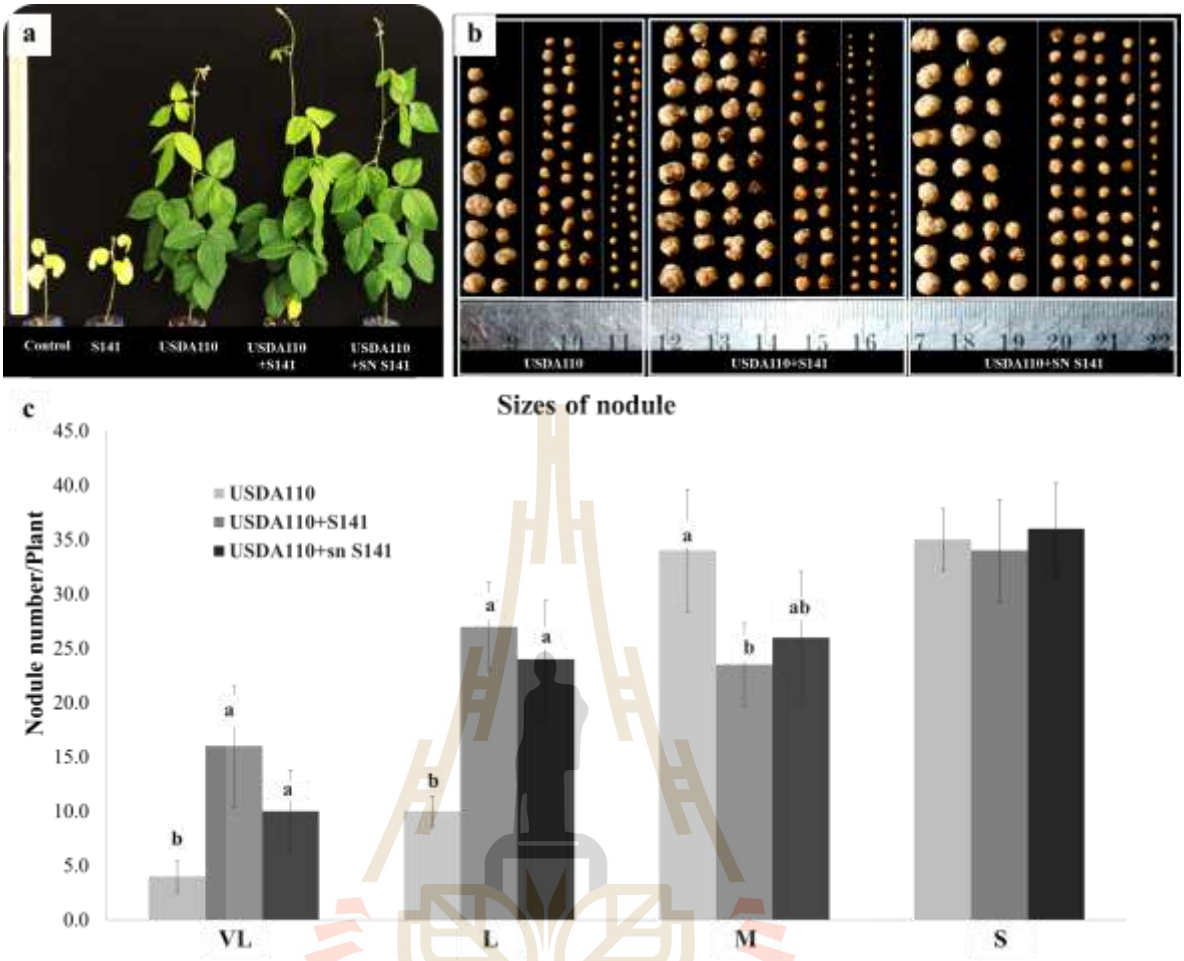

Figure 23. Soybean plant growth and the nodule sizes separation of soybean cultivar Chaing Mai 60 by co-inoculation with cells or supernatant of S141 and USDA110 at 45 DAI: (a) soybean cultivar CM60 growth under Leonard's jar experiments, (b) photograph of soybean nodule and (c) nodule sizes separation of soybean. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=10).

Moreover, co-inoculation of neither cells nor supernatant of S141 with USDA110 also showed significantly different between single inoculation and co-inoculation of nodule number per plant, but nodule dry weight demonstrated significantly different which indicated the sizes of the nodule are different, this was concurrence with the result of

soybean cultivars Chaing Mai 60 and Enrei. The nodule sizes separation revealed significantly different between single inoculation and co-inoculation in both cells and supernatant of S141 (Figure 23). The co-inoculation of either cells or supernatant of S141 with USDA110 showed the highest number of VL size nodule and L size nodule, which increased 150-300% and 140-170%, respectively when compared with single inoculation treatment. Besides, the single inoculation of USDA110 showed the highest number of S size nodules when compared with co-inoculation treatments because of not significantly different in nodule number per plant.

Furthermore, S141 and its supernatant was tested for their plant growth promotion using soybean by co-inoculation with *B. diazoefficiens* CB1809. CB 1809 has been selected for higher efficiency of N_2 fixation and higher adaptation to tropical soils. CB 1809 is the commercial strains that are broadly applied to soybean crops in the tropics, and known for its high efficiency in fixing nitrogen, but less competitiveness. The strain has been used in commercial inoculants in Brazil since 1992 (Siqueira et al., 2014). Leonard's jar experiment was conducted to evaluate the co-inoculation effects of CB1089 with cells or supernatant of S141 on soybean-*Bradyrhizobium* symbiosis. S141 and its supernatant performed the ability to promote one or more plant parameters including nodule dry weight, root dry weight, shoot dry weight and total plant dry weight when co-inoculated with CB1809 over the single inoculation of CB1809 on soybean at 45 DAI ($P<0.05$). The variations in plant growth parameters of soybean at 45 DAI based on single inoculation and co-inoculation were presented in Figures 24 and 25. The co-inoculation of either with cells or supernatant of S141 with CB1809 showed the highest nodule dry weight, root dry weight, shoot dry weight and total plant dry weight weight when compared with single inoculation treatment and control. On the other hand, co-inoculation of CB1809 with supernatant of S141 also showed not significantly different when compared with single inoculation of CB1809 in terms of nodule dry weight, root dry weight, shoot dry weight

and total plant dry weight on soybean-*Bradyrhizobium* symbiosis. The results indicated that co-inoculation of CB1809 with supernatant of S141 trend to increased nodule dry weight, root dry weight and total plant dry weight on soybean-*Bradyrhizobium* symbiosis at 45 DAI, but the results showed indistinctly when compared with co-inoculation of USDA110 with supernatant of S141. It is possible that supernatant of S141 can compatible with USDA110 more than CB1809, and able to enhance soybean growth better than CB1809. In some cases, multiple strains are synergistic for plants (İçgen et al., 2002), while in others, mixed inoculations result in plants that perform worse than predicted (Heath and Tiffin, 2007).

The co-inoculation of either cells or supernatant of S141 with CB1809 also did not showed significantly different between single inoculation and co-inoculation in term of nodule number per plant which was consistent with co-inoculation of cells or supernatant of S141 with USAD110, but nodule dry weight showed significantly different, indicated that sizes of the nodule are the difference. The nodule sizes separation demonstrated significantly different between single inoculation and co-inoculation of both cells and supernatant of S141 (Figure 26). The co-inoculation of either cells or supernatant of S141 with CB1809 showed the highest number of VL size nodule, which increased 59.25-74.07% when compared with single inoculation treatment, while L, M and S size nodule of co-inoculation between supernatant of S141 with CB1809 did not showed significantly different when compared with single inoculation treatment.

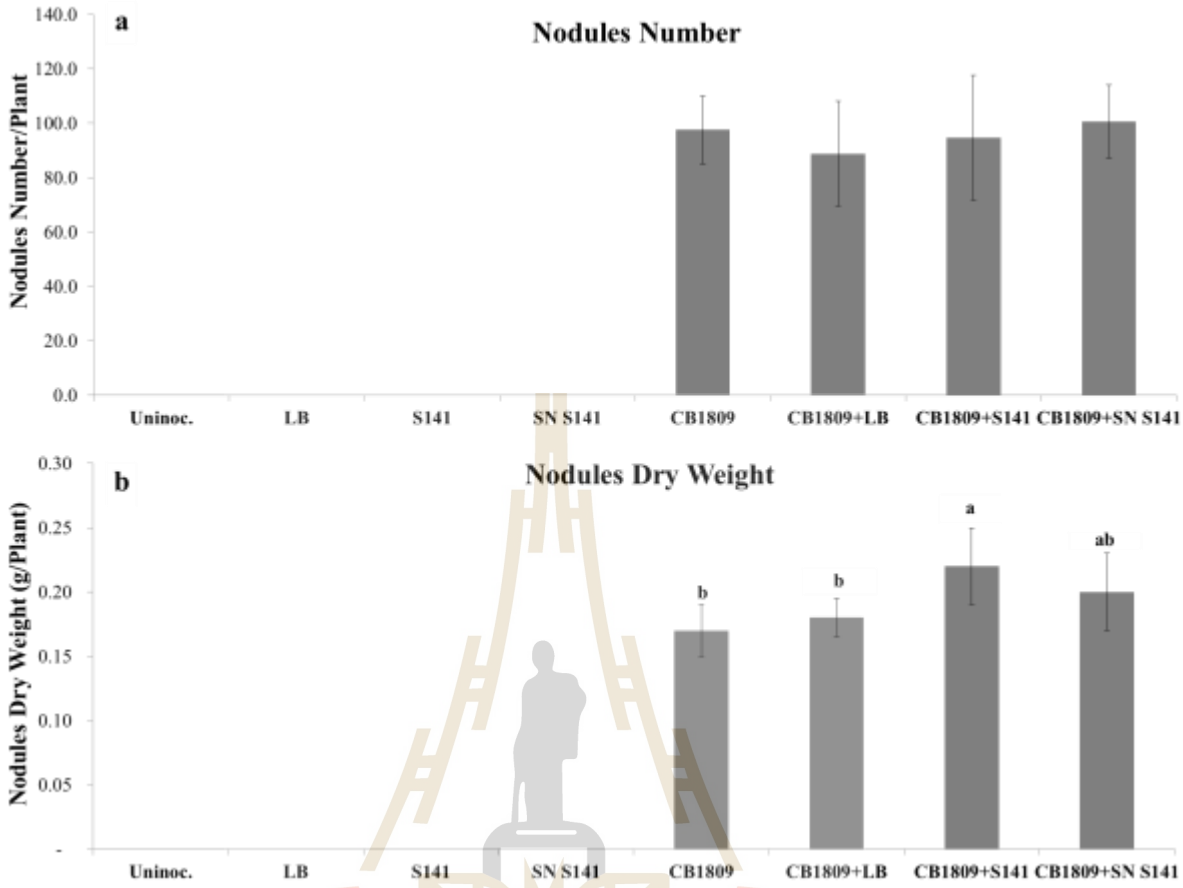

Figure 24. Plant growth parameters of soybean cultivar Chaing Mai 60 by co-inoculation with cells or supernatant of S141 and CB1809 at 45 DAI: (a) nodule numbers per plant and (b) nodule dry weight. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=8).

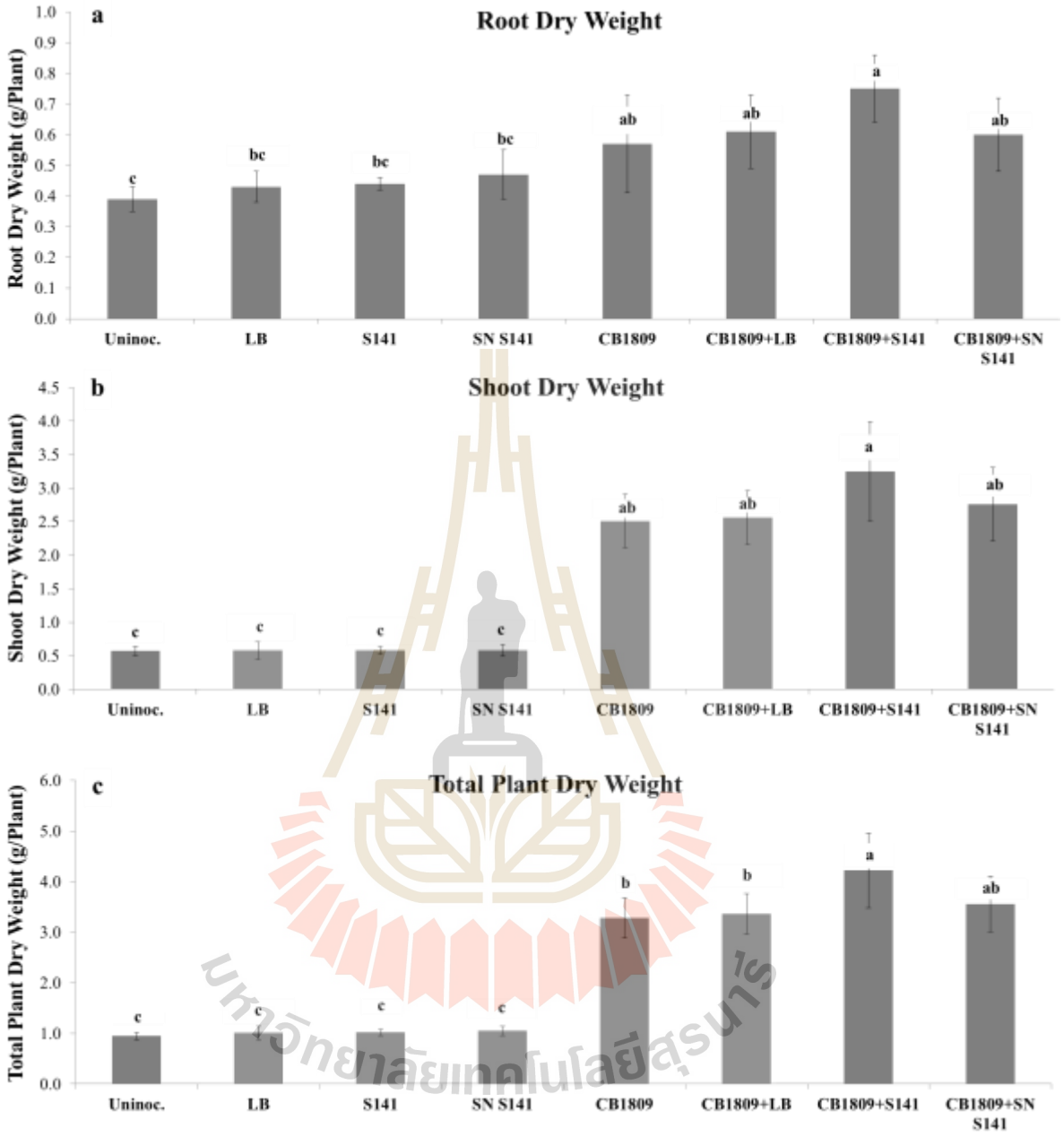

Figure 25. Plant growth parameters of soybean cultivar Chaing Mai 60 by co-inoculation with cells or supernatant of S141 and CB1809 at 45 DAI: (a) root dry weight, (b) shoot dry weight and (c) total plant dry weight. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=8).

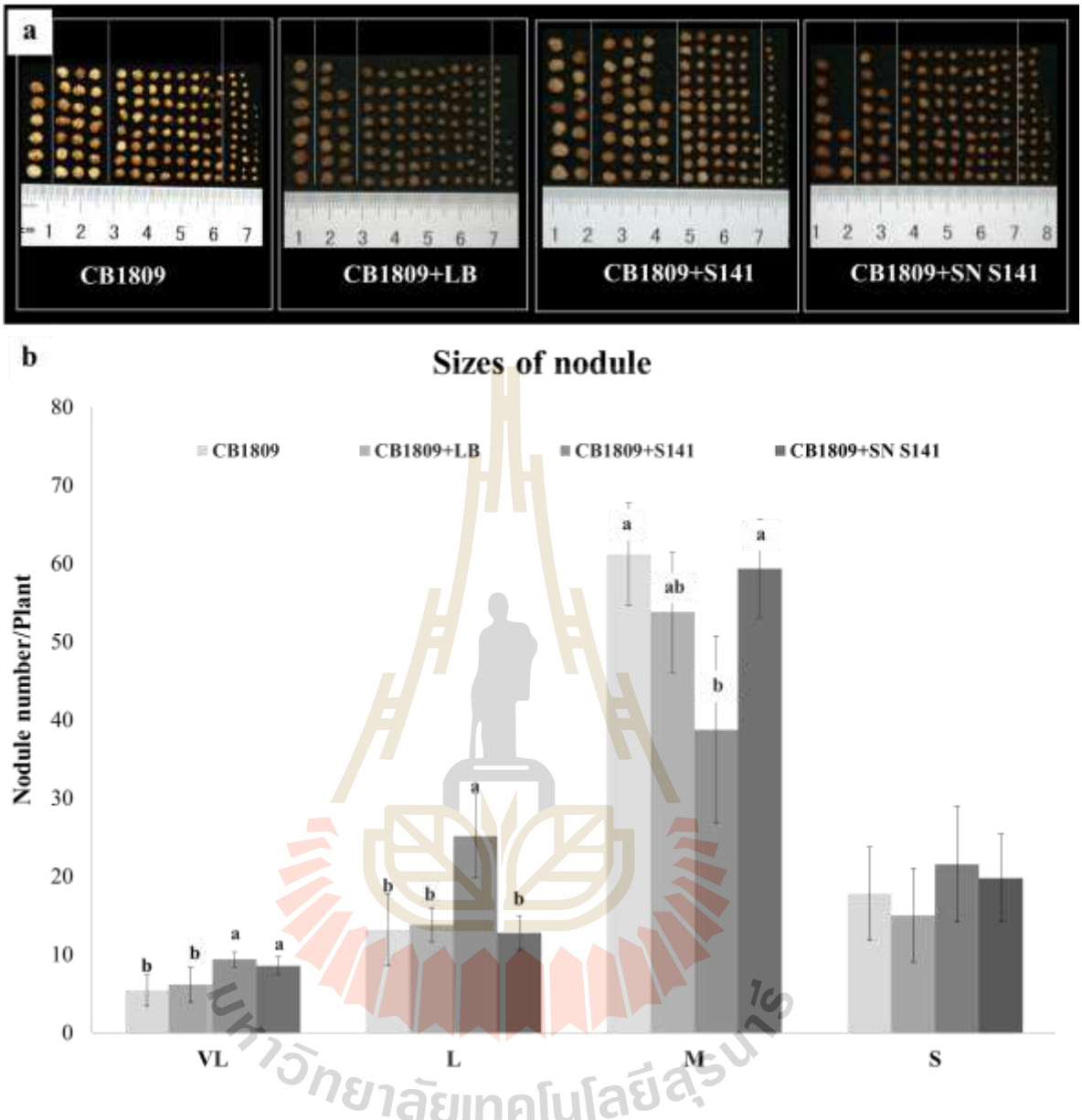

Figure 26. The nodule sizes separation of soybean cultivar Chaing Mai 60 by co-inoculation with cells or supernatant of S141 and CB1809 at 45 DAI: (a) photograph of soybean nodule and (b) nodule sizes separation of soybean. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=8).

Taken together these results of co-inoculation experiments between either cells or supernatant of S141 with *Bradyrhizobium* in soybean-*Bradyrhizobium* symbiosis, co-inoculation were significantly capable of promoting one or more plant growth and symbiotic parameters including nitrogen-fixing efficiency, nodule dry weight, root dry

weight, shoot dry weight and total plant dry weight. However, co-inoculation with supernatant of S141 were trend to promote soybean growth by increasing nodule dry weight. Furthermore, co-inoculation of either cells or supernatant of S141 enhanced soybean-*Bradyrhizobium* symbiosis to produced large size of nodules as the result of increasing nitrogen-fixing efficiency and promote soybean growth.

4.4 Effect of biological molecules secreted from S141 on inoculation dose of *Bradyrhizobium*

The inoculation dose of *Bradyrhizobium* and S141 co-inoculation is an important factor for the application of microbial inoculants to soybean. In addition, co-inoculation with supernatant of S141 trend to promote soybean growth by increasing nodule dry weight. To determine how the proportion of *Bradyrhizobium* strain in the inoculum mixtures might affect soybean production, the possible combinations involving *B. diazoefficiens* USDA110 and supernatant of S141 were used to co-inoculate soybean in six different ratios under Leonard's jar condition.

There were differences among USDA110 doses for all soybean growth parameters including nodule numbers, nodule dry weight, root dry weight, shoot dry weight and total plant dry weight (Figures 27, 28 and 29). The results of the single inoculation of USDA110 were not different when compared with co-inoculation in all inoculant doses tested (10^3-10^8 CFU ml^{-1}) on soybean nodule numbers, root dry weight, shoot dry weight and total plant dry weight. All inoculant doses tested (10^3-10^8 CFU ml^{-1}) of single inoculation of USDA110 or co-inoculation treatments produced higher root dry weight, shoot dry weight and total plant dry weight than those obtained from uninoculated control treatments. In case of nodule dry weight, co-inoculation treatments of USDA110 at inoculant doses tested 10^5-10^8 CFU ml^{-1} produced higher nodule dry weight than 10^3-10^4 CFU ml^{-1} indicated that co-

inoculation can help the soybean to produced large size of nodules as the result of increasing nitrogen-fixing efficiency.

Remarkably, the nodule sizes separation of co-inoculation treatments (Figures 30 and 31) with supernatant of S141 and USDA110 at inoculant doses tested 10^3-10^6 CFU ml^{-1} produced the highest number of L size nodule when compared with single inoculation treatment in each inoculant doses tested, while increasing of inoculant doses tested 10^7-10^8 CFU ml^{-1}, the number of L size nodule did not showed significantly different when compared with the numbers of nodule derived from single inoculation and co-inoculation treatments. For the numbers of M size and S size nodules also showed no different when compared with the numbers of nodule derived from single inoculation and co-inoculation treatments in each inoculant doses tested. These results suggested that co-inoculation of supernatant with S141 and USDA110 can promote soybean growth by enhancing production of number of L size nodules as the result of increasing nitrogen-fixing efficiency. Moreover, it implied that the concentration of cells of USDA110 can be reduced when co-inoculated with supernatant of S141. This finding greatly effect on inoculant production, by adding PGPR low number of rhizobia in inoculation could be sufficient for nodulation.

