การเพิ่มประสิทธิภาพการตรึงไนโตรเจนของถั่วเหลืองโดยการใช้หัวเชื้อร่วมระหว่าง PGPR และ *Bradyrhizobium japonicum*

นางสาวจันทร์เพ็ญ ประกำแหง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2556

MAXIMIZATION OF N_2-FIXING EFFICIENCY IN SOYBEAN (*Glycine max*) USING COINOCULATION WITH PGPR AND *Bradyrhizobium japonicum*

Janpen Prakamhang

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy in Biotechnology

Suranaree University of Technology

Academic Year 2013

MAXIMIZATION OF N_2-FIXING EFFICIENCY IN SOYBEAN (*Glycine max*) USING COINOCULATION WITH PGPR AND *Bradyrhizobium japonicum*

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

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จันทร์เพ็ญ ประกำแหง : การเพิ่มประสิทธิภาพการตรึงไนโตรเจนของถั่วเหลืองโดยการใช้หัวเชื้อร่วมระหว่าง PGPR และ *Bradyrhizobium japonicum* (MAXIMIZATION OF N_2-FIXING EFFICIENCY IN SOYBEAN (*Glycine max*) USING COINOCULATION WITH PGPR AND *Bradyrhizobium japonicum*) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.หนึ่ง เตียอำรุง, 137 หน้า.

วัตถุประสงค์ของงานวิจัยนี้ เพื่อคัดเลือกสายพันธุ์ของกลุ่มแบคทีเรีย PGPR ที่เหมาะสม และประเมินอิทธิพลในการส่งเสริมการสร้างปม และการเพิ่มประสิทธิภาพการตรึงไนโตรเจนในถั่วเหลือง (*Glycine max*) โดยการใช้หัวเชื้อร่วมกันระหว่าง *Bradyrhizobium japonicum* และแบคทีเรีย PGPR พบว่า แบคทีเรีย PGPR ที่ผ่านการคัดเลือกทั้งหมด 12 ไอโซเลต มีประสิทธิภาพในการส่งเสริมการตรึงไนโตรเจน การเพิ่มจำนวนปม การเพิ่มน้ำหนักแห้งของปม และน้ำหนักแห้งของต้นถั่วเหลือง เมื่อมีการใช้หัวเชื้อร่วมกับ *B. japonicum* สายพันธุ์ THA6 และ USDA110 ($p<0.05$) นอกจากนี้ PGPR 2 ไอโซเลตที่ดีที่สุด คือ ไอโซเลต S141 และ S222 ที่มีความสัมพันธ์อย่างใกล้ชิดกับแบคทีเรีย *Bacillus subtilis* และ *Staphylococcus* sp. นั้น ได้ถูกคัดเลือกเพื่อใช้เป็นเชื้อร่วมกับแบคทีเรีย *B. japonicum* สายพันธุ์ THA6 และ USDA110 สำหรับการทดลองต่อไป ปริมาณหัวเชื้อ PGPR : *B. japonicum* ทั้งสองสายพันธุ์ ที่มีประสิทธิภาพในการส่งเสริมการเจริญของถั่วเหลืองมากที่สุดเมื่อใช้เป็นหัวเชื้อร่วม คือที่ 10^6 : 10^6 CFU ml^{-1} การใช้หัวเชื้อร่วมกันในสภาพแปลงปลูกจริง สามารถเพิ่มผลผลิตเมล็ดถั่วเหลืองต่อเฮกตาร์ สูงกว่าการใช้หัวเชื้อ PGPR หรือ *B. japonic*um เพียงชนิดเดียวถึง 9.7-43.6% การวิเคราะห์การแสดงออกของยีนที่ตอบสนองต่อการใช้หัวเชื้อร่วมกัน ด้วยเทคนิค RT- PCR ทั้งยีนของพืชและของเชื้อแบคทีเรีย ทั้งในรากและปมถั่วเหลือง พบว่า ยีนที่เกี่ยวข้องในช่วงแรกของการสร้างปม ได้แก่ยีน *nodD1, GmCaMK1* และ *GmNIN1A* ในรากถั่วเหลืองอายุ 7 วันหลังการใส่หัวเชื้อ (DAI) ร่วมระหว่าง *B. japonicum* และ PGPR มีการแสดงออกเพิ่มขึ้นอย่างเห็นได้ชัด โดยเฉพาะยีน *nodD1* และ *GmCCaMK* แต่ระดับการแสดงออกของยีน *GmNIN1A* ไม่แตกต่างอย่างมีนัยสำคัญ เมื่อเทียบกับการใส่หัวเชื้อเพียงชนิดเดียว ระดับการแสดงออกของยีนที่เกี่ยวกับการทำงานของ PGPR ได้แก่ ยีน *iaaH* และ *ipdC* พบว่า ยีนดังกล่าวถูกควบคุมโดยการใส่หัวเชื้อร่วมระหว่าง *B. japonicum* และ PGPR นอกจากนี้ เมื่อทดลองใส่หัวเชื้อเพียงชนิดเดียว เปรียบเทียบกับการใส่หัวเชื้อร่วมระหว่าง *B. japonicum* และ PGPR ที่ช่วงเวลาที่แตกต่างกัน ต่อการแสดงออกของยีนของทั้งถั่วเหลืองและเชื้อแบคทีเรีย (ยีน *GmMyb* , *otsA* , *phbC* , *dctA* และ *nifH*) ที่พบในปมถั่วเหลือง พบว่า ยีนดังกล่าวมีการแสดงออกทั้งที่เพิ่มขึ้นและลดลง แสดงถึงการถูกควบคุมโดยผลของการใส่เชื้อร่วมระหว่าง *B. japonicum* และ PGPR ผล