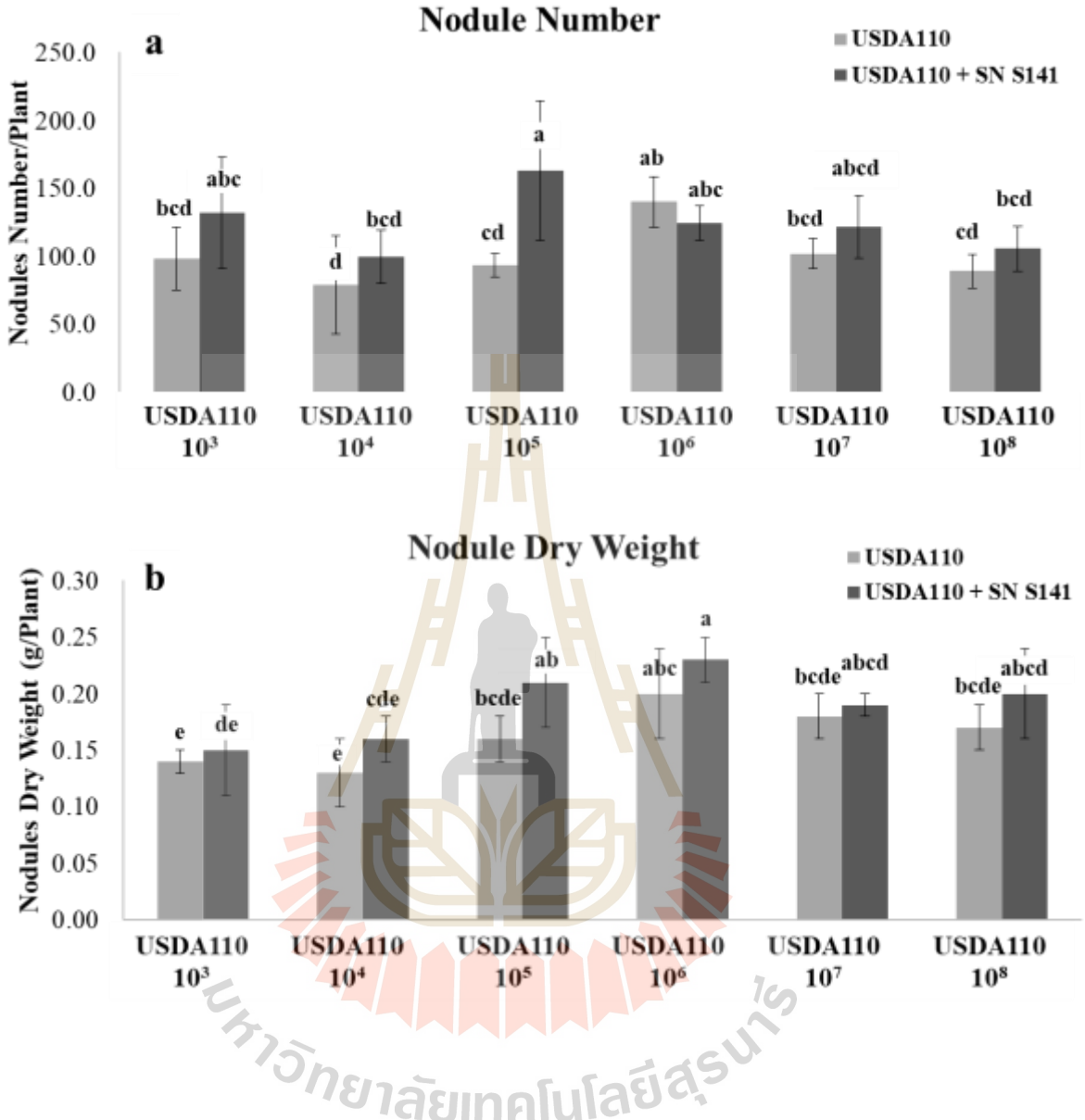

Figure 27. Effects of inoculation dose between supernatant of S141 with various doses of USDA110 on plant growth parameters of soybean cultivar Chaing Mai 60 at 45 DAI: (a) nodule numbers per plant and (b) nodule dry weight. The number at the x-axis symbolized the varied inoculation doses of USDA110 are 10^3, 10^4, 10^5, 10^6, 10^7 and 10^8 CFU ml^{-1}. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=5).

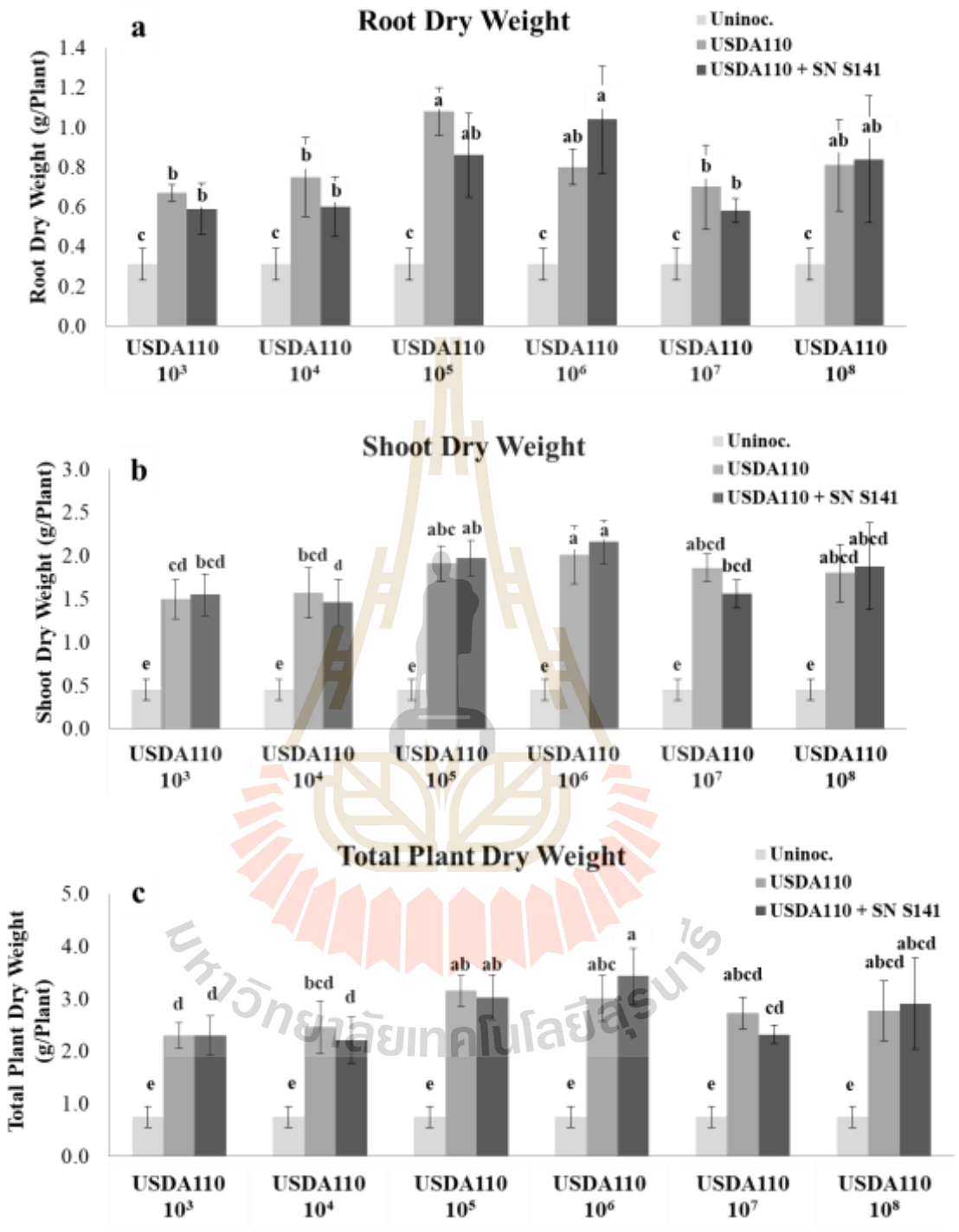

Figure 28. Effects of inoculation dose between supernatant of S141 with various doses of USDA110 on plant growth parameters of soybean cultivar Chaing Mai 60 at 45 DAI: (a) root dry weight, (b) shoot dry weight and (c) total plant dry weight. The number at the x-axis symbolized the varied inoculation doses of USDA110 are 10^3, 10^4, 10^5, 10^6, 10^7 and 10^8 CFU ml^{-1}. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=5).

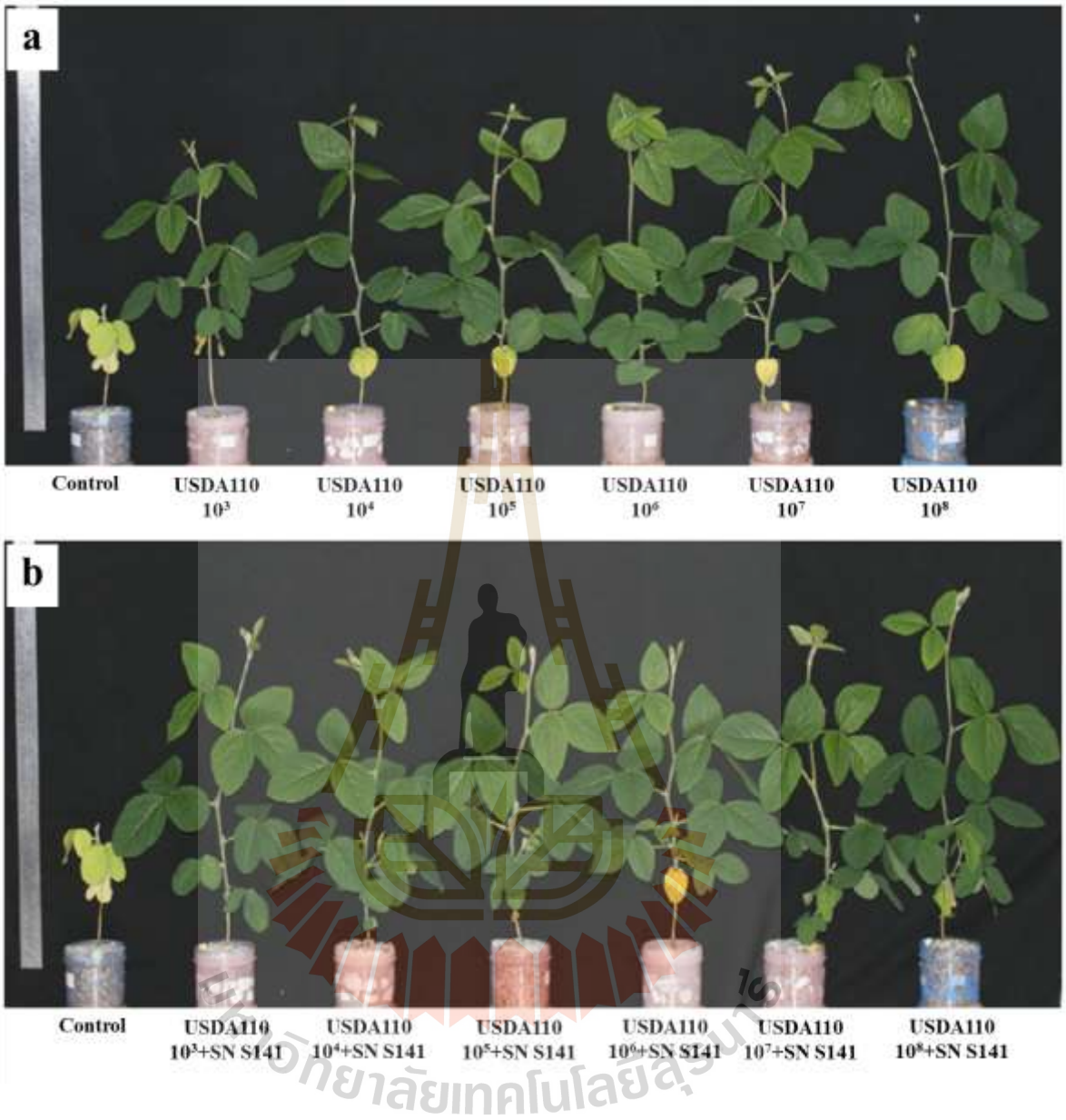

Figure 29. Effects of inoculation dose between supernatant of S141 with various doses of USDA110 on plant growth parameters of soybean cultivar Chaing Mai 60 at 45 DAI: (a) single inoculation with various doses of USDA110 on soybean growth under Leonard's jar experiments and (b) co-inoculation between supernatant of S141 with various doses of USDA110 on soybean growth under Leonard's jar experiments.

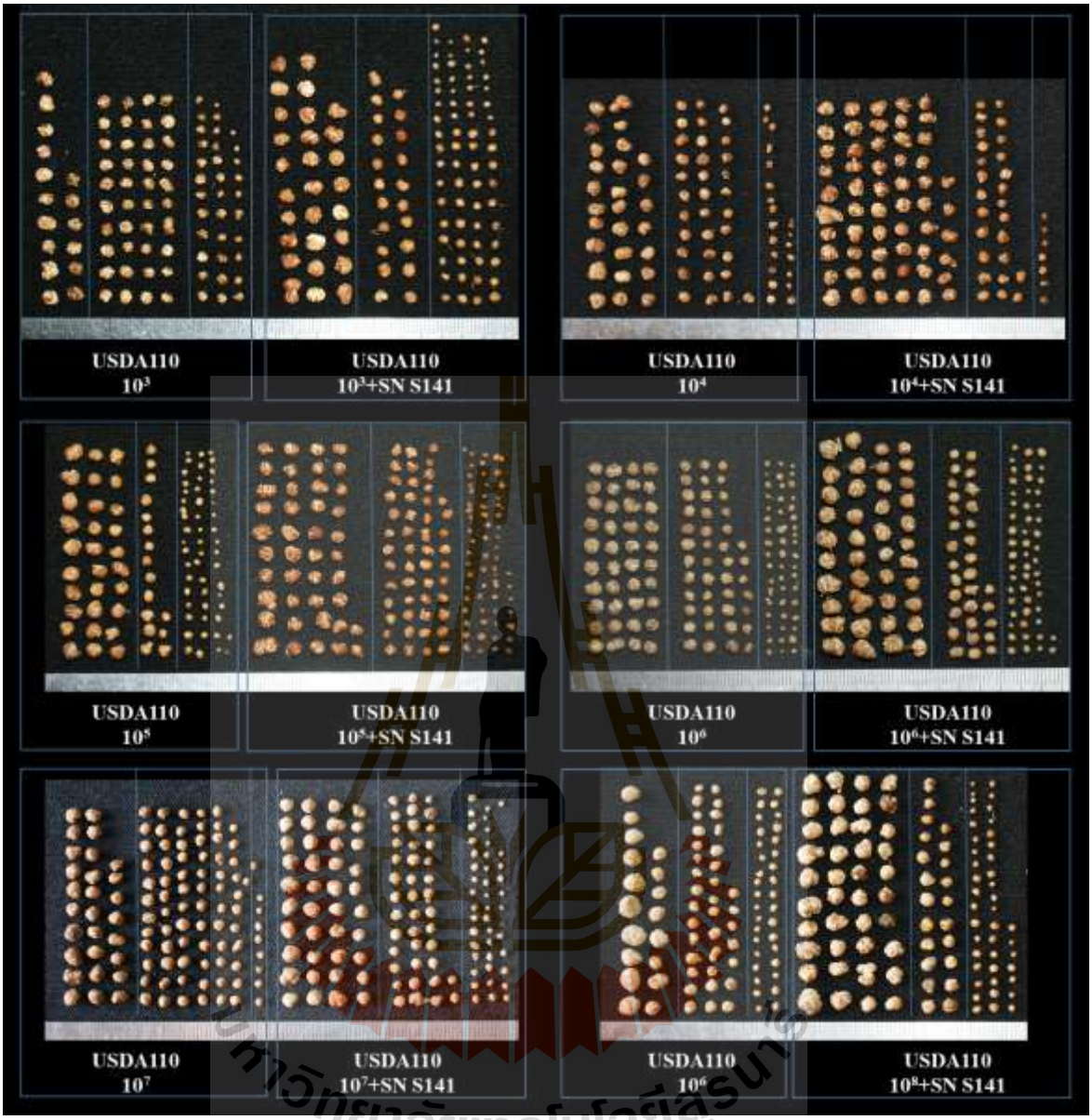

Figure 30. Photograph of soybean nodule sizes separation of single inoculation various doses of USDA110 and co-inoculation of supernatant of S141 and various doses of USDA110 on soybean cultivar Chaing Mai 60 at 45 DAI.

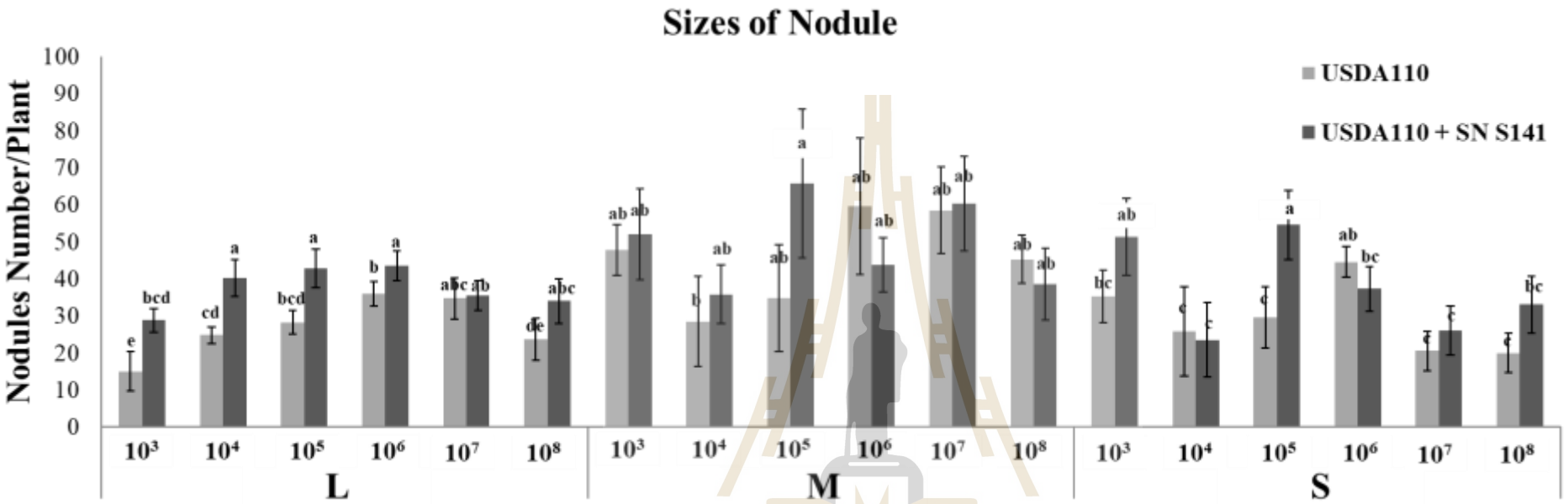

Figure 31. Soybean nodule sizes separation of single inoculation with various doses of USDA110 and co-inoculation with supernatant of S141 and various doses of USDA110 on soybean cultivar Chaing Mai 60 at 45 DAI. The number at the x-axis symbolized the varied inoculation doses of USDA110 are 10^3, 10^4, 10^5, 10^6, 10^7 and 10^8 CFU ml^{-1}. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=5).

4.5 Co-inoculation effect of S141 on *Bradyrhizobium* competition

The important factor that a significant limitation to the agricultural use of improved rhizobial inoculant strains is competition from the indigenous soil population. To demonstrate the effect of co-inoculation of bradyrhizobial strains (*B. diazoefficiens* GUS-tagged USDA110, and *Bradyrhizobium* sp. dsRed-tagged SUTN9-2) and S141 on legume-*Bradyrhizobium* symbiosis competition, the competitive ability of inoculated and indigenous *Rhizobium* or *Bradyrhizobium* spp. to nodulate and fix N_2 in grain legumes including soybean cultivar Chaing Mai 60 and mung bean cultivar SUT4 were investigated in pots with local soils collected from fields on the basis of cropping history. Tap-root nodules were harvested 45 days after planting, and nodule occupancies were determined for the bradyrhizobial strains originally applied.

In soybean, there were differences among single inoculation and co-inoculation for all soybean growth parameters including nodule numbers, root dry weight and shoot dry weight (Figures 32 and 33). The results of co-inoculation of GUS-tagged USDA110 and S141 showed significantly different in nodule numbers, root dry weight and shoot dry weight compared with single inoculation treatments and control. Interestingly, co-inoculation of GUS-tagged USDA110 and S141 demonstrated the highest of nodule numbers, most of nodules were formed by GUS-tagged USDA110 when compared with single inoculation, but the percentage of nodule occupancy did not show significantly different when compared with single inoculation. These results suggested the co-inoculation of GUS-tagged USDA110 and S141 consistently altered nodulation among certain combinations of bradyrhizobia strains. This alteration typically reflected enhanced nodulation by co-inoculation of S141 with GUS-tagged USDA110.

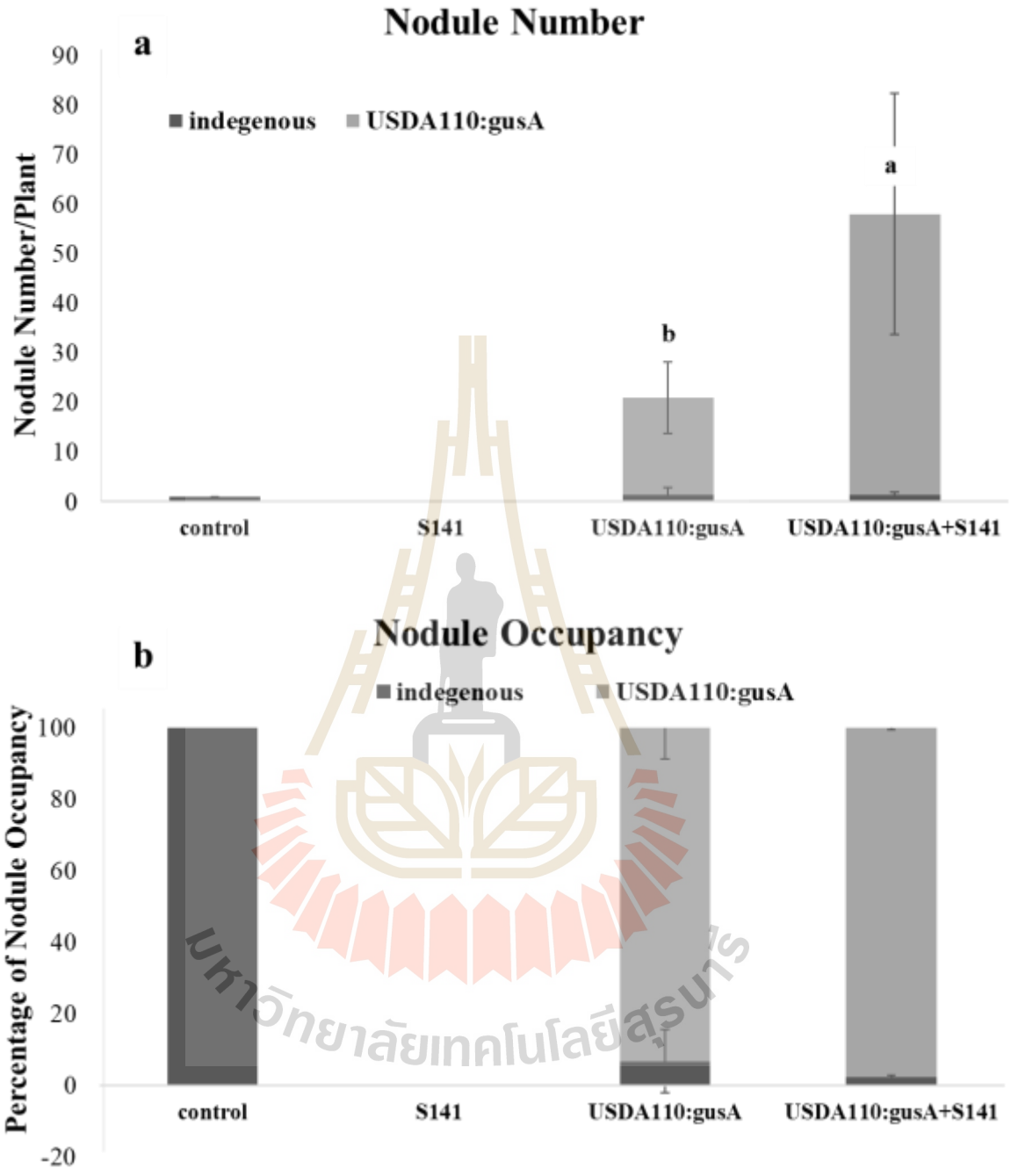

Figure 32. Plant growth parameters of soybean cultivar Chaing Mai 60 by co-inoculation with S141 and GUS-tagged USDA110 at 45 DAI: (a) nodule numbers per plant and (b) percentage of nodule occupancy. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=5).

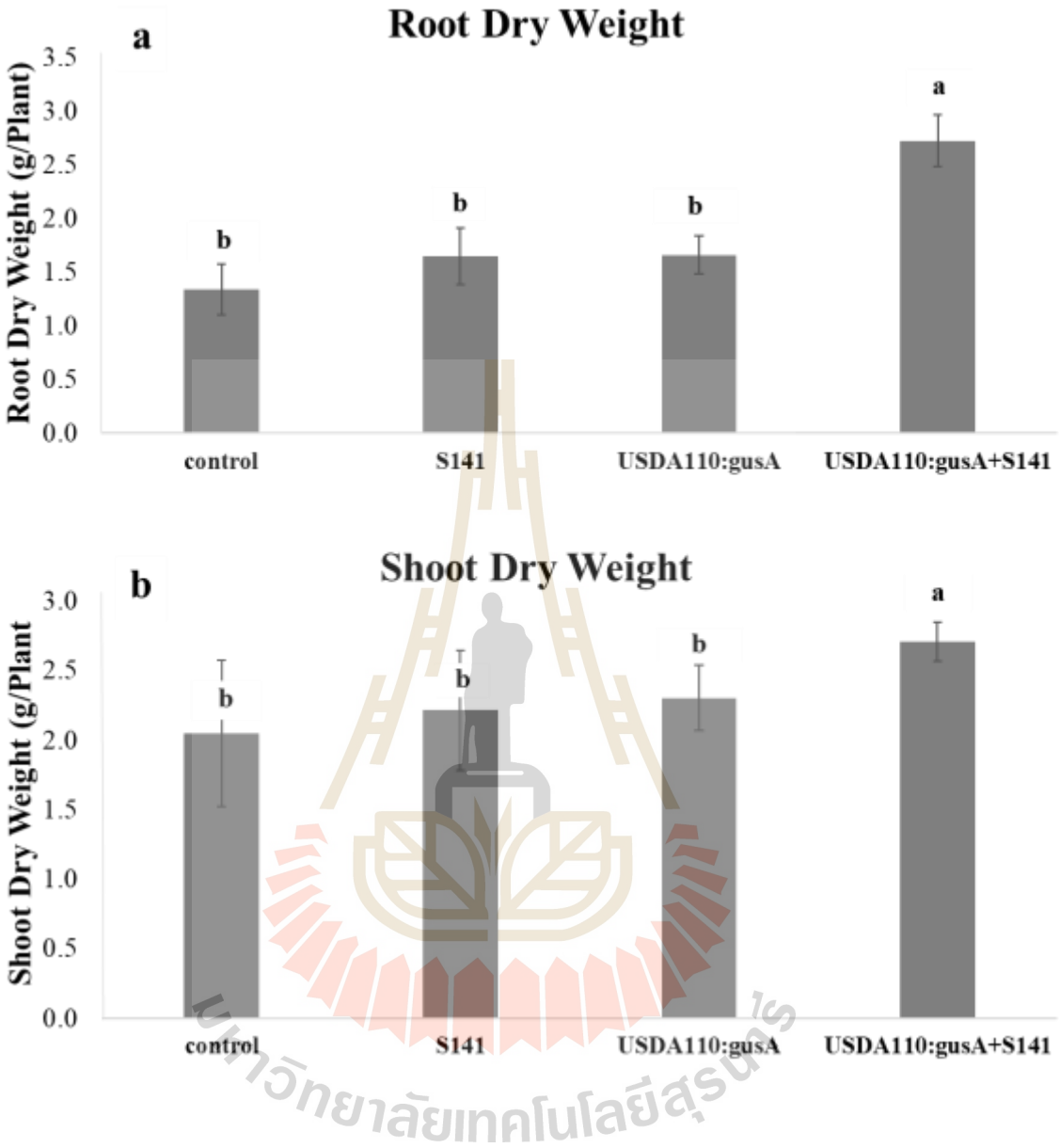

Figure 33. Plant growth parameters of soybean cultivar Chaing Mai 60 by co-inoculation with S141 and GUS-tagged USDA110 at 45 DAI: (a) root dry weight and (b) shoot dry weight. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=5).

The nodule sizes separation indicated significantly different between single inoculation and co-inoculation as presented in Figure 34. The co-inoculation of S141 with GUS-tagged USDA110 performed the highest nodule number in all size of soybean nodules when compared with single inoculation treatment, because co-inoculation of S141

with GUS-tagged USDA110 showed significantly different of nodule numbers when compared with single inoculation treatment.

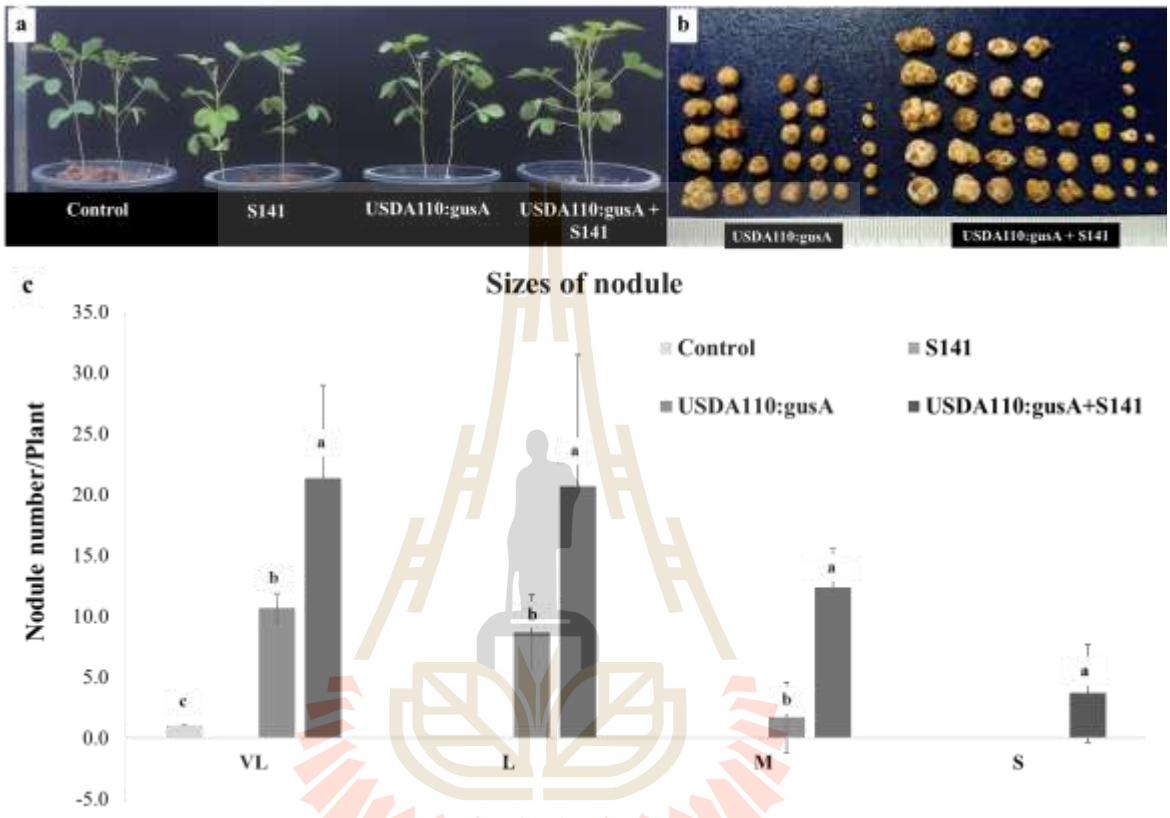

Figure 34. Soybean plant growth and the nodule sizes separation of soybean cultivar Chaing Mai 60 by co-inoculation with S141 and GUS-tagged USDA110 at 45 DAI: (a) soybean cultivar CM60 growth under pot experiments, (b) photograph of soybean nodule and (c) nodule sizes separation of soybean. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=5).

In mung bean, there were differences among single inoculation and co-inoculation which was consistent with co-inoculation between GUS-tagged USDA110 and S141 in soybean for all mung bean growth parameters including nodule numbers, root dry weight and shoot dry weight (Figures 35 and 36). The results of co-inoculation of dsRed-tagged

SUTN9-2 and S141 performed significantly different in nodule numbers, root dry weight and shoot dry weight when compared with single inoculation treatments and control. In addition, co-inoculation of dsRed-tagged SUTN9-2 and S141 demonstrated the highest of nodule numbers, most of nodules were occupied by dsRed-tagged SUTN9-2 when compared with single inoculation, but the percentage of nodule occupancy did not show significantly different when compared with single inoculation. These results also suggested that co-inoculation of dsRed-tagged SUTN9-2 and S141 promote nodulation in mung bean. This alteration typically reflected enhanced nodulation by co-inoculation of S141 with dsRed-tagged SUTN9-2.

The nodule sizes separation indicated the significantly different between single inoculation and co-inoculation as depicted in Figure 37. The co-inoculation of S141 with dsRed-tagged SUTN9-2 showed the highest nodule numbers in VL size and L size nodule of mung bean when compared with single inoculation treatment. Besides, the nodule number in S size of mung bean did not show differences between single inoculation and co-inoculation, because co-inoculation of S141 with dsRed-tagged SUTN9-2 indicated significantly different of nodule numbers when compared with single inoculation treatment.

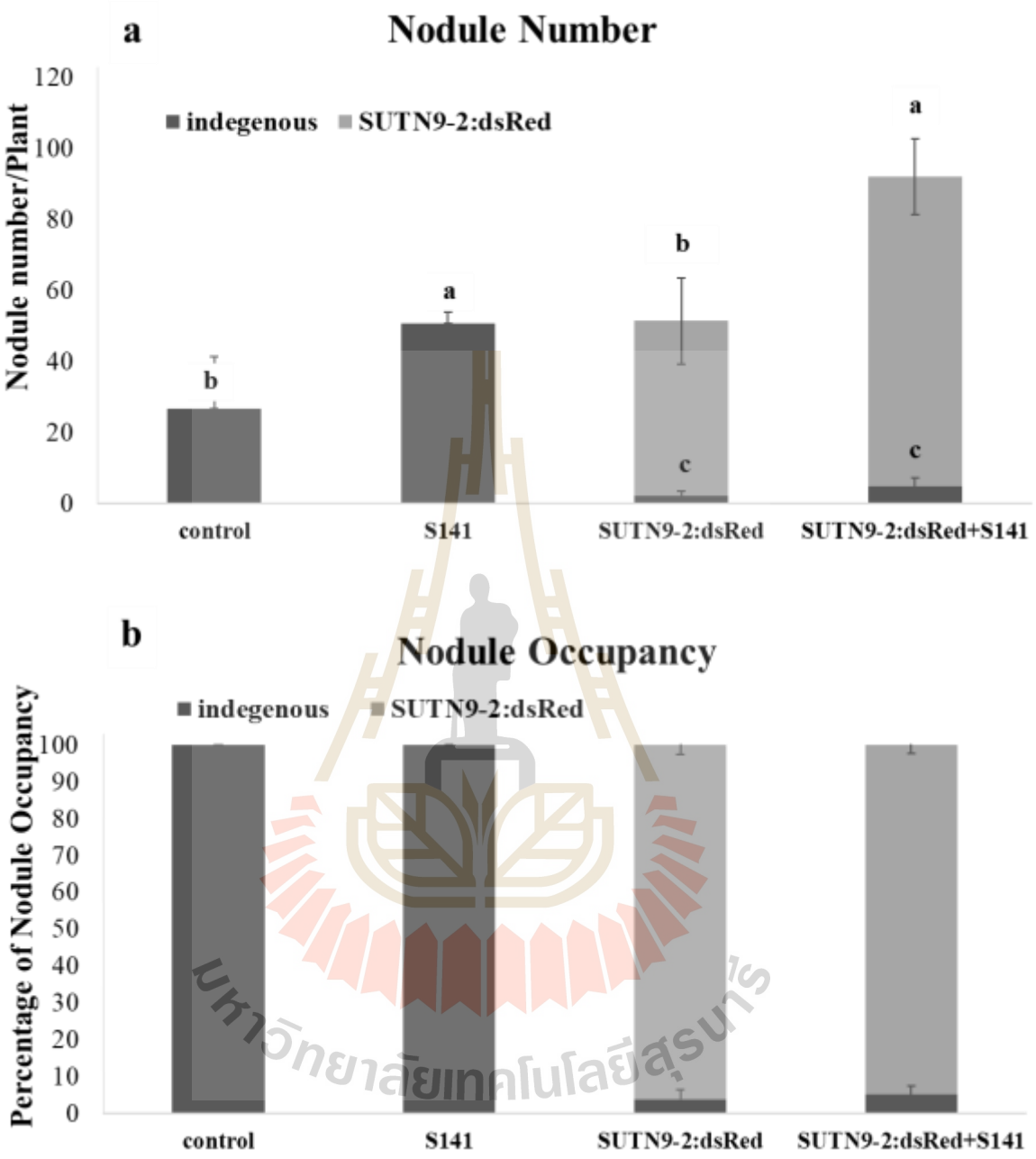

Figure 35. Plant growth parameters of mung bean cultivar SUT4 by co-inoculation with S141 and dsRed-tagged SUTN9-2 at 45 DAI: (a) nodule numbers per plant and (b) percentage of nodule occupancy. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=5).

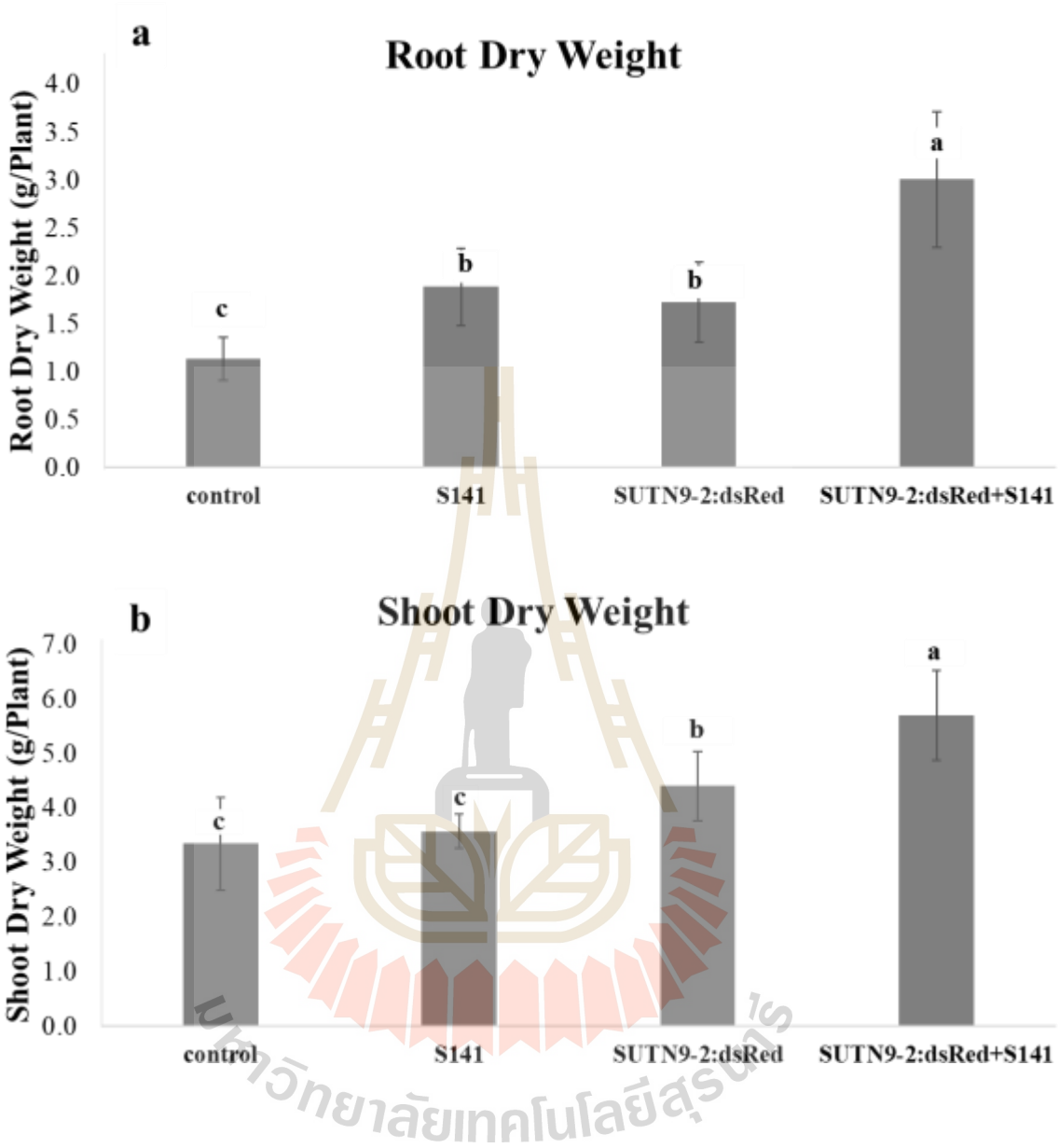

Figure 36. Plant growth parameters of mung bean cultivar SUT4 by co-inoculation with S141 and dsRed-tagged SUTN9-2 at 45 DAI: (a) root dry weight and (b) shoot dry weight. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=5).

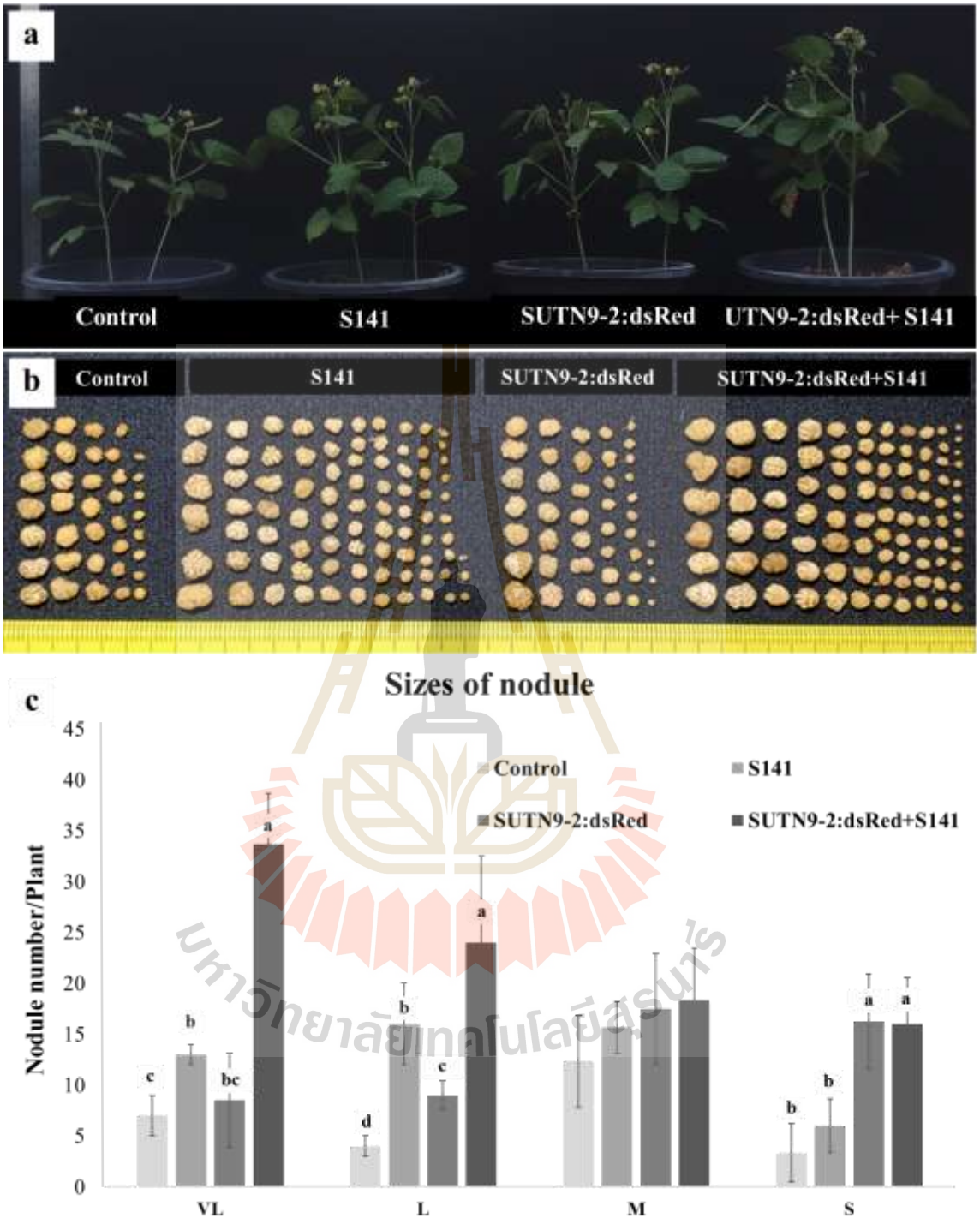

Figure 37. Mung bean plant growth and the nodule sizes separation of mung bean cultivar SUT4 by co-inoculation with S141 and dsRed-tagged SUTN9-2 at 45 DAI: (a) mung bean cultivar SUT4 growth under pot experiments, (b) photograph of soybean nodule and (c) nodule sizes separation of soybean. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=5).

These results indicated that co-inoculation of bradyrhizobial strains and S141 did not affected nodulation competition among applied bradyrhizobial strains and indigenous strains, co-inoculation of S141 cannot enhanced the competitiveness of *Bradyrhizobium* on legumes nodulation. Nevertheless, co-inoculation of bradyrhizobial strains and S141 was significant in capable of promoting legumes growth by enhancing legumes nodulation in term of increasing nodule number and producing the large size of nodules rendering increase of nitrogen-fixing efficiency and promote soybean growth. The effect of Rhizobium–PGPR co-inoculation has been observed in different symbiotic and plant growth parameters. Compared to single *Rhizobium* inoculation, co-inoculation of *Rhizobium* spp. and *Azospirillum* spp. can enhance the number of root hairs, the amount of flavonoids exuded by the roots and the number of nodules formed (Remans et al., 2008). The beneficial influence of PGPR on nodulation of legumes by *Rhizobium* has been variously attributed to their ability to produce phytohormones (Schmidt, 2008), toxins (Zaidi et al., 2012), or antibiotics (Yuttavanichakul et al., 2012), as well as by other unidentified mechanisms (Paulucci et al., 2012). For example, Argaw (2011) demonstrated that some *Pseudomonas* strains, colonized the root and increased nodule number and acetylene reduction in soybean plants inoculated with *B. japonicum*.

4.6 Extra/intracellular proteome of S141 or USDA110

Total cellular proteins from S141 were separated using SDS-PAGE as shown in Figure 38. Total cellular protein pattern bands from S141 demonstrated different bands when induced by supernatant of USDA110 and/or root exudates of soybean in both 24 and 48 hours. The protein pattern bands showed up and down expression after induced by supernatant of USDA110 and/or root exudates of soybean in both 24 and 48 hours. The polymorphic band in the size approximately 20-70 kDa were extracted and digested according to Yuan et al. (2006) and analyzed using Nano-liquid chromatography

electrospray ionization quadrupole-time of flight MS (nano-LC–ESI–MSMS), and proteins identification by MASCOT search engine used MS data to identify the proteins from primary sequence databases as described previously by Yuan et al. (2006).

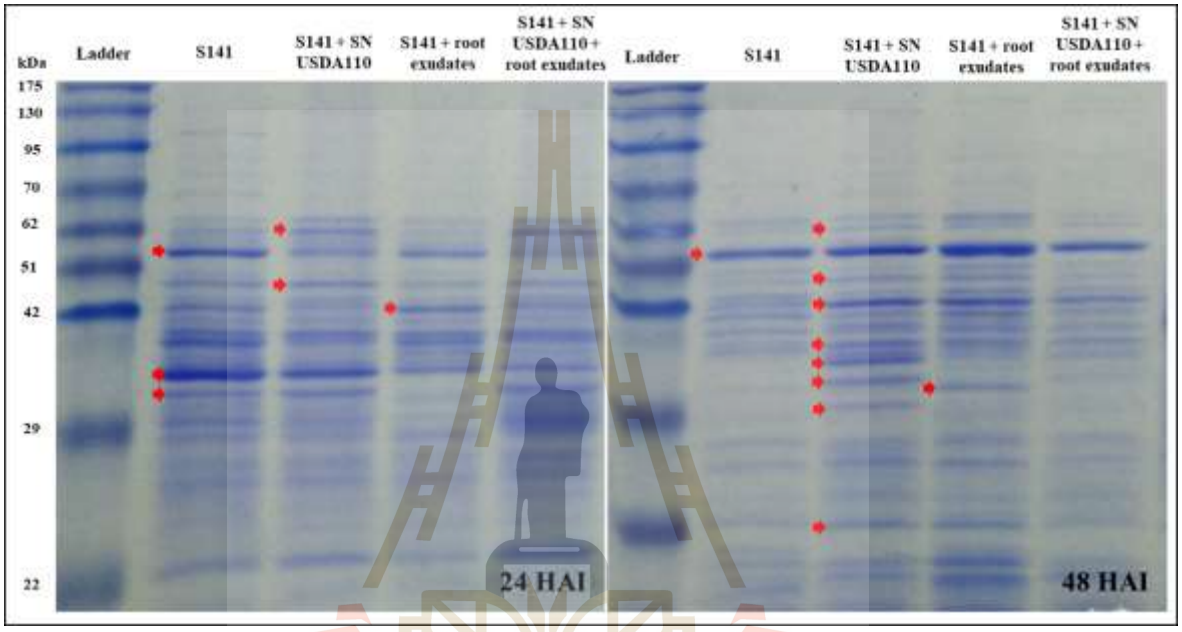

Figure 38. Electrophoretic profile of total cellular proteins from S141 after induced by supernatant and/or root exudates for 24 and 48 hour using 12% SDS-PAGE.

The results of protein identification after induced by supernatant of USDA110 and/or root exudates of soybean for 24 hours (Table 5) and 48 hours (Table 6) demonstrated that malate dehydrogenase was up-regulated in S141 after induced by supernatant of USDA110 and/or root exudates of soybean in both 24 and 48 hours, flagellin A and 1-pyrroline-5-carboxylate dehydrogenase were down-regulated at 24 hours, but increasing the expression after induced by supernatant of USDA110 and/or root exudates of soybean at 48 hours, 2, 3-bisphosphoglycerate-independent phosphoglycerate mutase and glucose-6-phosphate isomerase were up-regulated in S141 after induced by supernatant of USDA110 for 24 hours, phosphopyruvate hydratase (enolase) was up-regulated after induced by root

exudates of soybean for 24 hours, while up-regulation shown in all inductions at 48 hours. Moreover, at 48 hours after induction by supernatant of USDA110 and/or root exudates of soybean, ABC transporter substrate-binding protein and catalase were up-regulated.

The electrophoretic profile of total cellular proteins from S141and USDA110 after induced by supernatant of S141 or USDA110 for 24 and 48 hours were analyzed as depicted in Figure 39. Total cellular protein pattern bands from S141 and USDA110 displayed different with total cellular protein from S141 and USDA110 after induced by supernatant of S141 or USDA110 for both 24 and 48 hours. The protein pattern bands were up and down expressed after induced by supernatant of S141 or USDA110 for both 24 and 48 hours. The polymorphic band in the size approximately 10-150 kDa were extracted, digested and analyzed.

Table 5. Protein expression of S141 after induced by supernatant of USDA110 and/or root exudates of soybean for 24 hours.

No.	Protein Prediction	Protein expression of S141 when compare with control			NCBI ID	Organism
		SN USDA110	RE CM-60	SN USDA110 and RE CM-6		
1	1-pyrroline-5-carboxylate dehydrogenase	down	down	down	gi|385147932	*Bacillus* sp. 5B6
2	flagellin A	down	down	down	gi|549783040	*B. subtilis* group
3	malate dehydrogenase	up	up	up	gi|385150300	*Bacillus* sp. 5B6
4	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	up	same level	up	gi|48924342	*Bacillus*
5	glucose-6-phosphate isomerase	up	same level	up	gi|489243924	*B. velezensis*
6	phosphopyruvate hydratase (enolase)	same level	up	same level	gi|489243426	Bacillaceae

SN; Supernatant and RE; Root exudates

Table 6. Protein expression of S141 after induced by supernatant of USDA110 and/or root exudates of soybean for 48 hours.

No.	Protein Prediction	Protein expression of S141 when compare with control			NCBI ID	Organism
		SN USDA110	RE CM-60	SN USDA110 and RE CM-6		
1	ABC transporter substrate-binding protein	up	up	up	gi|745765591	*B. amyloliquefaciens*
2	1-pyrroline-5-carboxylate dehydrogenase	up	same level	up	gi|387170925	*B. amyloliquefaciens*
3	phosphopyruvate hydratase (enolase)	up	up	up	gi|489277787	*B. licheniformis*
4	flagellin A	up	up	up	gi|14278900	*B. subtilis*
5	malate dehydrogenase	up	up	up	gi|38515030	*Bacillus* sp. *5B6*
6	fructose-bisphosphate aldolase	up	same level	same level	gi|48924289	*Bacillus*
7	catalase	up	up	up	gi|696298641	*B. subtilis* group
8	glucose-6-phosphate isomerase	same level	up	same level	gi|489243924	*B. velezensis*
9	Hypothetical protein	same level	up	same level	gi|983385355	*B. velezensis*

SN; Supernatant and RE; Root exudates

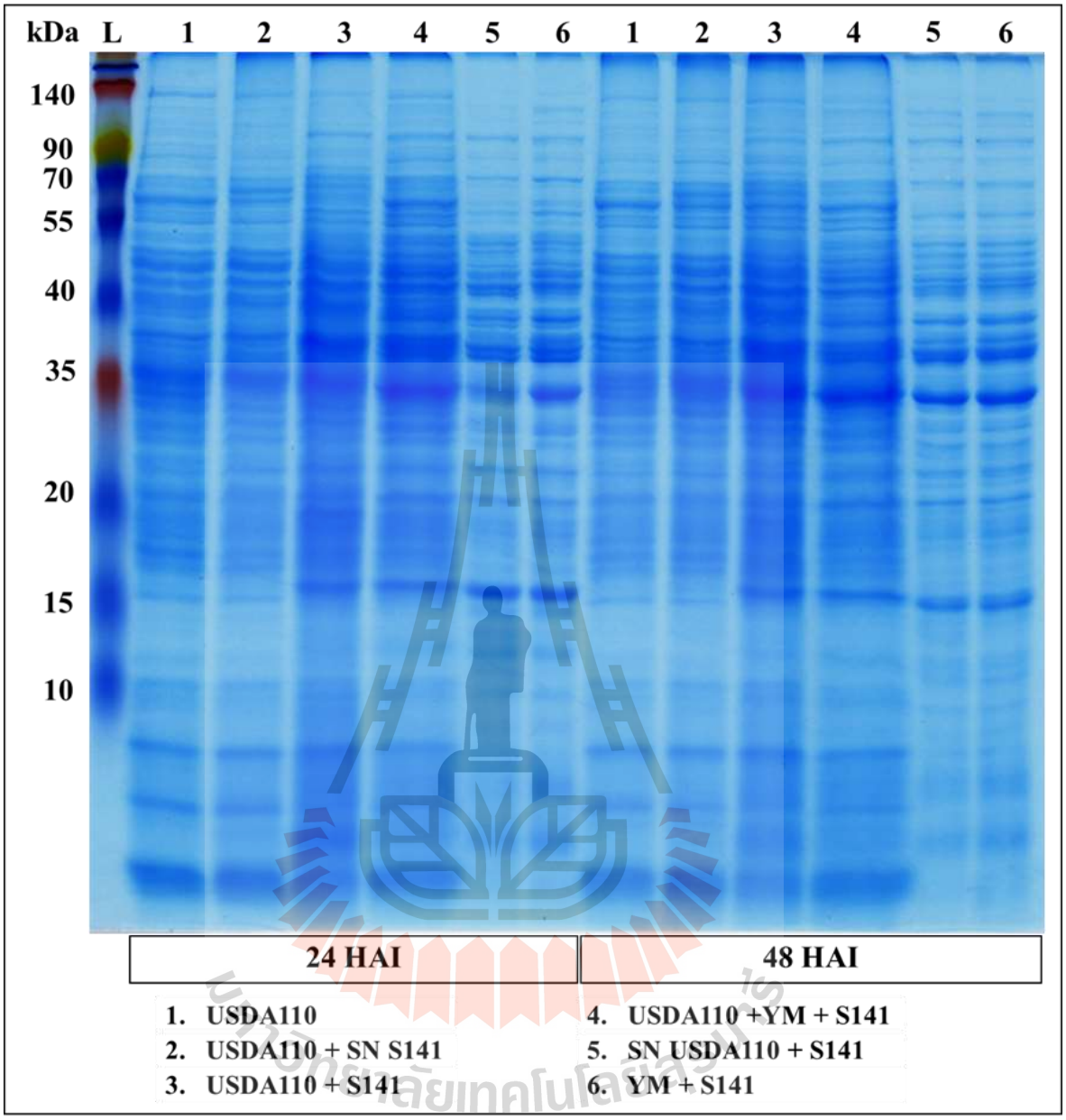

Figure 39. Electrophoretic profile of total cellular proteins from S141and USDA110 after induced by supernatant of S141 or USDA110 for 24 and 48 hour using 12% SDS-PAGE.

After induction of USDA110 by supernatant of S141 and induction of S141 by supernatant of USDA110 for 24 hours and 48 hours, the results of protein expressions were summarized in (Tables 7 and 8). The glutamate synthase (NADH), pyruvate carboxylase, nucleotidyl transferase, elongation factor Tu and fructose-bisphosphate aldolase were

down-regulated in S141 after induced by supernatant of USDA110 for 24 hours, and up-regulation of 3-phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase and malate/lactate dehydrogenase were detected. In USDA110 after induced by supernatant of S141 for 24 hours, down-regulation of glutamine synthetase, 50S ribosomal protein, transcriptional regulatory protein and DNA biding proteins were detected, while malate synthase, enoyl-CoA hydratase, ABC transporter substrate binding protein, outer membrane protein and acetoacetyl-CoA reductase were up-regulated. In addition, acetyl-coenzyme A synthetase was found more than one analog and up-regulated in USDA110. Furthermore, at 48 hours after induction by USDA110, down regulations of pyruvate carboxylase and succinate dehydrogenase flavoprotein subunit were detected, while malate/lactate dehydrogenase and putative intracellular protease/amidase were up-regulated in S141. On the other hand, at 48 hours after induction by S141, down regulations of ABC transporter substrate binding protein and glutamate synthase were presented, but putative outer membrane protein, malate synthase, malate dehydrogenase, acetoacetyl-CoA reductase and peroxiredoxin were up-regulated in USDA110.

Interestingly, malate synthase is up-regulated in USDA110, which is an enzyme that catalyzes the acetyl-CoA, and glyoxylate to synthesize the malate and CoA, then malate may secrete out of the cells. Beside, malate dehydrogenase (MDH) is up-regulated in S141, which are components of the malate-aspartate shuttle, are present in the 20 μm residue as well as in the soluble fraction (which is mainly of plant origin) of the nodule homogenate (Akkermans et al., 1981). After malate reaches, it is converted by malate dehydrogenase into oxaloacetate, during which NAD^+ is reduced with two electrons to form NADH. Oxaloacetate is then transformed into aspartate by aspartate aminotransferase. Since aspartate is an amino acid, an amino radical needs to be added to the oxaloacetate. This is supplied by glutamate, which in the process is transformed into alpha-ketoglutarate by the same enzyme (Appels and Haaker, 1991). The presence of glutamate, aspartate, and

dicarboxylic acids in appreciable concentrations in soybean seed and root exudates indicated that these compounds likely represent natural chemoattractants for *B. japonicum* (Barbour et al., 1991), may play a key role in enhancing nodulation as the result of high nodule number in soybean-*Bradyrhizobium* symbiosis.

Table 7. Protein expression of S141 after induced by supernatant of USDA110 and USDA110 after induced by supernatant of S141 for 24 hours.

No.	Protein prediction	Protein expression	Organism
1	Glutamate synthase (NADH) large chain	down	S141
2	Pyruvate carboxylase	down	S141
3	Nucleotidyl transferase superfamily	down	S141
4	Elongation factor Tu	down	S141
5	3-Phosphoglycerate kinase	up	S141
6	Glyceraldehyde-3-phosphate dehydrogenase	up	S141
7	Fructose-bisphosphate aldolase	down	S141
8	Malate/lactate dehydrogenase	up	S141
9	Malate synthase	up	USDA110
10	Acetyl-coenzyme A synthetase	up	USDA110
11	Enoyl-CoA hydratase	up	USDA110
12	Acetyl-coenzyme A synthetase	up	USDA110
13	Acetyl-coenzyme A synthetase	up	USDA110
14	Glutamine synthetase	down	USDA110
15	ABC transporter substrate binding protein	up	USDA110
16	50S ribosomal protein L5	down	USDA110
17	Outer membrane protein	up	USDA110
18	Acetoacetyl-CoA reductase	up	USDA110
19	Transcriptional regulatory protein	down	USDA110
20	DNA biding protein	down	USDA110

Table 8. Protein expression of S141 after induced by supernatant of USDA110 and USDA110 after induced by supernatant of S141 for 48 hours.

No.	Protein prediction	Protein expression	Organism
1	Pyruvate carboxylase	down	S141
2	Succinate dehydrogenase flavoprotein subunit	down	S141
3	Malate/lactate dehydrogenase	up	S141
4	Putative intracellular protease/amidase	up	S141
5	Putative outer membrane protein	up	USDA110
6	malate synthase	up	USDA110
7	ABC transporter substrate binding protein	down	USDA110
8	Malate dehydrogenase	up	USDA110
19	Glutamate synthase	down	USDA110
10	Acetoacetyl-CoA reductase	up	USDA110
11	peroxiredoxin	up	USDA110
12	50S ribosomal protein L7/L12	down	USDA110

4.7 Genetics analysis of S141

Microbial whole-genome sequencing is an important tool for mapping genomes of novel organisms, finishing genomes of known organisms, or comparing genomes across multiple samples. Sequencing the entire microbial genome is important for generating accurate reference genomes, for microbial identification, and other comparative genomic studies. Whole genome sequence of S141 was aimed to determine the genetic background of its soybean growth-promoting capacity involving symbiotic N_2 fixation with *B. diazoefficiens* USDA110.

The whole genome of S141 was presented in Figure 40, S141 genome was identified to comprise a 3,974,582 bp-long circular chromosome without a plasmid. Its genome sequence encoding at least 3,817 proteins-coding genes. General information about the genomes of S141 was showed 46.51% G+C content, 9 copies of ribosomal ribonucleic acid (rRNA) and 86 transfer ribonucleic acid (tRNA). *B. velezensis* S141 genome sequence was deposited in DDBJ/EMBL/GenBank under accession number AP018402 (Sibponkrung et al., 2017).

Figure 40. Circular representation of the chromosome of S141

Based on 16S rRNA gene analysis using BLAST searches, the highest sequence identities (up to 100%) were identified with *B. amyloliquefaciens*, *B. velezensis*, *B. subtilis*, and *B. methylotrophicus*, but blast data showed the highest score with *B. amyloliquefaciens* subsp. *plantarum* NAU-B3 (Figure 41). Average nucleotide identity (ANI) analysis using

the Map Reads to Reference tool indicated that among all related *Bacillus* species, the *B. methylotrophicus* NAU-B3 (*B. amyloliquefaciens* subsp. *plantarum* NAU-B3) genome sequence (Dunlap et al., 2015) showed the highest identity (96.44%) to that of strain S141. Strain NAU-B3 was recently reclassified as *B. velezensis* (Dunlap et al., 2016), and thus we decided to name strain S141 *B. velezensis* S141.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus velezensis GH1-13, complete genome	2870	25759	100%	0	100%	CP019040.1
Bacillus subtilis strain J-5, complete genome	2870	25770	100%	0	100%	CP018295.1
Bacillus velezensis 9912D, complete genome	2870	25815	100%	0	100%	CP017775.1
Bacillus amyloliquefaciens B15, complete genome	2870	21453	100%	0	100%	CP014783.1
Bacillus amyloliquefaciens UMAF6614, complete genome	2870	25781	100%	0	100%	CP006960.1
Bacillus methylotrophicus B25 genome assembly, chromosome : BAMMD1	2870	19698	100%	0	100%	LN999829.1
Bacillus subtilis strain ATCC 19217, complete genome	2870	20023	100%	0	100%	CP009749.1
Bacillus velezensis SQR9, complete genome	2870	20034	100%	0	100%	CP006890.1
Bacillus amyloliquefaciens subsp. *plantarum* NAU-B3, complete genome	2870	28567	100%	0	100%	HG514499.1
Bacillus amyloliquefaciens subsp. *plantarum* UCMB5036 complete genome	2870	26881	100%	0	100%	HF563562.1
Bacillus velezensis AS43.3, complete genome	2870	26560	100%	0	100%	CP003838.1

Figure 41. Ribosomal RNA of S141 nucleotide BLAST in NCBI

Considering based on genomic analysis, the genomic of S141 annotation data were used to categorized gene clusters responsible for the biosynthesis of numerous bioactive metabolites by the Kyoto Encyclopedia of Genes and Genomes (KEGG) system, 80.90% (3088 ORFs) of the ORFs of S141 comprise 16.94% of environmental information processing, 16.71% of genetic information processing, 8.23% of carbohydrate metabolism, 7.42% of amino acid metabolism, 7.12% of cellular processes, 5.21% of metabolism of

cofactors and vitamins, 3.89% of enzyme families, 3.79% of energy metabolism, 2.88% of nucleotide and 2.82% lipid metabolism (Figure 42).

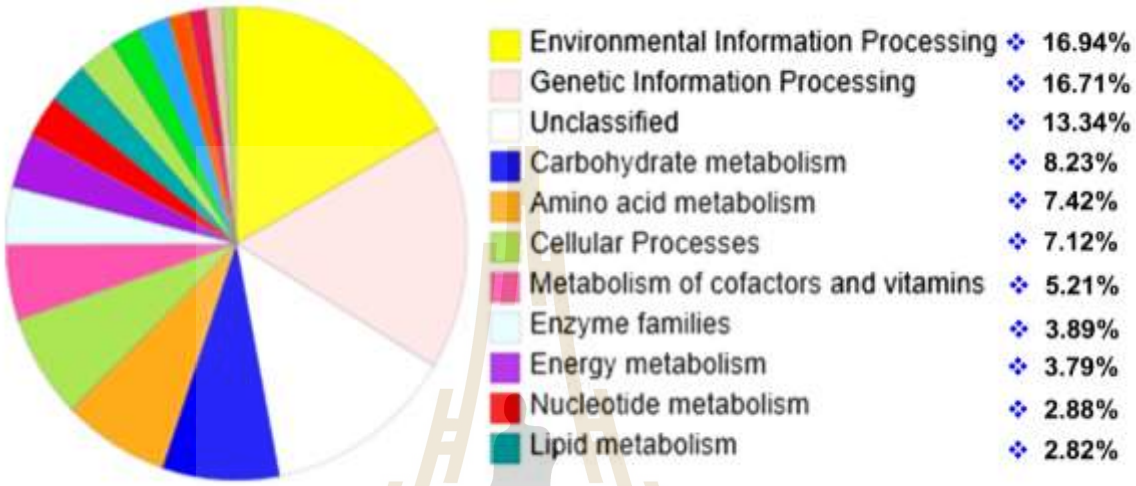

Figure 42. Gene functional annotation of *B. velezensis* S141 categorized using Kyoto Encyclopedia of Genes and Genomes (KEGG) system.

The survival of many bacteria, including pathogenic species, depends on the ability to synthesize the amino acid L-tryptophan whenever it is not available from the environment. The pathway for L-Trp biosynthesis is typically organized in a single strictly regulated *trp* operon and most often include *trpE*, *trpG*, *trpD*, *trpC*, *trpB* and *trpA* genes (Michalska et al., 2017). The enzyme TrpC catalyzes the formation of Indole-3-glycerol phosphate (IGP) from 1-(o-carboxyphenylamino) 1-deoxyribulose 5-phosphate as part of the tryptophan biosynthesis pathway. Based on molecular phylogenetic analysis by maximum likelihood method, *trpC* gene (Indole-3-glycerol phosphate synthase) was analyzed as shown in Figure 43. The result showed low similarity with NAU-B3 strain, while high similarity with FZB42 strain. Moreover, Idris et al. (2007) presented additional evidence for the existence of a tryptophan-dependent pathway as the main route of IAA biosynthesis in *B.*

amyloliquefaciens FZB42. The production of IAA in tryptophan-dependent IAA biosynthesis was dependent on tryptophan.

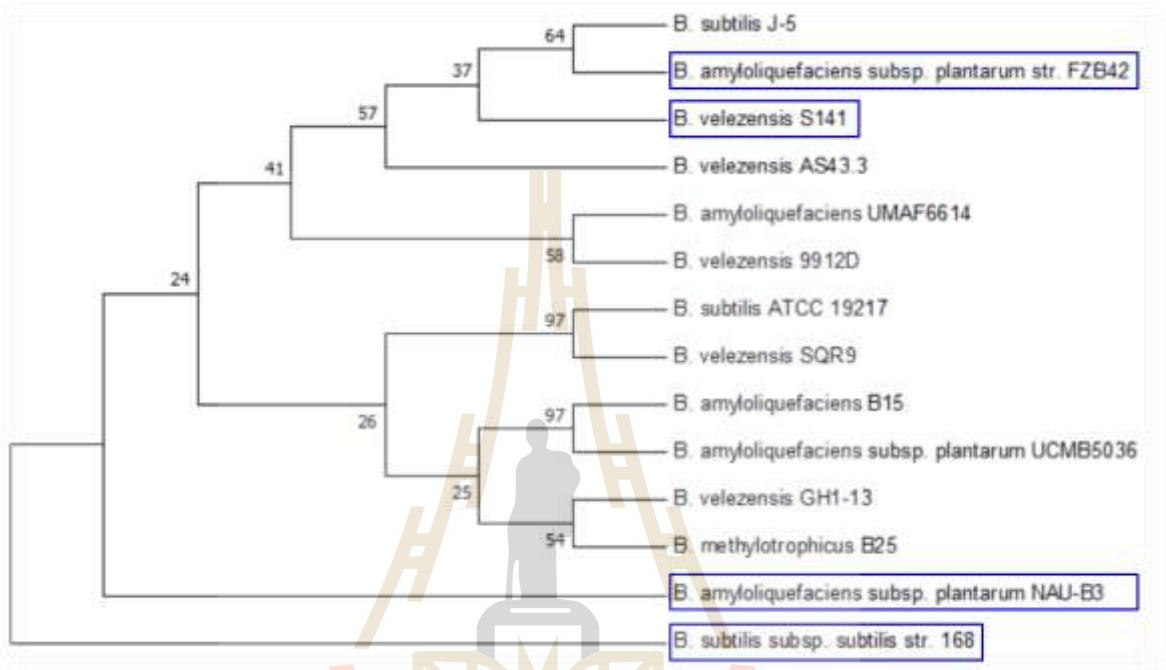

Figure 43. Molecular phylogenetic analysis of *trpC* gene (Indole-3-glycerol phosphate synthase) by maximum likelihood method.

According to Prakamhang et al. (2015) reported that isolate S141 was produced amounts of IAA into 19.33 ug/ml. Compared with another PGPR *Bacillus* strain, *B. velezensis* FZB42 (*B. amyloliquefaciens* subsp. *plantarum* FZB42) (Dunlap et al., 2016), S141 contains putative genes involved in indole-3-acetic acid (IAA) production were summerized in Table 9, including *IPyAD* and *dhaS* encoding indole-3-pyruvate decarboxylase and indole-3-acetaldehyde dehydrogenase, respectively, to synthesize IAA from indole-3-pyruvic acid; putative *iaaH* encoding indole-3-acetamide hydrolase to synthesize IAA from indole-3-acetamide; and *yhcX* encoding nitrilase to synthesize IAA from indole-3-acetonitrile. Additionally, *ysnE* was, which putatively encodes IAA

transacetylase involved in the tryptophan-independent IAA biosynthesis pathway. Moreover, tryptophan transporter, Indole-3-glycerol phosphate and tryptophan-rich sensory protein were found in S141 genome gain the upper hand of IAA biosynthesis pathway in *B. velezensis* FZB42. Furthermore, not only IAA biosynthesis pathway was found in S141 but also cytokinin biosynthesis pathway. The translated *IPT* gene products were predicted via the genome database of S141 to possess a tRNA delta (2)-isopentenyl pyrophosphate (IPP) transferase domain (protein_id BBA76310.1), which is critical for the enzyme's function in cytokinin biosynthesis. Isopentenyl transferases (IPTs) carry out the first and rate limiting step in cytokinin biosynthesis, where an isopentenyl group is transferred to either AMP, ADP or ATP (Mens et al., 2018). Besides, isopentenyl pyrophosphate isomerase (IPI) (protein_id BBA76708.1) was also found in S141, is an isomerase that catalyzes the conversion of the relatively un-reactive isopentenyl pyrophosphate (IPP) to the more-reactive electrophile dimethylallyl pyrophosphate (DMAPP). This isomerization is a key step in the biosynthesis of isoprenoids through the mevalonate pathway and the MEP pathway (Kaneda et al., 2001). Thus, these results suggested that *B. velezensis* S141 possesses multiple genes that are functionally related to auxin and cytokinin biosyntheses and play key roles in its ability to promote plant growth.

Table 9. Comparison of genes involved in indole-3-acetic acid (IAA) production between *B. velezensis* FZB42 with S141.

Gene in *Bacillus velezensis* S141		**Gene in *Bacillus velezensis* FZB42 (Reference strain)**	
Gene name	**Gene description**	**Gene name**	**Gene description**
menF	Isochorismate synthase	*trpE*	Anthranilate synthase
dhbC	Isochorismate synthase	*trpE*	Anthranilate synthase
trpA	tryptophan synthase (alpha subunit)	*trpA*	Tryptophan synthase (alpha subunit)
trpB	tryptophan synthase (beta subunit)	*trpB*	Tryptophan synthase (beta subunit)
trpF	phosphoribosylanthranilate isomerase	*trpF*	Phosphoribosyl anthranilate isomerase
trpC	indol-3-glycerol phosphate synthase	*trpC*	Indol-3-glycerol phosphate synthase
trpD	Anthranilate phosphoribosyltransferase	*trpD*	Anthranilate phosphoribosyl transferase
trpE	Anthranilate synthase	*trpE*	Anthranilate synthase
pabB	para-aminobenzoate synthase subunit A	*trpE*	anthranilate synthase
NADP-dependent glyceroldehyde 3-phosphate dehydrogenase	NADP-dependent glyceroldehyde 3-phosphate dehydrogenase	*dhaS*	NADP-dependent indole-3-aldehyde dehydrogenase

cont.

Table 9. Comparison of genes involved in indole-3-acetic acid (IAA) production between *B. velezensis* FZB42 with S141 (continued).

Gene in *Bacillus velezensis* S141		**Gene in *Bacillus velezensis* FZB42 (Reference strain)**	
Gene name	**Gene description**	**Gene name**	**Gene description**
aldehyde dehydrogenase	NAD-dependent aldehyde dehydrogenase	*dhaS*	NADP-dependent indole-3-aldehyde dehydrogenase
gbsA	NAD-dependent aldehyde dehydrogenase	*dhaS*	Indole-3-acetaldehyde dehydrogenase
gabD	NAD-dependent aldehyde dehydrogenase	*dhaS*	NADP-dependent indole-3-aldehyde dehydrogenase
aldehyde dehydrogenase	NAD-dependent aldehyde dehydrogenase	*dhaS*	NADP-dependent indole-3-aldehyde dehydrogenase
1-pyroline-5-carboxylate dehydrogenase	NAD-dependent aldehyde dehydrogenase	*dhaS*	NADP-dependent indole-3-aldehyde dehydrogenase
methylmalonate-semialdehyde dehydrogenase	NAD-dependent aldehyde dehydrogenase	*dhaS*	NADP-dependent indole-3-aldehyde dehydrogenase

cont.

Table 9. Comparison of genes involved in indole-3-acetic acid (IAA) production between *B. velezensis* FZB42 with S141 (continued).

Gene in *Bacillus velezensis* S141		**Gene in *Bacillus velezensis* FZB42 (Reference strain)**	
Gene name	**Gene description**	**Gene name**	**Gene description**
AldX	NAD-dependent aldehyde dehydrogenase	*dhaS*	NADP-dependent indole-3-aldehyde dehydrogenase
ywdH	Aldehyde dehydrogenase	*dhaS*	NADP-dependent indole-3-aldehyde dehydrogenase
1-pyroline-5-carboxylate dehydrogenase	1-pyroline-5-carboxylate dehydrogenase	*dhaS*	NADP-dependent indole-3-aldehyde dehydrogenase
acetyltransferase	Acetyltransferase	*ysnE*	IAA transacetylase
amidohydrolase	Amidohydrolase	*yhcX*	Nitrilase, hydrolase carbon-nitrogen family
yhcX	Carbon-nitrogen hydrolase protein family (amidohydrolase)	*yhcX*	Nitrilase, hydrolase carbon-nitrogen family
trpP	Tryptophan transporter		
B938_10225	Indole-3-glycerol phosphate		cont.

Table 9. Comparison of genes involved in indole-3-acetic acid (IAA) production between *B. velezensis* FZB42 with S141 (continued).

Gene in *Bacillus velezensis* S141		**Gene in *Bacillus velezensis* FZB42 (Reference strain)**	
Gene name	**Gene description**	**Gene name**	**Gene description**
hypothetical protein	Anthranilate/para-aminobenzoate		
pabA	para-aminobenzoate/anthranilate synthase glutamate transferase component II		
Translocator protein peripheral-receptor	Tryptophan-rich sensory protein		

4.8 Colonization of S141 on soybean-*Bradyrhizobium* symbiosis

The green fluorescent protein (GFP) was constructed into S141 and GFP gene was activated in S141 by insertion to *tuf* promoter gene (Figure 44), which was used as the GFP-tagged strain for the colonization on soybean-*Bradyrhizobium* symbiosis (Figure 45). The colonization of S141 on soybean-*Bradyrhizobium* symbiosis was demonstrated in Figure 46. The results showed S141 colonize around nodule and root surface of soybean. These results confirmed that S141 act closely in rhizosphere as plant growth promoting rhizobacteria.

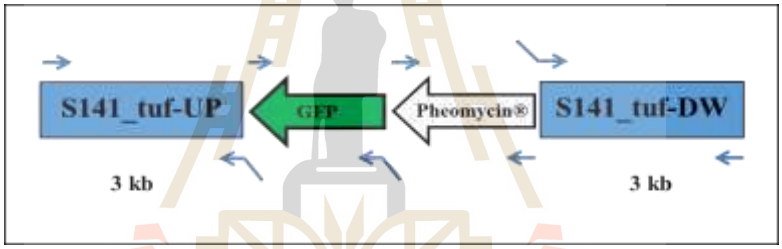

Figure 44. Construction of GFP and phleomycin resistance gene into S141 by insertion to *tuf* promoter gene.

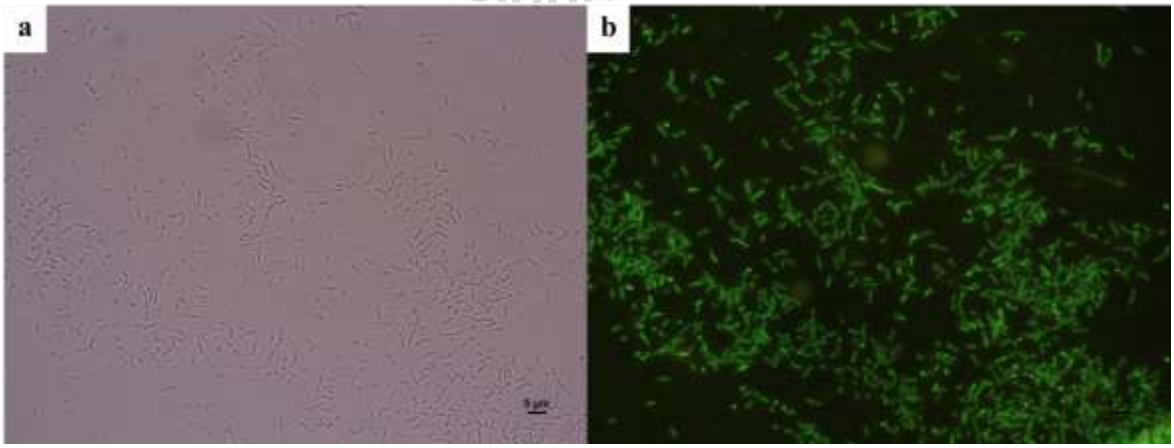

Figure 45. Visualization of green fluorescent protein (GFP) expression in S141: (a) bright-field and (b) fluorescence in free cells of S141.

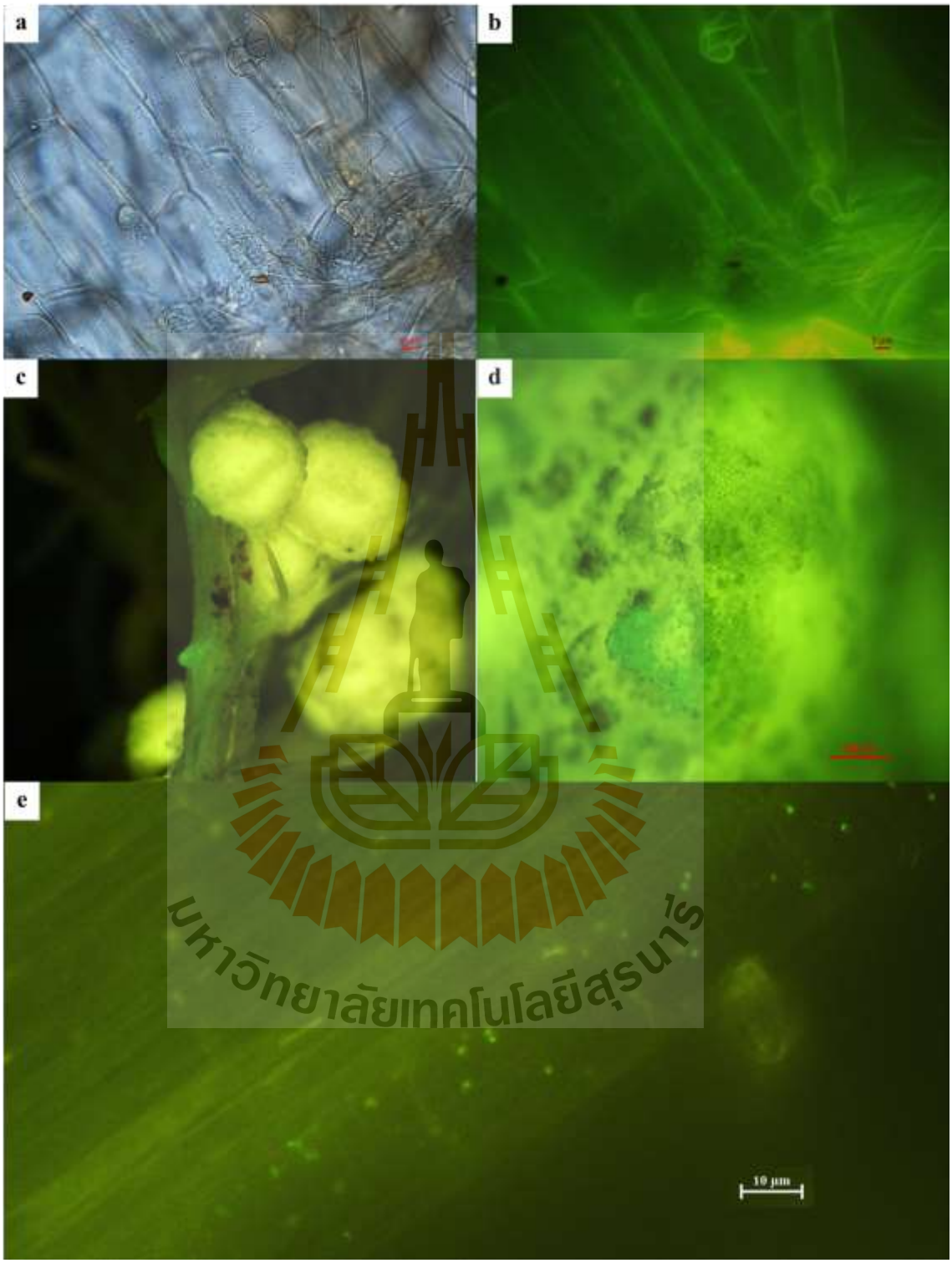

Figure 46. Colonization of S141 GFP-tagged strain on soybean rhizosphere: (a) bright-field and (b) fluorescence of S141 colonize around soybean root surface, (c) colonization of S141 around soybean root surface, (d) colonization of S141 around soybean nodule and (e) fluorescence of S141 colonize around soybean root surface.

4.9 Effect of S141 and plant growth hormone on soybean-*Bradyrhizobium* symbiosis

To demonstrate the effect of co-inoculation between USDA110 and S141 on soybean-*Bradyrhizobium* symbiosis in comparison with commercial plant growth hormone, the plant growth parameters including nitrogen-fixing efficiency, nodule dry weight, root dry weight, shoot dry weight and total plant dry weight in soybean cultivar Chaing Mai 60 were determined under Leonard's jar experiments. The results found that S141 showed significantly capable of promoting one or more plant parameters including nitrogen-fixing efficiency, nodule number, nodule dry weight, root dry weight, shoot dry weight and total plant dry weight when co-inoculated with strains of *B. diazoefficiens* USDA110 over the single inoculation on soybean at 45 DAI ($P<0.05$). Variations in plant growth parameters of soybean at 45 DAI as consequent of single inoculation, single inoculation supplemented with IAA and/or 6-BA and co-inoculation were presented in Figures 47 and 48. The co-inoculation of S141 with USDA110 showed the highest nitrogen-fixing efficiency, nodule dry weight, root dry weight, shoot dry weight and total plant dry weight when compared with all inoculation treatments and control. At the same time, nitrogen-fixing efficiency was not show different between co-inoculation of USDA110 with S141 and single inoculation of USDA110 supplemented with IAA but, it showed significantly different with single inoculation of USDA110 supplemented with 6-BA and 6-BA plus IAA treatments. In case of nodule dry weight, root dry weight, shoot dry weight and total plant dry weigh the results displayed the same pattern, which is the highest in co-inoculation of S141 with USDA110 followed by a single inoculation of USDA110 supplemented with IAA, single inoculation of USDA110, single inoculation of USDA110 supplemented with IAA plus 6-BA and single inoculation of USDA110 supplemented with 6-BA, respectively.

The co-inoculation of S141 with USDA110 also did not showed significantly different in nodule dry weight with single inoculation of USDA110 and single inoculation of USDA110 supplemented with IAA but, it showed significantly different with single inoculation of USDA110 supplemented with 6-BA and 6-BA plus IAA treatments. Meanwhile, nodule dry weight demonstrated the significantly different (Figure 47), indicated that sizes of the nodule are different. The nodule sizes separation showed significantly different between single inoculation and co-inoculation as presented in Figure 49. The co-inoculation of S141 with USDA110 showed the highest nodule number of L size nodules when compared with single inoculation treatment followed by a single inoculation of USDA110 supplemented with IAA, single inoculation of USDA110, single inoculation of USDA110 supplemented with IAA plus 6-BA and single inoculation of USDA110 supplemented with 6-BA, respectively. While nodule number of M size nodules, the single inoculation of USDA110 supplemented with IAA displayed the highest when compared with all inoculation treatments.

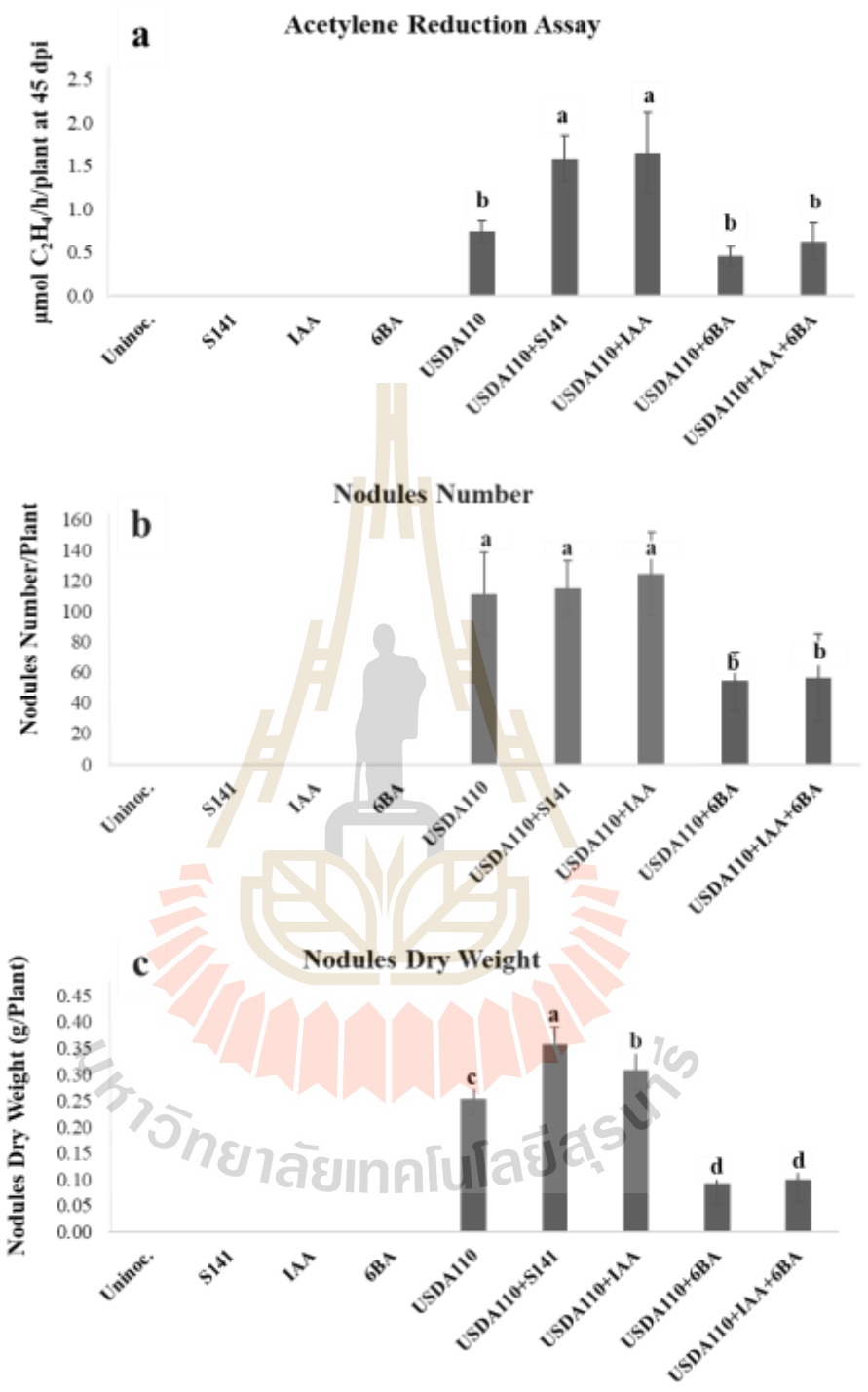

Figure 47. Plant growth parameters of soybean cultivar Chaing Mai 60 by co-inoculation with S141 and USDA110 compared with single inoculation supplemented with Indole-3-Acetic Acid and/or 6-Benzylaminopurine at 45 DAI: (a) nitrogenase activity was determined using the acetylene reduction assay, (b) nodule numbers per plant and (c) nodule dry weight. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=8)

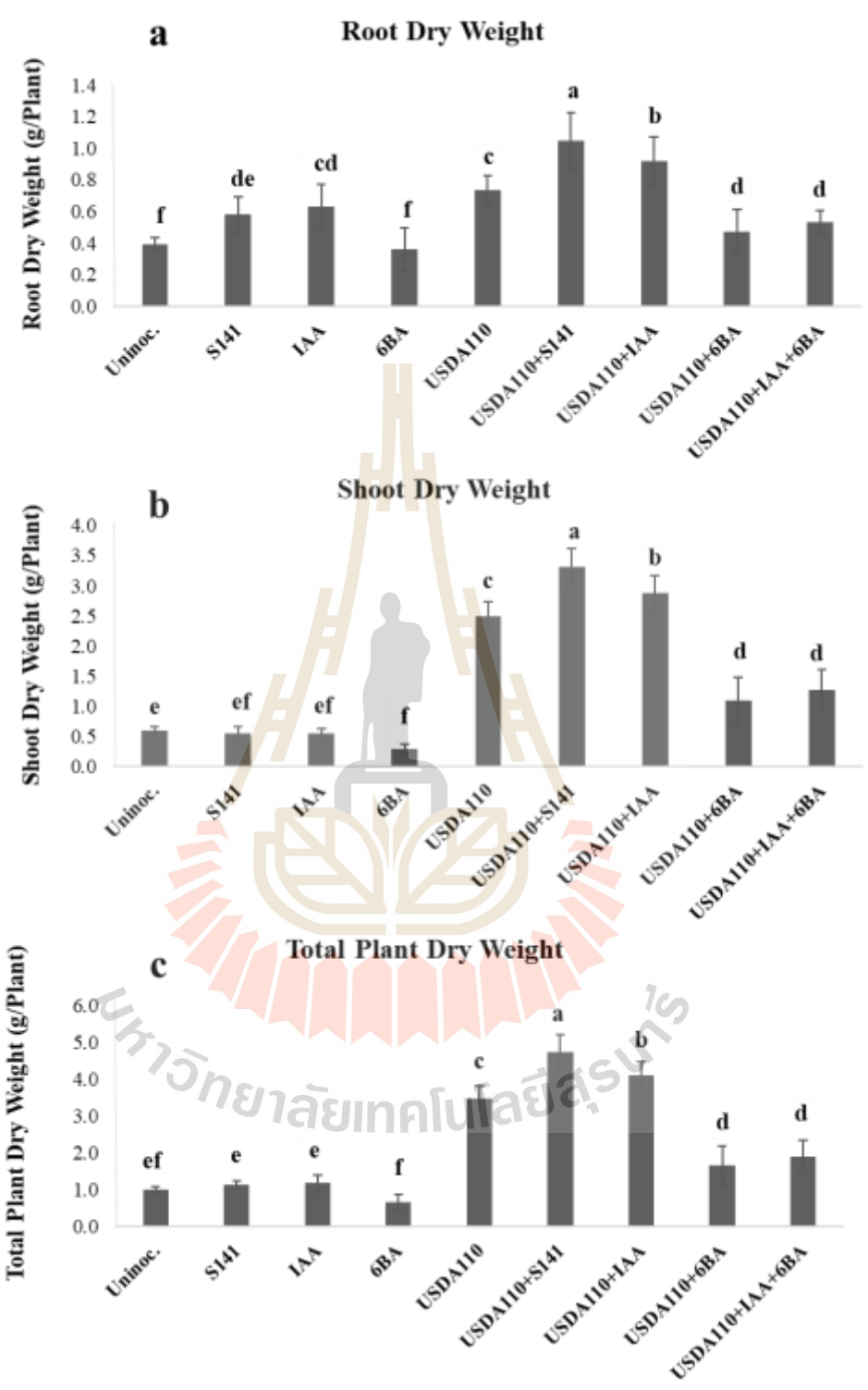

Figure 48. Plant growth parameters of soybean cultivar Chaing Mai 60 by co-inoculation with S141 and USDA110 compared with single inoculation supplemented with Indole-3-Acetic Acid and/or 6-Benzylaminopurine at 45 DAI: (a) root dry weight, (b) shoot dry weight and (c) total plant dry weight. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=8)

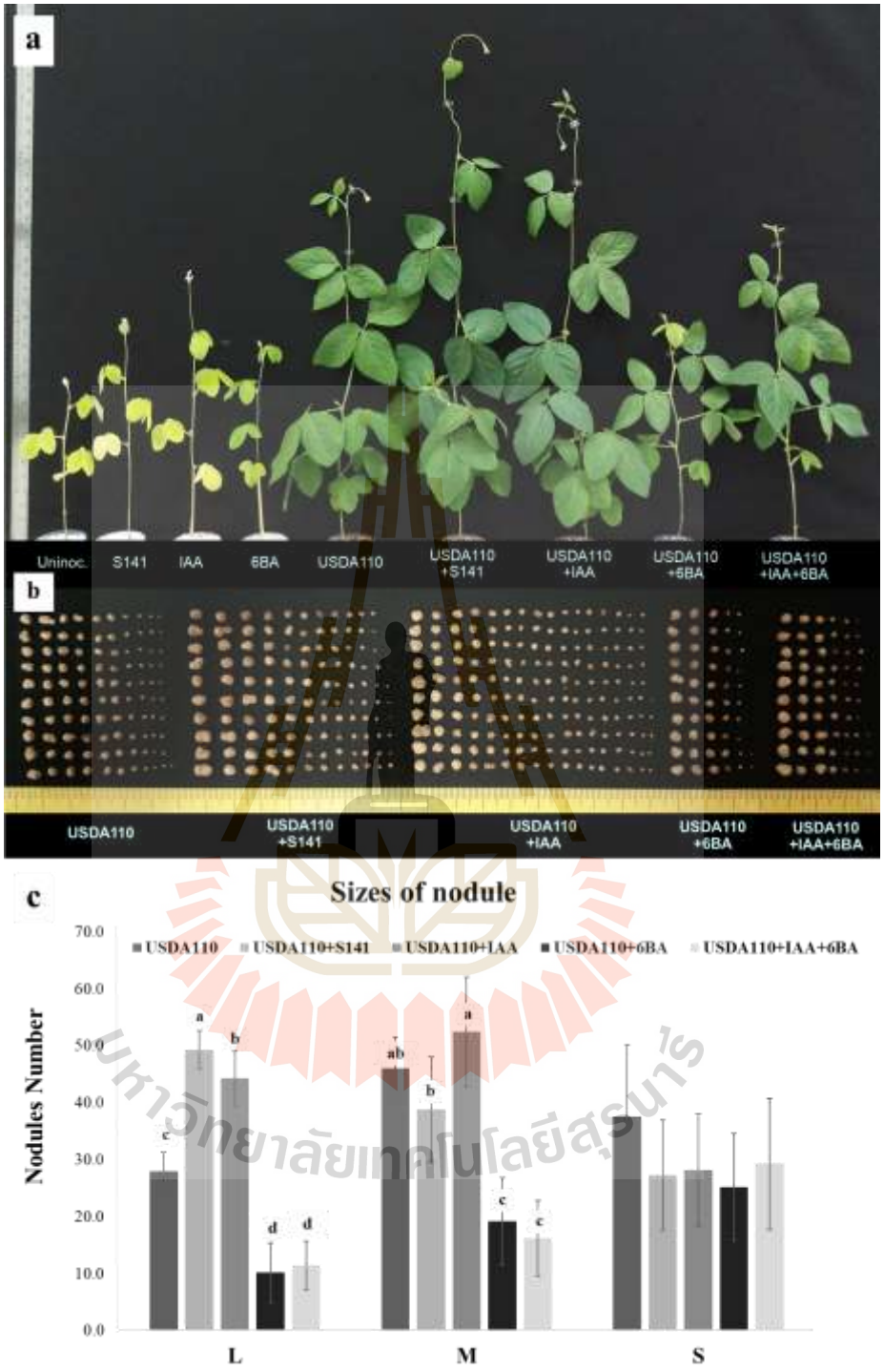

Figure 49. Soybean plant growth and the nodule sizes separation of soybean cultivar Chaing Mai 60 by co-inoculation with S141 and USDA110 and/or supplemented with IAA and/or 6BA at 45 DAI: (a) soybean growth under Leonard's jar experiments, (b) photograph of soybean nodule and (c) nodule sizes separation of soybean. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=8).

The nodule sizes separation was changed to the percentage of each nodule size. Consideration the percentage of each nodule size regardless of nodule number demonstrated the significantly different between single inoculation and co-inoculation as presented in Figure 50. The co-inoculation of S141 with USDA110 and single inoculation of USDA110 supplemented with IAA performed the highest percentage of nodule number of L size nodule and showed significantly different with single inoculation of USDA110, single inoculation of USDA110 supplemented with IAA plus 6-BA and single inoculation of USDA110 supplemented with 6-BA, but the nodule number in M size nodule was not different in all treatments. In contrast, the nodule number of S size nodule showed the highest in the single inoculation of USDA110 supplemented with IAA plus 6-BA and single inoculation of USDA110 supplemented with 6-BA followed by single inoculation of USDA110, co-inoculation of S141 with USDA110 and single inoculation of USDA110 supplemented with IAA, respectively.

The results indicated that co-inoculation of USDA110 with S141 promoted soybean growth by increasing nitrogen-fixing efficiency, nodule dry weight, root dry weight, shoot dry weight and total plant dry weight on soybean-*Bradyrhizobium* symbiosis at 45 DAI. Interestingly, co-inoculation of USDA110 with S141 and single inoculation of USDA110 supplemented with IAA enhanced soybean-*Bradyrhizobium* symbiosis to produced L size of nodules as the result of increasing nitrogen-fixing efficiency. It seems like IAA and also IAA produced from S141 had the effect with cell elongation of nodules. Auxin was the first identified phytohormone that plays essential roles to regulate diverse aspects of cell proliferation and differentiation (Chapman and Estelle 2009). The results was consistent with Liu et al. (2018) indicated that the auxin signaling pathway is required for both infection thread formation and nodule development. Auxin signaling initiates this infection thread and cytokinin signaling is required for infection thread elongation. This symbiotic signal induces cortex cell division and initiates nodule organogenesis. Auxin and cytokinin

are key regulators of cortical cell proliferation and differentiation and are essential for further nodule development. However, the commercial cytokinin; 6-Benzylaminopurine (6-BA) was not promoted soybean growth and nodulation in these experiments because it seems high concentration of cytokinin the retard growth of soybean-*Bradyrhizobium* symbiosis. A wide range of microorganisms found in the rhizosphere are able to produce substances that regulate plant growth and development. PGPR produce phytohormones such as auxins and cytokinins can affect cell proliferation in the root architecture by overproduction of lateral roots and root hairs with a subsequent increase of nutrient and water uptake (Arora et al., 2013). Moreover, scientists have uncovered the critical role of cytokinin signaling in root nodule symbiosis. It has been shown that rhizobia can produce cytokinin, which was proposed to be important for nodule formation (Sturtevant and Taller, 1989). Liu et al. (2018) suggested that an appropriate level of cytokinin is required for nodule development. Carimi et al. (2003) reported that high concentrations of cytokinins block cell proliferation and induce programmed cell death (PCD) in both carrot (*Daucus carota* L.) and *Arabidopsis thaliana* (L.) Heynh. Also Mens et al, (2018) proposed that cytokinins are important regulators of cell proliferation and differentiation in plant development, a role for this phytohormone group in soybean nodulation is shown through the exogenous application of cytokinins (6-benzylaminopurine, N6-(12-isopentenyl)-adenine and trans-zeatin) via either root drenching or a petiole feeding technique. Overall, nodule numbers were reduced by treatment with high cytokinin concentrations, but increased with lower concentrations.

The cross section of nodules from co-inoculation of USDA110 with S141 and single inoculation of USDA110 in each size were depicted in Figure 51. Co-inoculation of USDA110 with S141 nodules cross-section showed a thinner layer of the cortex and larger area of the infection zone than the nodules of single inoculation. It is obviously that IAA might play an important role in cortex cell elongation and proliferation. This result

consistent with Liu et al. (2018) who revealed that auxin and cytokinin are key regulators of cortical cell proliferation and differentiation and are essential for further nodule development.

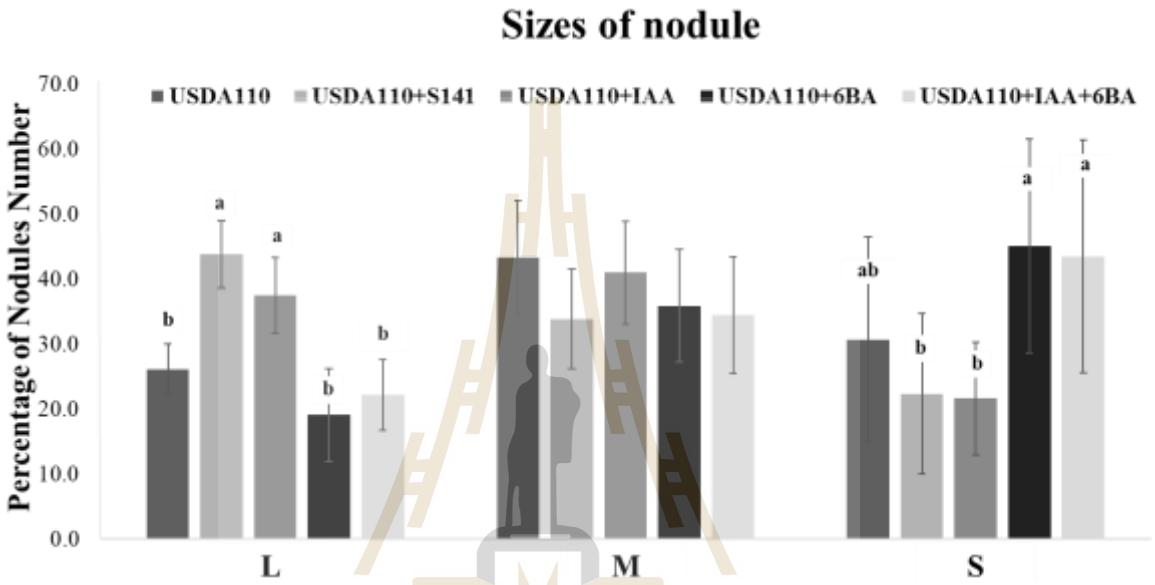

Figure 50. The nodule sizes separation of soybean cultivar Chaing Mai 60 by co-inoculation with S141 and USDA110 and/or supplemented with IAA and/or 6BA at 45 DAI. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=8).

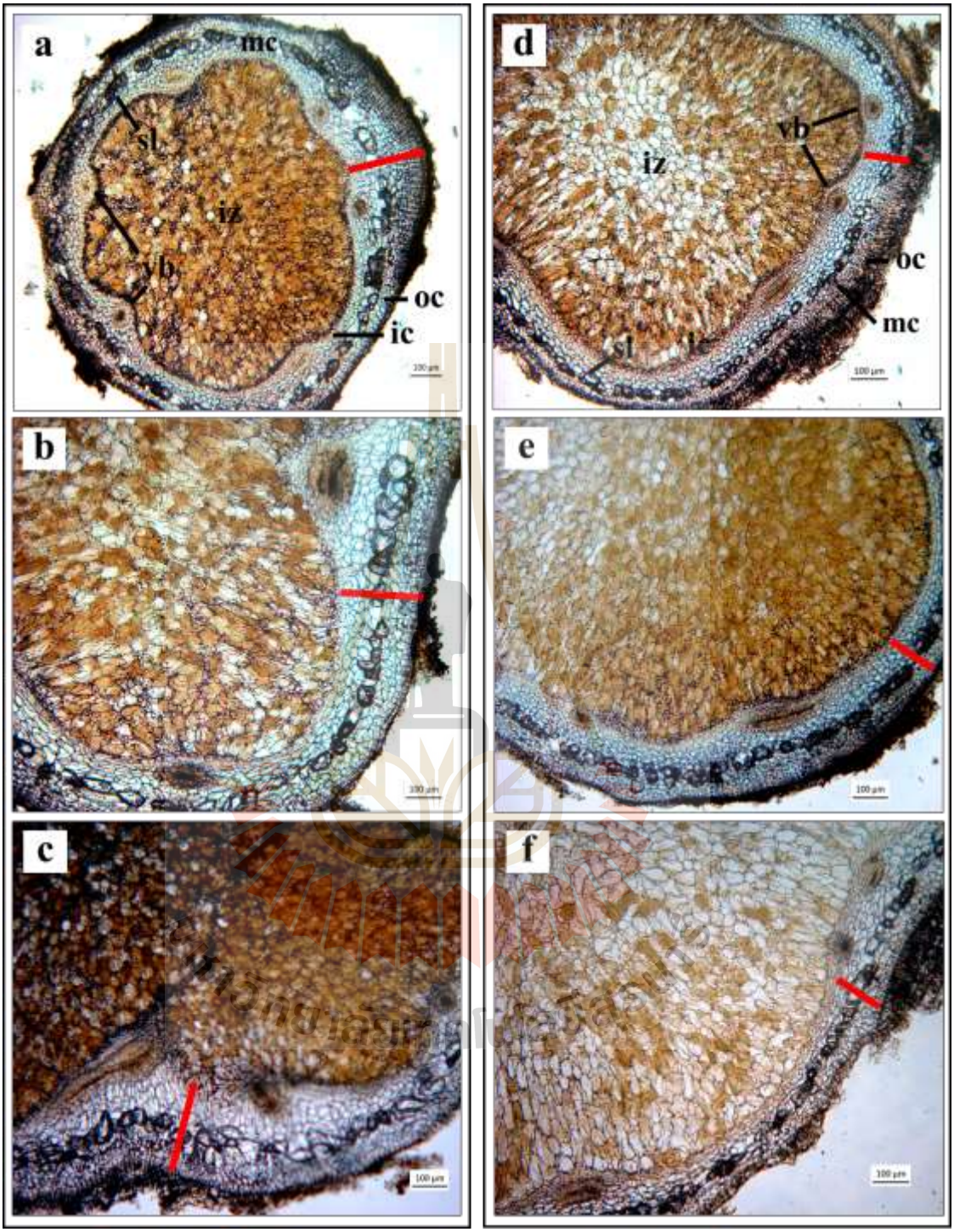

Figure 51. Comparison of soybean nodule cross section under light compound microscope between single inoculation of USDA110 (a) small nodule (b) medium nodule and (c) large nodule and co-inoculation of USDA110 with S141 (d) small nodule (e) medium nodule and (f) large nodule at 45 DAI: iz, infected zone; ic, inner cortex; mc, middle cortex; oc, outer cortex; vb, vascular bundles and sl, sclerid layer. Red bar indicated length of cortex zone.

4.10 Effect of plant growth hormone biosynthesis in S141 on soybean-*Bradyrhizobium* symbiosis

To determine the genes involved in biological determinant as IAA biosynthesis of S141 the related genes were disrupted, the verifications of S141 mutant strains were revealed in Figure 52.

Figure 52. The verification of S141 mutant strains in *dhaS*, *yhcX* and *IPyAD* genes in auxin biosynthesis pathways and *IPT* and *IPI* genes in cytokinin biosynthesis pathways of S141. Red boxes indicated the DNA insertion band to disruption of each genes.

The putative gene related to IAA biosynthesis pathway including: *dhaS*, *yhcX* and *IPyAD* were disrupted (Figure 53). In order to replace the respective wild-type genes, appropriate gene cassettes consisting of the antibiotics resistance determinant flanked by the respective gene sequences were constructed. The IAA production of S141 and S141 IAA related mutant strains were determined as described by (Costacurta et al., 1998).

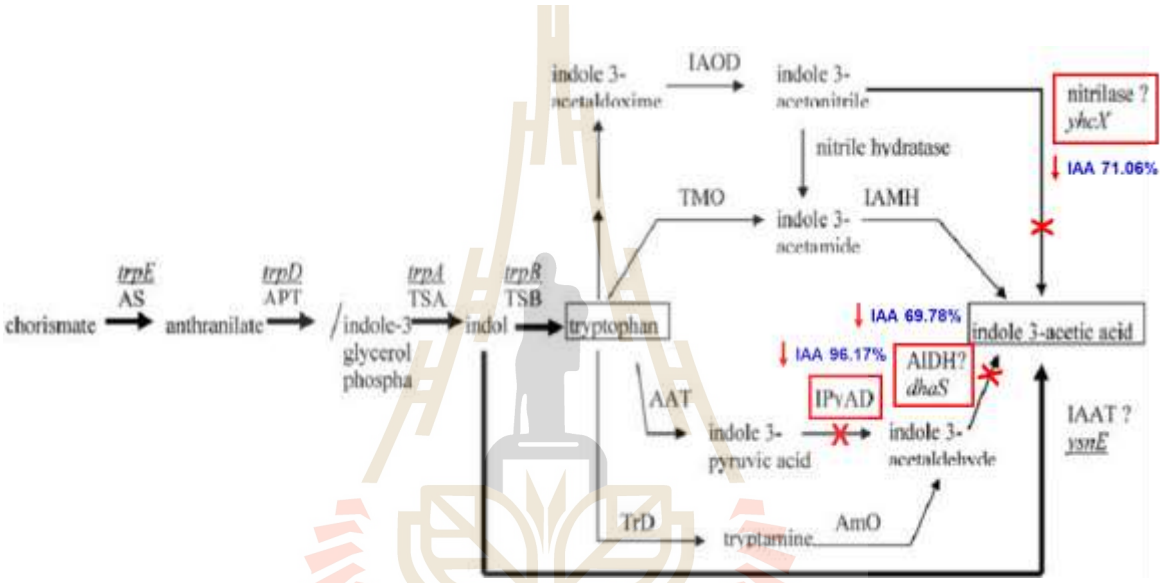

Figure 53. Disruption of *dhaS*, *yhcX* and *IPyAD* genes in tryptophan-dependent pathways of S141 indole-3-acetic acid (IAA) synthesis (Modified from Idris et al. (2007)). Red arrow indicated percentage of reduction of IAA production in S141 IAA related mutant strains.

The result of IAA production in S141 and S141 IAA related mutant strains was presented in Figure 54. Quantification of the IAA amounts presented in culture filtrates of strains S141*ΔdhaS*, S141*ΔyhcX* and S141*ΔIPyAD* by the colorimetrically methodology revealed reduction amounts of IAA. The S141*ΔIPyAD* mutant strain formed only 3.83% of the amount produced by the wild type, while the strain bearing the mutation S141*ΔyhcX* produced 28.94% of the amount of the wild type. Strain S141*ΔdhaS* produced 30.22% of

the amount of the wild type, all of S141 IAA related mutant strains reduced amounts of IAA production more than 50% when compared with wild type. The results suggested that all of S141 IAA related mutant strains are related with IAA biosynthesis pathway. However, Idris et al. (2007) reported that strain E104 (*ΔdhaS*) was not significantly affected in its IAA production, suggesting no participation of the *dhaS* gene product in IAA synthesis.

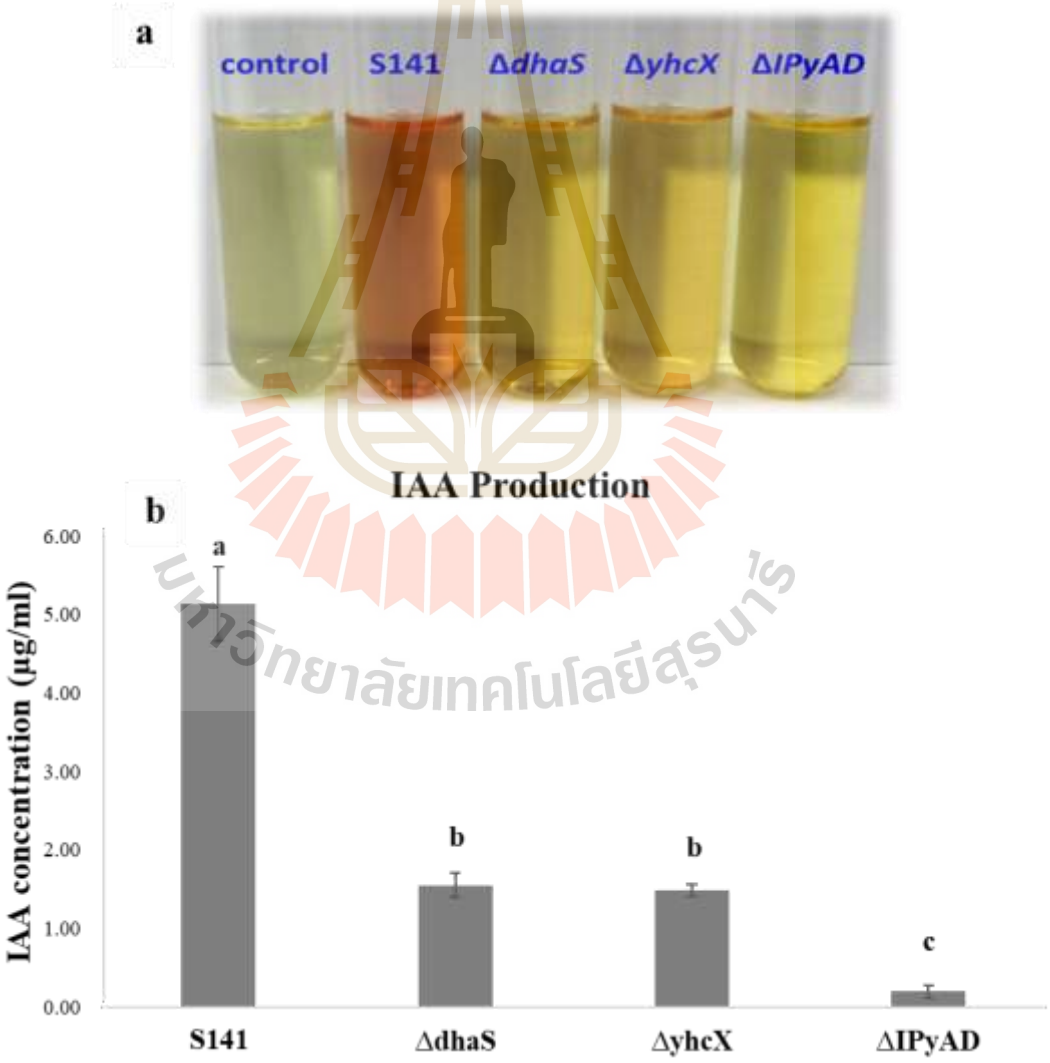

Figure 54. Quantification of the IAA production in S141 and S141 IAA related mutant strains (a) colorimetrically of IAA production (b) IAA concentration in S141 and S141 IAA related mutant strains.

In S141, the gene related to cytokinin biosynthesis pathway including: *IPT*-gene coding for isopentenyl transferase and *IPI*-gene coding for isopentenyl isomerase were disrupted (Figure 55). In order to replace the respective wild-type genes, appropriate gene cassettes consisting of the antibiotics resistance determinant flanked by the respective gene sequences were constructed.

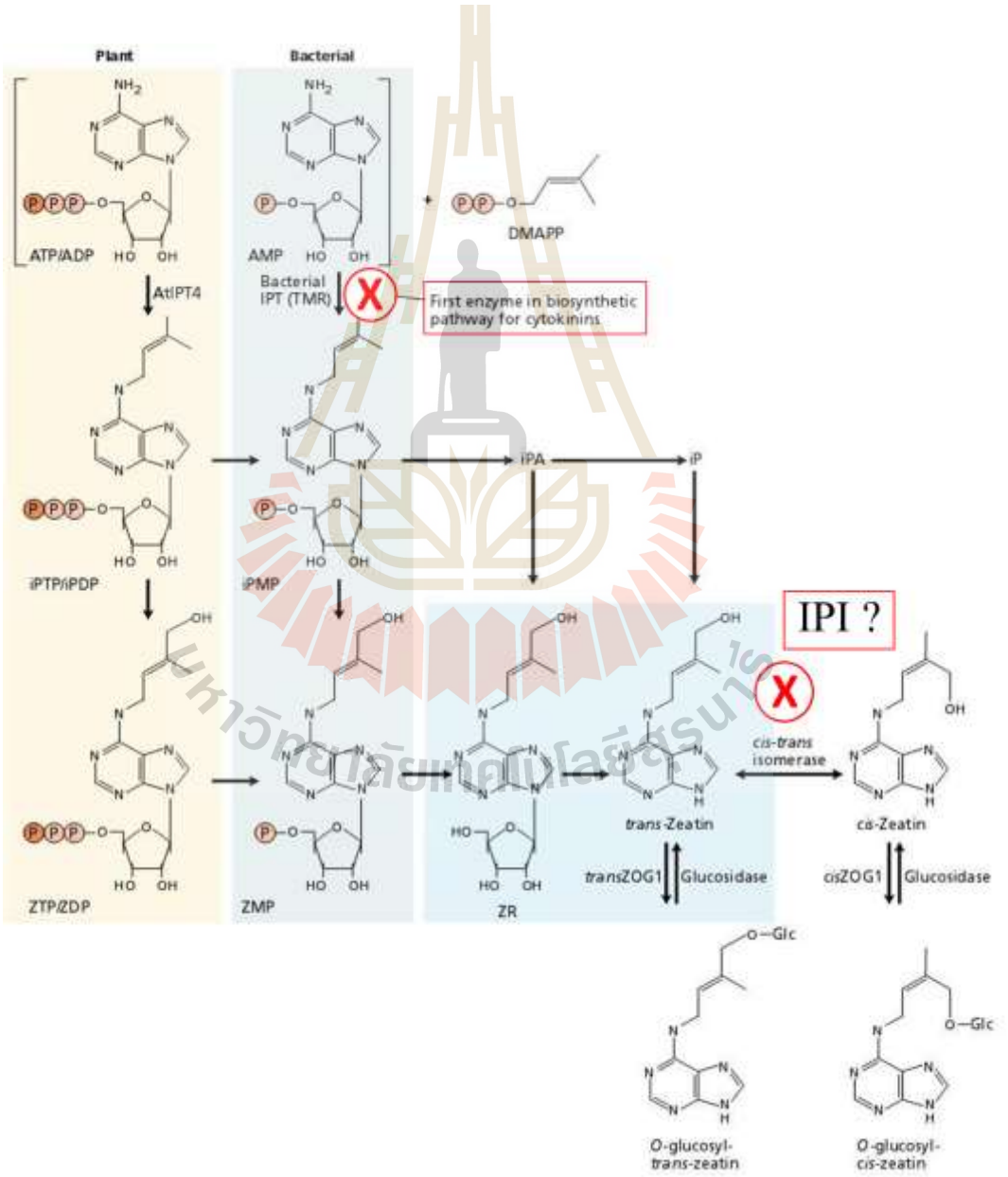

Figure 55. Disruption of *IPT* and *IPI* gene in cytokinin biosynthesis pathways of S141 (Modified from Kakimoto, (2003)).

The mutant strains including IAA related mutant strains (S141*ΔdhaS*, S141*ΔyhcX* and S141*ΔIPyAD*) and cytokinin related mutant strains (S141*ΔIPT* and S141*ΔIPI*) were analyzed for plant growth promoting activity in soybean cultivar Chaing Mai 60 under Leonard's jar experiments. Plant growth conditions in laboratory designed to verify and further characterize these effects. The results found that S141 performed significantly capable of promoting one or more plant parameters including nitrogen-fixing efficiency, nodule number, nodule dry weight, root dry weight, shoot dry weight and total plant dry weight when co-inoculated with strains of *B. diazoefficiens* USDA110 over the single inoculation and co-inoculation with S141 mutant strains on soybean at 45 DAI ($P<0.05$). Variations in plant growth parameters of soybean at 45 DAI as consequent of single inoculation, co-inoculation with S141 mutant strains were presented in Figures 56, 57 and 58. The co-inoculation of S141 with USDA110 showed the highest nitrogen-fixing efficiency, nodule dry weight, root dry weight, shoot dry weight and total plant dry weight compared with all inoculation treatments and control. The nitrogen-fixing efficiency was not different in co-inoculation with USDA110 and S141, co-inoculation of USDA110 with S141*ΔIPT* and co-inoculation of USDA110 and S141*ΔIPI* but showed significantly different with single inoculation of USDA110, and co-inoculation of USDA110 with IAA related mutant strains S141*ΔdhaS* and S141*ΔIPyAD* and showed the lowest nitrogen-fixing efficiency on co-inoculation of USDA110 with S141*ΔyhcX* treatments.

In case of nodule dry weight, it showed the highest value when co-inoculated USDA110 with S141, co-inoculation of USDA110 with S141*ΔIPT*, co-inoculation of USDA110 with S141*ΔIPI* and co-inoculation of USDA110 with S141*ΔIPyAD* showed significantly different when compared with co-inoculation of USDA110 with S141*ΔyhcX*, co-inoculation of USDA110 with S141*ΔdhaS* and single inoculation of USDA110. For root dry weight, it showed the hightest value when co-inoculated USDA110 with S141 followed by co-inoculation of USDA110 with all of mutant strains and single inoculation of

USDA110 and the lowest numbers were found in S141*ΔdhaS,* S141*ΔyhcX* and control. The results of shoot dry weight and total plant dry weigh showed the same pattern, which is the highest values were found when co-inoculated S141 with USDA110 followed by co-inoculation of USDA110 with both cytokinin related mutant strains (S141*ΔIPT* and S141*ΔIPI*), co-inoculation of USDA110 with S141*ΔIPyAD,* co-inoculation of USDA110 with S141*ΔdhaS* and single inoculation of USDA110, co-inoculation of USDA110 with S141*ΔyhcX* and showed the lowest in S141*ΔdhaS* and S141*ΔyhcX,* respectively.

The co-inoculation of S141 with USDA110 also did not showed significantly different in nodule dry weight with single inoculation of USDA110 and co-inoculation of USDA110 with S141*ΔIPT,* S141*ΔIPI,* S141*ΔIPyAD* and S141*ΔdhaS* but, it displayed significantly different with co-inoculation of USDA110 with S141*ΔyhcX.* Meanwhile, nodule dry weight showed significantly different (Figure 57), indicated that sizes of the nodule are the differences. The nodule sizes separation performed significantly different between single inoculation and co-inoculation as illustrated in Figure 59. The co-inoculation of S141 with USDA110 showed the highest nodule number of VL size nodules similarly with co-inoculation of USDA110 with S141*ΔIPT,* while co-inoculations of USDA110 with S141*ΔIPI,* S141*ΔIPyAD,* S141*ΔyhcX* and S141*ΔdhaS* reduced nodule number of VL size nodules. Moreover, co-inoculation of USDA110 with S141*ΔyhcX* also reduced nodule number of L size nodules.

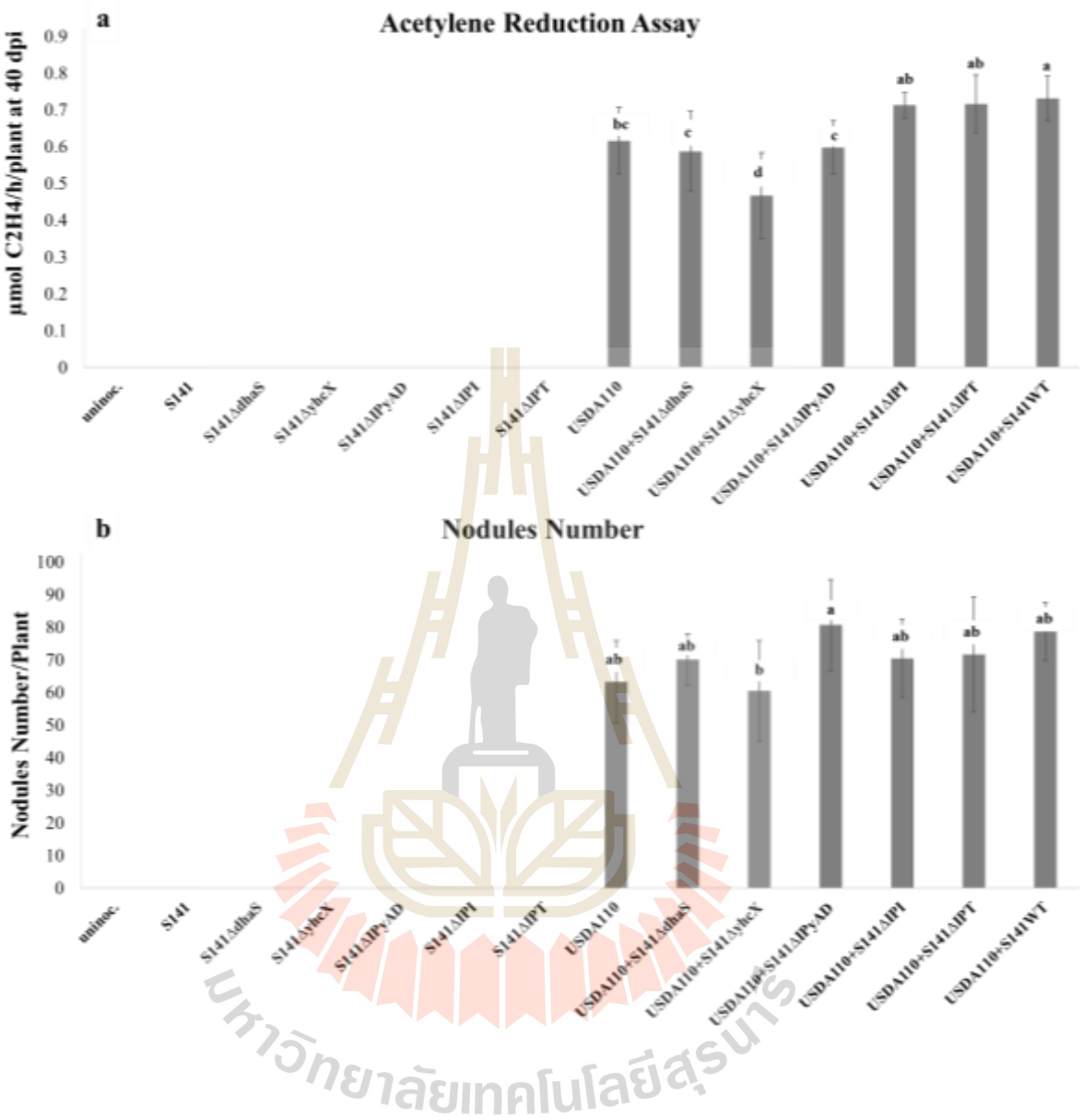

Figure 56. Plant growth parameters of soybean cultivar Chaing Mai 60 by co-inoculation with USDA110 and S141 or S141 mutant strains compared with single inoculation at 45 DAI: (a) nitrogenase activity was determined using the acetylene reduction assay and (b) nodule numbers per plant. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=8).

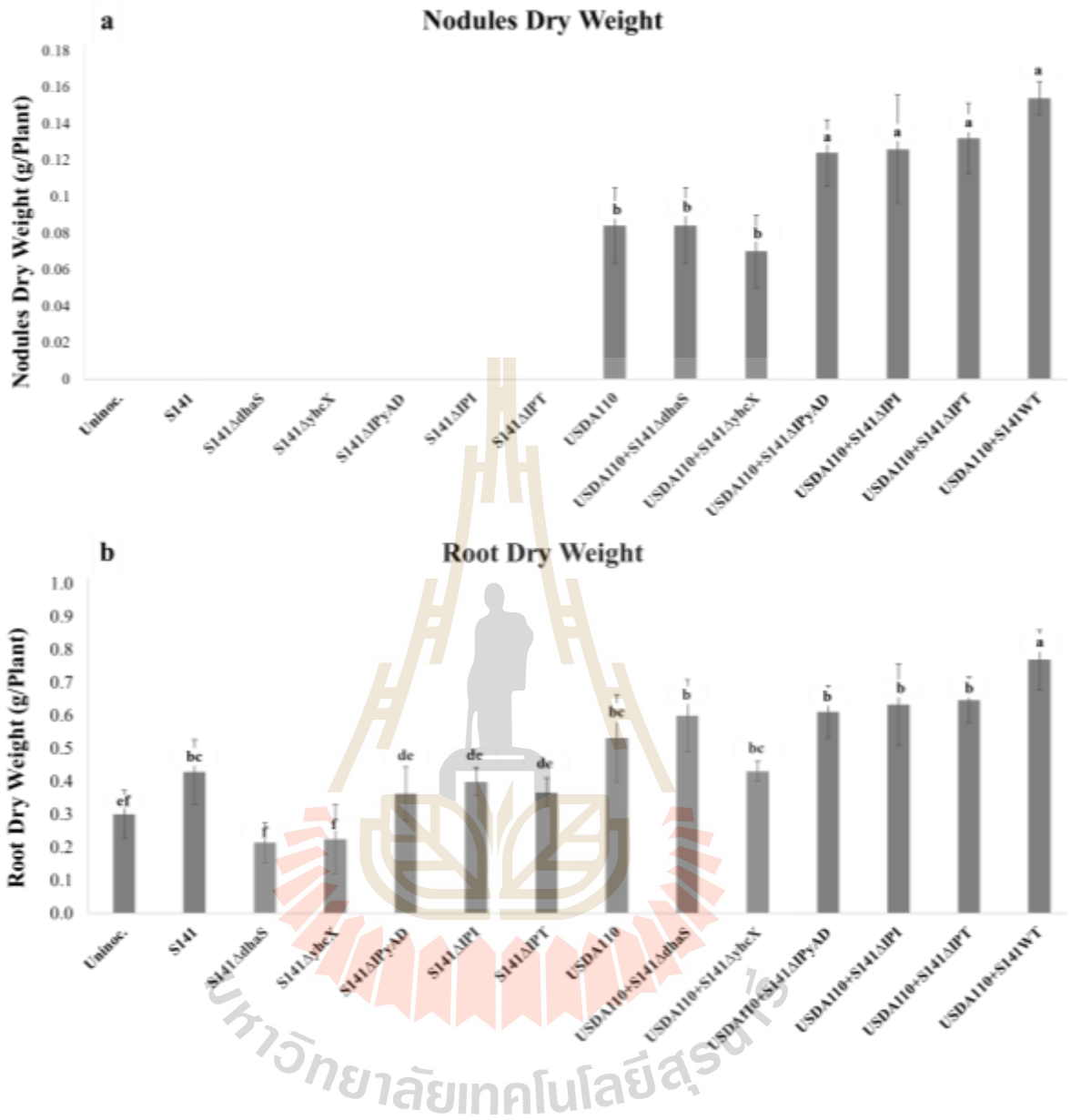

Figure 57. Plant growth parameters of soybean cultivar Chaing Mai 60 by co-inoculation with USDA110 and S141 or S141 mutant strains compared with single inoculation at 45 DAI: (a) nodule dry weight and (b) root dry weight. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=8).

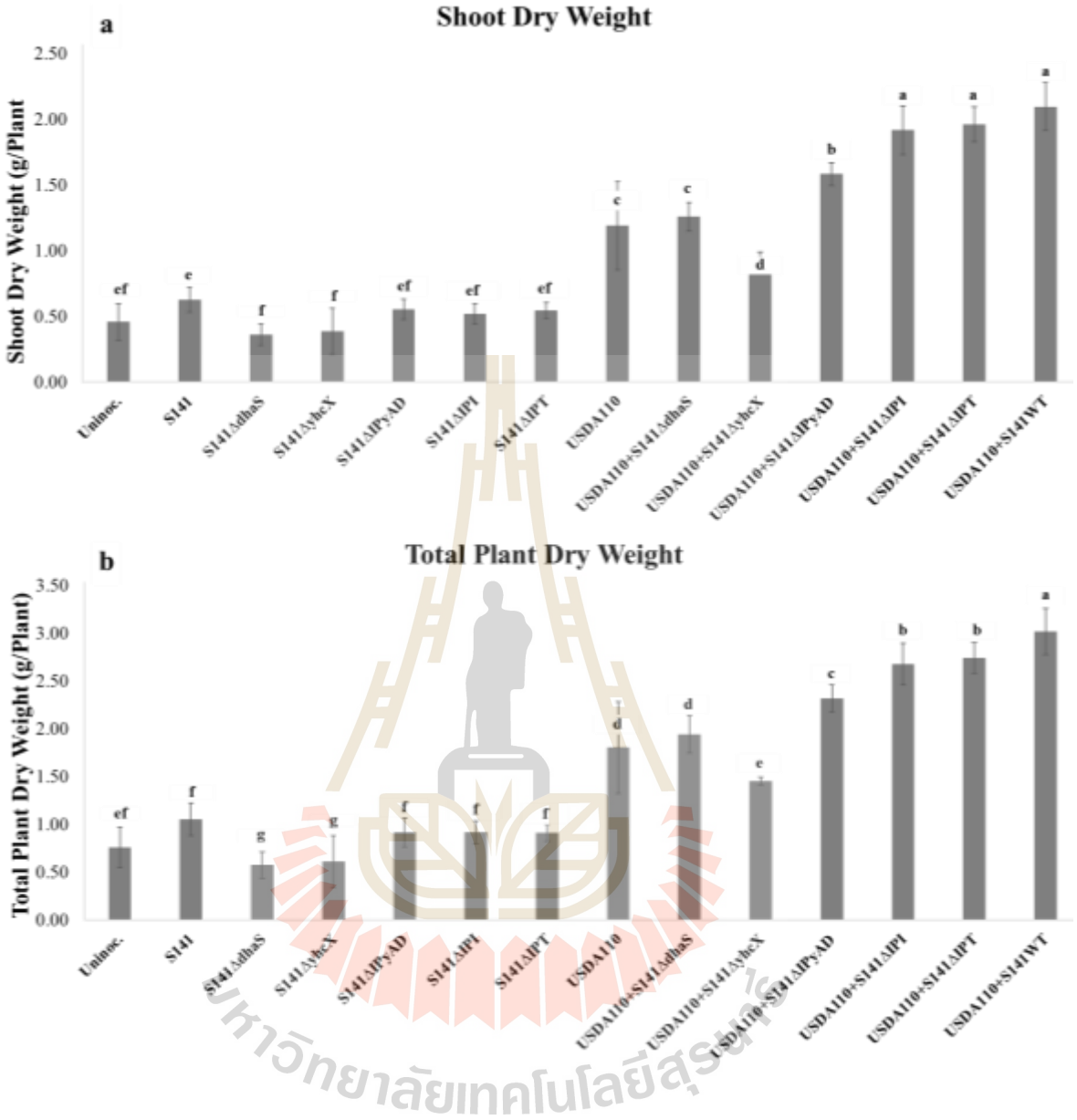

Figure 58. Plant growth parameters of soybean cultivar Chaing Mai 60 by co-inoculation with USDA110 and S141 or S141 mutant strains compared with single inoculation at 45 DAI: (a) shoot dry weight and (b) total plant dry weight. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=8).

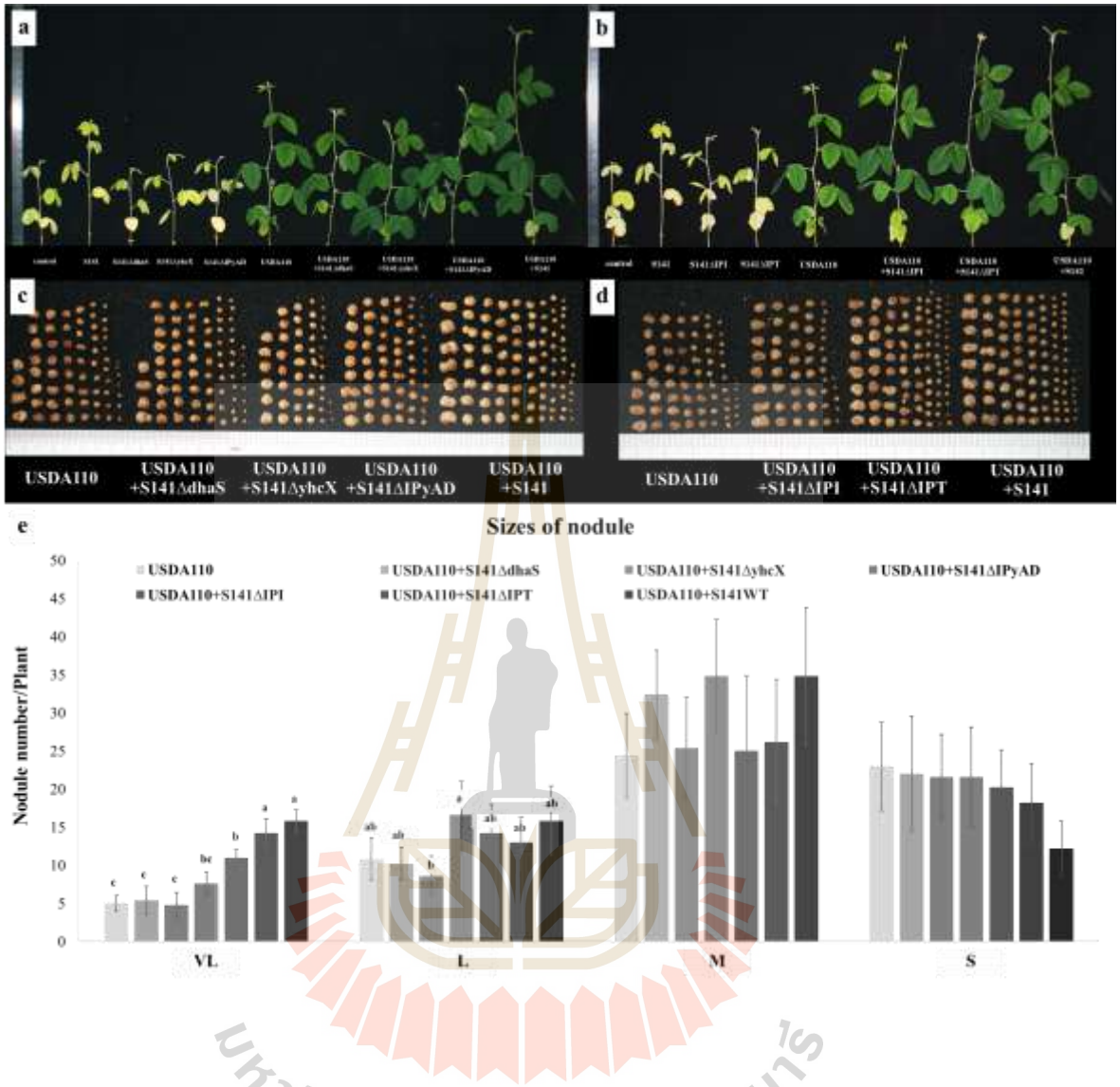

Figure 59. Soybean plant growth and the nodule sizes separation of soybean cultivar Chaing Mai 60 by co-inoculation with USDA110 and S141 or S141 mutant strains compared with single inoculation at 45 DAI: (a) and (b) soybean growth under Leonard's jar experiments, (c) and (b) photograph of soybean nodule and (e) nodule sizes separation of soybean. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=8).

The results taken together indicated that co-inoculation of USDA110 with S141 promoted soybean growth by increasing nitrogen-fixing efficiency, nodule dry weight, root dry weight, shoot dry weight and total plant dry weight on soybean-*Bradyrhizobium*

symbiosis at 45 DAI. Interestingly, co-inoculation of USDA110 with S141 enhanced soybean-*Bradyrhizobium* symbiosis to produced VL size of nodules as the result of increasing nitrogen-fixing efficiency, it seems like IAA produced from S141 had the effect with cell elongation and cortical cell proliferation of nodules. Compared with another PGPR *Bacillus* strain, *B. velezensis* FZB42 (*B. amyloliquefaciens* subsp. *plantarum* FZB42) (Dunlap et al., 2016), strain S141 contains putative genes involved in indole-3-acetic acid (IAA) production including *IPyAD* and *dhaS* encoding indole-3-pyruvate decarboxylase and indole-3-acetaldehyde dehydrogenase, respectively, to synthesize IAA from indole-3-pyruvic acid and *yhcX* encoding nitrilase to synthesize IAA from indole-3-acetonitrile. Additionally, ysnE was, which putatively encodes IAA transacetylase involved in the tryptophan-independent IAA biosynthesis pathway. Furthermore, not only IAA biosynthesis pathway was found in S141 but, cytokinin biosynthesis pathway was also found in S141. The translated IPT gene products were predicted via the genome database of S141 to possess a tRNA delta (2)-isopentenyl pyrophosphate (IPP) transferase domain (protein_id BBA76310.1), which is critical for the enzyme's function in cytokinin biosynthesis. Besides, isopentenyl pyrophosphate isomerase (IPI) (protein_id BBA76708.1) was also found in S141, is an isomerase that catalyzes the conversion of the relatively un-reactive isopentenyl pyrophosphate (IPP) to the more-reactive electrophile dimethylallyl pyrophosphate (DMAPP). This isomerization is a key step in the biosynthesis of isoprenoids through the mevalonate pathway and the MEP pathway (Kaneda et al., 2001). Mutants bearing *ΔysnE*, *ΔdhaS* and *ΔIPyAD* deletions impaired IAA production. Despite, all of IAA related mutant strains (S141*ΔdhaS*, S141*ΔyhcX* and S141*ΔIPyAD*) could not completed abolish IAA production, but co-inoculation of USDA110 with all of IAA related mutant strains (S141*ΔdhaS*, S141*ΔyhcX* and S141*ΔIPyAD*) also reduced nodule number of VL size nodule.

Moreover, co-inoculation of USDA110 with S141*ΔyhcX* also reduced nodule number of L size nodule, it is most likely that *yhcX* may play an important role in IAA biosynthesis on soybean growth promotion. The deduced *yhcX* gene product is similar to nitrilase 2 of *Arabidopsis thaliana*, which catalyzes the direct conversion of indole 3-acetonitrile to IAA (Hillebrand et al., 1998). The *ysnE* gene is similar to a putative IAA acetyl transferase gene localized within the tryptophan biosynthesis gene cluster of the plant-growth-promoting α proteobacterium *Azospirillum brasilense* (Zimmer et al., 1991). Its deduced gene product belongs to a widely distributed family of acetyl transferases that catalyze the transfer of an acetyl group to a nitrogen atom on the acceptor molecule. *YsnE* has been suggested to participate in the tryptophan dependent IAA production (Zimmer et al. 1991). Taken together, the results present additional evidence for the existence of a tryptophan-dependent pathway as the main route of IAA biosynthesis in *B. velezensis* S141. However, minor tryptophan-independent pathways for IAA biosynthesis may exist in S141, because IAA biosynthesis was not abolished completely in the S141*ΔdhaS*, S141*ΔyhcX* and S141*ΔIPyAD* mutant strains. Double mutants might be useful to prove the presence of alternative Trp-independent pathways. The results of nodule size confirmed that IAA had the effect with nodule size and involved in cell elongation and cortical cell proliferation of nodule. The results was consistent with Liu et al. (2018) suggested that the auxin signaling pathway is required for both infection thread formation and nodule development. Auxin signaling initiates this infection thread and cytokinin signaling is required for infection thread elongation. This symbiotic signal induces cortex cell division and initiates nodule organogenesis.

Considering about cytokinin biosynthesis, co-inoculation of USDA110 with S141 enhanced soybean-*Bradyrhizobium* symbiosis to produced high nodule dry weight similar with co-inoculation of USDA110 with S141*ΔIPT* or S141*ΔIPI* as the result of increasing nitrogen-fixing efficiency, but co-inoculation of USDA110 with S141*ΔIPI* showed the

reduction of nodule number of VL size nodule. It seems like isopentenyl pyrophosphate isomerase (IPI) may play an important role in controlling nodule size of soybean-*Bradyrhizobium* symbiosis.

CHAPTER V

CONCLUSION

Bacillus sp. S141 strain is a PGPR isolated from soybean rhizosphere soil in Thailand by Prakamhang et al. (2015), and is closely related to *B. subtilis* GB03 based on 16S rRNA gene sequencing. S141 strain were performed significantly capable of promoting nitrogen-fixing efficiency, nodule number, nodule dry weight, size of nodules and total plant dry weight with both soybean and mung bean-*Bradyrhizobium* symbiosis by co-inoculation with *B. diazoefficiens* USDA110 and *Bradyrhizobium* sp. SUTN9-2, respectively. Furthermore, co-inoculation between supernatant of S141 with USDA110 performed the same results with the cells of S141. The effective inoculation doses of *B. diazoefficiens* USDA110 on soybean were 10^6-10^8 cells/seed. However, the concentration of soybean inoculant USDA110 can be reduced when co-inoculation with cells or supernatant of S141. Besides, the competition for nodulation of co-inoculation between bradyrhizobial strains (GUS-tagged USDA110 or dsRed-tagged SUTN9-2) and S141 did not affected on nodulation competition among applied bradyrhizobial strains and indigenous bradyrhizobial strains, co-inoculation of S141 cannot enhance the competitiveness of *Bradyrhizobium* on legumes nodulation, while co-inoculation of bradyrhizobial strains and S141 significant was capable of promoting legumes growth by enhancing legumes nodulation in terms of increasing nodule number and producing the large size of nodules.

SDS-PAGE and protein identification of S141 proteome demonstrated the significantly altered expression levels in response to supernatant of USDA110 after an interaction period of 24 and 48 h, the majority of which are involved in metabolism and

transport of nutrients, motility, stress responses and chemotaxis.These results suggested that the PGPR may facilitate the induction of numerous compounds such as dicarboxylic acids and the amino acids which were strong attractants stimulating chemotactic response and metabolism, resulting in the enhancement of nodulation and nitrogen-fixing efficiency in soybean.

The whole genome of S141 identified to comprise a 3,974,582 bp-long circular chromosome without a plasmid. Its genome sequence encoding at least 3,817 proteins-coding genes. General information about the genomes of S141 showed 46.51% G+C content, 9 copies of ribosomal ribonucleic acid (rRNA) and 86 transfer ribonucleic acid (tRNA). Based on 16S rRNA gene analysis and average nucleotide identity (ANI) indicated strain S141 classified as *B. velezensis* thus S141 was decided to name strain S141 *B. velezensis* S141. S141 genome sequence was deposited in DDBJ/EMBL/GenBank under accession number AP018402. Moreover, S141 possesses multiple genes that are functionally related to auxin and cytokinin biosyntheses and play key roles in its ability to promote plant growth. Colonization of S141 around nodule and root surface of soybeans confirmed that S141 act closely with rhizosphere as plant growth promoting rhizobacteria.

IAA and also IAA produced from S141 had the effect with cell elongation and cell proliferation of nodules when co-inoculated with USDA110. This symbiotic signal induces cortex cell division and initiates nodule organogenesis. Cytokinin was proposed to be important for nodule formation, but the 6-BA was not promote soybean growth and nodulation in these experiments because it seems high concentration of cytokinin may retard growth of soybean-*Bradyrhizobium* symbiosis. The cross section of nodules from co-inoculation of USDA110 with S141 displayed a thinner layer of the cortex and larger area of the infection zone than the nodules derived from single inoculation. It is obviously that IAA might play an important role in cortex cell elongation and proliferation. The disruption of putative genes related to IAA biosynthesis pathway including: *dhaS*, *yhcX* and *IPyAD*

and quantification of the IAA presented in culture filtrates of strains S141*ΔdhaS*, S141*ΔyhcX* and S141*ΔIPyAD* revealed the reduction amounts of IAA, suggested that, all of S141 IAA related mutant strains were related with IAA biosynthesis pathway.

The co-inoculation of USDA110 with S141 enhanced soybean-*Bradyrhizobium* symbiosis to produced very large size of nodules as the result of increasing nitrogen-fixing efficiency, it seems like IAA produced from S141 had the effect with cell elongation and cortical cell proliferation of nodules. Mutants bearing *ΔysnE*, *ΔdhaS* and *ΔIPyAD* deletions were impaired in IAA production. Despite, all of IAA related mutant strains (S141*ΔdhaS*, S141*ΔyhcX* and S141*ΔIPyAD*) could not completed abolish IAA production, but co-inoculation of USDA110 with all of IAA related mutant strains (S141*ΔdhaS*, S141*ΔyhcX* and S141*ΔIPyAD*) also reduced nodule number of VL size nodule. Moreover, co-inoculation of USDA110 with S141*ΔyhcX* also reduced nodule number of L size nodule. This is the first reported that *yhcX* may play a major role in IAA biosynthesis in *Bacillus velezensis* as well as provide major impact on soybean growth promotion. In case of cytokinin biosynthesis, the disruption of genes related to cytokinin biosynthesis pathway including: *IPT* and *IPI* genes were generated and applied in this study. Co-inoculation of USDA110 with S141 enhanced soybean-*Bradyrhizobium* symbiosis to produced high nodule dry weight similar with co-inoculation of USDA110 with S141*ΔIPT* or S141*ΔIPI* as the result of increasing nitrogen-fixing efficiency, but co-inoculation of USDA110 with S141*ΔIPI* reduced nodule number of VL size nodule, it seems like isopentenyl pyrophosphate isomerase (IPI) may play an important role in controlling the nodule size of soybean-*Bradyrhizobium* symbiosis.

These are the first observation demonstrated the mechanism of *B. velezensis* S141 on mung bean and soybean-*Bradyrhizobium* symbioses. The co-inoculation with *Bradyrhizobium* and S141 leads to an increased number of the most active nodules,

producing the large size of nodules and promoting legumes growth, therefore, to a greater nitrogen fixation. Nevertheless, S141 may facilitate the induction of numerous compounds which present an increase in metabolism, resulting in the enhanced nodulation, size of nodule, N_2-fixation in soybean and mung bean. However, it is possible that not only IAA and cytokinin but also some other substance secreted from S141 might facilitate *Bradyrhizobium* to make bigger nodules rendering the higher efficiency of N_2-fixation. Therefore, the efficiency to enhance soybean and mung bean N_2-fixation by PGPR S141 with *Bradyrhizobium* co-inoculation strategy could be developed for supreme *Bradyrhizobium*-soybean and mung bean inoculants.

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APPENDICES

APPENDICES

Appendix 1. Yeast Extract Mannitol (YEM) medium (Somasegaran and Hoben, 1994)

D-Mannitol	10.0	g
K_2HPO_4	0.5	g
$MgSO_4 \cdot 7H_2O$	0.2	g
NaCl	0.1	g
Yeast Extract	0.5	g
Distilled Water	1.0	liter
Adjust pH to 6.8 with 0.1 N NaOH		

Appendix 2. LB medium (Bertani, 1951)

Tryptone	10.0	g
Yeast extract	5.0	g
NaCl	10.0	g
Distilled Water	1.0	liter

Appendix 3. LG medium (Lipman, 1904)

Glucose	10.000	g
KH_2PO_4	0.410	g
K_2HPO_4	0.520	g
$CaCl_2$	0.200	g
Na_2SO_4	0.050	g
$MgSO_4 \cdot 7H_2O$	0.100	g
$FeSO_4 \cdot 7H_2O$	0.005	g
$Na_2MoO_4 \cdot 2H_2O$	0.0025	g
Distilled Water	1.0	liter

Appendix 4. Glucuronidase (GUS) assay solution

20 $mg \cdot ml^{-1}$ X-Gluc in N, N-Dimethylformamide	40.0	µl
SDS	20.0	mg
Methanol	2.0	ml
1 M Sodium phosphate buffer	0.2	ml
Distilled Water	7.76	ml

Appendix 5. N-free Nutrient Solution (Broughton and Dillworth, 1971)

Stock Solutions	Elements	Chemical Formula	MW	g/liter
1	Ca	$CaCl_2 \cdot 2H_2O$	147.03	294.1
2	P	KH_2PO_4	136.09	136.1
3	Fe	Fe-citrate	355.04	6.7
	Mg	$MgSo_4 \cdot 7H_2O$	246.5	123.3
	K	K_2SO_4	174.06	87.0
	Mn	$MnSO_4 \cdot H_2O$	169.02	0.338
4	B	H_3BO_3	61.84	0.247
	Zn	$ZnSO_4 \cdot 7H_2O$	287.56	0.288
	Cu	$CuSO_4 \cdot 5H_2O$	249.69	0.100
	Co	$CoSO_4 \cdot 7H_2O$	281.12	0.056
	Mo	$Na_2MoO_2 \cdot 2H_2O$	241.98	0.048

Appendix 6. Primers used for combining the contigs in whole genome sequence of *B. velezensis* S141

Primer	Sequences (5′→3′)
contig 1 F_PRIMER	TGATTAAGAGGTCGCTTCGAG
contig 1 R_PRIMER	GGTATGCAAAGCGGATGATT
contig 2 F_PRIMER	AACCCAAAAAGATTGCAACG
contig 2 R_PRIMER	TTACCGCCTACACGATTTCC
contig 3 F_PRIMER	GGATTATCCGTTTCCGGTTT
contig 3 R_PRIMER	GTGTATCCGCGATGGATCTT
contig 4 F_PRIMER	AGGCGTGCGAGTAACGTATT
contig 4 R_PRIMER	CTCACGACCCCGTCTACTTT
contig 5 F_PRIMER	CGATCCGTTTTTCCATGACT
contig 5 R_PRIMER	TTAATGCTCCCCTTGTGGTC
contig 6 F_PRIMER	TGAAAAAGTCGCCTCGAGAT
contig 6 R_PRIMER	ATTCCGGTCGCTCCAATATC
contig 7 F_PRIMER	TCGCTTGAACGAAAGACAAA
contig 7 R_PRIMER	GATCAGAGGTCGGAGTGGAA
contig 8 F_PRIMER	ACTCAACCCCCTCCTTCATT
contig 8 R_PRIMER	CTTTCGTCTCCCCTTTTGAA
contig 9 F_PRIMER	CCATCATCACCGATCCCTAC
contig 9 R_PRIMER	GTTGTGGTATCCGGTCATCC
contig 10 F_PRIMER	CTTGTCCGGGAATTAAACGA
contig 10 R_PRIMER	AACGTATGGTTCGGTTTTGC
contig 11 F_PRIMER	TACGTCGCTGATGACGAAAC

cont.

Appendix 6. Primers used for combining the contigs in whole genome sequence of *B. velezensis* S141 (continued)

Primer	Sequences (5′→3′)
contig 11 R_PRIMER	TTTTGAGGCTGACCCGATAG
contig 12 F_PRIMER	GGACGGGATAAGTGCTGAAA
contig 12 R_PRIMER	CTACGCATTTCACCGCTACA
contig 13 F_PRIMER	CAACCGCTTGAAGGAAAAAG
contig 13 R_PRIMER	TGTTGCGAAAACTCTTGCTG
contig 14 F_PRIMER	TCGGAAAAGCTTCTGCATCT
contig 14 R_PRIMER	GGCCAACTGCAGAAAGAGTC
contig 15 F_PRIMER	AAGGAGAGGGTGATGTGCTG
contig 15 R_PRIMER	ACGACTTTCTCGACGTGCTT
contig 16 F_PRIMER	GCATATGCAATGAGCGTCTG
contig 16 R_PRIMER	TTCCAAGCTGATGTCGTGAG
contig 17 F_PRIMER	CGCGCGAGTAACGTATTGTA
contig 17 R_PRIMER	GACAGGGGCTGTCTCTGAAG
contig 18 F_PRIMER	GAGCCATGAAGGACTCGAAC
contig 18 R_PRIMER	CGAGGACAAGCTGAAAGAGG
contig 19 F_PRIMER	GGTGCCGTTACAGGACATCT
contig 19 R_PRIMER	TGACTTGTATCCGCAGCTTG
contig7_F (new)	CCGCCTTGTCATGCTGTTAA
contig8_F (new)	AGCGATTTCACTGCGTTTGA
contig8_R (new)	ACGCCTCCTTATCTTGTCCA
contig10_R (new)	ATCCCGTACAAACTGCGTTC

Appendix 7. Primers used for Sanger sequencing to closed gaps between the contig in whole genome sequence

Primer	Sequences (5′→3′)
2_1R	TGGAAAGCAATACACTTACGGA
2_2F	GCAGCTGATCAAGTGTGTGT
2_2R	CAACCCTTGGGACCGACTAC
2_3F	TCTGTGACCGAAAACGGGA
2_3R	AGGCAGAGATTCGTTTACCTGA
2_5F	CTTTGCGGGACATGCTCAT
2_5R	ACACTGGGACTGAGACACG
2_7R	ACTTATCCCGTCCGCACATA
2_9F	CACACTTCCACCTCAGACCT
2_9R	CTGTGTCCTGCTTATCTCCAA
2_10F	GATGGAGGCATTATCAGCGG
2_11F	GAGGCAGCAGTAGGGAATCT
2_13F	CACAGCACAATGACCGTACA
2_14R	GAAATGAGATTGGCAGGCGT
2_15F	CCCATCCTTCTTGCGCTTTT
2_15R	CCTTTGCGGGACATGCTCAT
2_16F	GATCATCCCAAGCGCCATTT
2_16R	GACGCCACCAAGCTTTTCTA
2_17F	ACATACATCCGTTTCTGCGC
2_17R	GCGTTTTCTCGATGATCCGT
2_18F	GCATGGGAACGGGTGTGA

cont.

Appendix 7. Primers used for Sanger sequencing to closed gaps between the contig in whole genome sequence (continued)

Primer	Sequences (5′→3′)
2_18R	AGGTCCAAAAGCTCGAATGG
2_19R	CGAAAGGGGACAAACGGTTT
rRNA_23s_12F	AGGTCTGAGGTGGAAGTGTG
rRNA_16s_12R	ACGGCACTTGTTCTTCCCTA
rRNA_16s_F	AGGTGGGGATGACGTCAAAT
rRNA_23s_R	GCTTTCTCTTCCTCCGGGTA
7F8FnRNA1+23s_12F_2	GCCCTCGGCATTCATTATTA
7F8FRNA1+16s_12R_2	GAGGAGTCGAACCCCTAACC
8R10RRNA1+23s_12F_2	GCTGTATTCTGGCCATGCTT
8R10RRNA1+16s_12R_2	GAGAATGGTCGGGAAGACAG
12R_SNV41-98	CCGTCAGACTTTCGTCCATT
12F_SNV132-152	CTGGGATAACTCCGGGAAAC
12R_SNV132-152	CGGCACTTGTTCTTCCCTAA
12F_SNV434-521	CAATGGACGAAAGTCTGACG
12F_MNV1328-1329	GAGGTTAAGCCAATCCCACA
12F_SNV1840	GGACGAACACCGATATGCTT
12F_SNV3466-3766	CGTTAAGGAACTCGGCAAAA
12F_MNV1328-1329 (new)	AGGTGGGGATGACGTCAAAT

Appendix 8. Primers used for confirm the transconjugants of *B. velezensis* S141

Primer	Sequences (5′→3′)
78293(dhaS)_insert_F	GTCCGTTTCCAATCTTGATGTCC
78293(dhaS)_insert_R	AACATCCGGATATTAAAGCCATCTC
75506(yhcX)_insert_F	TAAATATGCGACATTAATGCAATGG
75506(yhcX))_insert_R	AGGCTGATATAGTCCTCGGTATATTCG
77145(IPyAD)_insert_F	GAGGTTTCAAGCCCTCTTCTCTG
77145(IPyAD)_insert_R	TTTACCACTGTGACTTGCTGATCAG

Appendix 9. Soil characteristic

Soil characteristic	
Texture	sand
pH	7.064
EC (ms/cm)	0.238
Organic matter content (%)	0.722
Nitrogen (%)	0.036
Available P (ppm)	50.236
Exchangeable K (ppm)	69.00
Ca (ppm)	172.00

Appendix 10. Protein extraction buffer

Tris HCl pH 8.0	20.0	mM
Triton X-100	1.0	%
Lysozyme	400.0	µg/ml
DNase	40.0	µg/ml

Appendix 11. 5% polyacrylamide gel

30% acrylamide solution (29:1; acrylamide:Bis-acrylamide)	166.0	µl/ml
0.5 M Tris pH 6.8	250.0	µl/ml
10% sodium dodesyl sulfate (SDS)	10.0	µl/ml
10% ammonium persulfate (APS)	10.0	µl/ml
TEMED	1.0	µl/ml

Appendix 12. 12% polyacrylamide gel

30% acrylamide solution (29:1; acrylamide:Bis-acrylamide)	400.0	µl/ml
1.5 M Tris pH 8.6	250.0	µl/ml
10% sodium dodesyl sulfate (SDS)	10.0	µl/ml
10% ammonium persulfate (APS)	10.0	µl/ml
TEMED	0.6	µl/ml

Appendix 13. SDS-PAGE staining solution

Coomassie Brilliant Blue G-250	1.0	g/L
Methanol	400.0	ml/L
Glacial acetic acid	100.0	ml/L

Appendix 14. SDS-PAGE destaining solution

Methanol	400	ml/L
Glacial acetic acid.	100	ml/L

BIOGRAPHY

Mr. Surachat Sibponkrung was born in 1985, Nakhon Ratchasima, Thailand. He graduated with a Bachelor's degree of science with first class honor in 2006 from School of Crop Production Technology, Institute of Agricultural Technology, Suranaree University of Technology. He obtained SUT scholarship (The achievement is outstanding) for continue his studied in the same field and recieved a Master's degree in 2011. He was awarded a scholarship from the Thailand Research Fund under the Royal Golden Jubilee program in 2013 to pursue a Ph.D degree in School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology. During his Ph.D., he presented research at the 4th Asian Conference on plant-microbe symbiosis and nitrogen fixation, October 16-19, 2016 (Poster presentation; in "Determinants derived from PGPR capable of increasing soybean production via *Bradyrhizobium diazoefficiens* USDA110 inoculation"). RGJ-Ph.D. Congress 19, June 7-9, 2018, Jomtien Palm beach Hotel & Resorrt, Pattaya, Thailand (Oral presentation; in "Genetic analysis of PGPR (*Bacillus velezensis* S141) on increasing N_2 fixation efficiency of soybean- *Bradyrhizobium* symbiosis"; recognition of outstanding oral presentation). While he was studies, he received a scholarship JASSO (Japan Student Service Organization) during November 16, 2015-January 21, 2016 for acquired his expertise in cloning of a fluorescent gene at Tokyo University of Agriculture and Technology and has an experience to improve his knowledge regarding genome analysis at Kobe University, Kobe, Japan for 9 months. He conducted to research in the topic of Determinants derived from PGPR capable of increasing soybean and mung bean production via *Bradyrhizobium* inoculation. In 2017, he published his work in a title of Genome sequence of *Bacillus velezensis* S141, a new strain of plant growth-promoting rhizobacterium isolated from soybean rhizosphere.