การศึกษาดังกล่าวสอดคล้องกับการทดลองด้านกายภาพ ทั้งการทดลองใน Leonard's jar และในสภาพแปลงปลูกจริง อีกทั้งยังสอดคล้องกับการศึกษาด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่าน (TEM) ในปมถั่วเหลือง ที่แสดงให้เห็นถึงความหนาแน่นของแกรนูล poly-ß-hydroxybutyrate (PHB) ภายใน bacteroids ซึ่ง PHB นี้ พบมากในปมแก่ของตัวอย่างปมที่ใส่หัวเชื้อร่วม ในขณะปมของตัวอย่างที่ใส่หัวเชื้ออย่างเดียวกลับมีการเสื่อมสภาพ เมื่อนำผลการศึกษาทั้งหมดนี้มารวมกัน ทำให้สรุปได้ว่า เชื้อจุลินทรีย์ PGPR อาจช่วยให้เกิดการชักนำการสะสมของน้ำตาล Trehalose และเพิ่มการขนส่ง C4-dicarboxylic acid ซึ่งบ่งบอกถึงการเพิ่มขึ้นของการสะสม PHB ในปม ส่งผลต่อเนื่องในการส่งเสริมการสร้างปม และการตรึงไนโตรเจนในถั่วเหลือง ดังนั้น การเพิ่มประสิทธิภาพการตรึงไนโตรเจนของถั่วเหลือง โดยใช้กลยุทธ์การใช้หัวเชื้อร่วมระหว่าง PGPR ไอโซเลต S141 และ S222 ร่วมกับแบคทีเรีย *B. japonicum* สายพันธุ์ USDA110 และ THA6 นั้น สามารถพัฒนาเพื่อเป็นใช้เป็นหัวเชื้อที่มีประสิทธิภาพสำหรับถั่วเหลืองได้

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2556 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

JANPEN PRAKAMHANG : MAXIMIZATION OF N_2-FIXING EFFICIENCY IN SOYBEAN (*Glycine max*) USING COINOCULATION WITH PGPR AND *Bradyrhizobium japonicum*. THESIS ADVISOR : ASSOC. PROF. NEUNG TEAUMROONG, Dr.rer.nat., 137 PP.

SOYBEAN/COINOCULATION/*Bradyrhizobium japonicum*/PGPR/MECHANISM

The objectives of this research are to select the appropriate PGPR and evaluate its influence on promoting nodulation and N_2-fixing efficiency of soybean (*Glycine max*) by coinoculation with *Bradyrhizobium japonicum* strain. The selected 12 appropriate PGPR had significant capability of promoting N_2-fixation, nodule number, nodule and plant dry weight with both commercial Bradyrhizobial strains, *B. japonicum* THA6 and USDA110 (P<0.05). Furthermore, the best two PGPR, isolates S141 and S222 which are closely related to *Bacillus subtilis* and *Staphylococcus* sp., were selected for coinoculation with *B. japonicum* USDA110 and THA6. The effective coinoculation doses of PGPR:Bradyrhizobium on soybean were 10^6:10^6 CFU ml^{-1}. The effect of coinoculation experiment under field condition could increase 9.7-43.6% of seed yield per hectare which is higher than those of uninoculated or single inoculation of PGPR or *B. japonicum*. The effects of simultaneous presence of coinoculation on the plant and bacterial response by the gene expression analyses were identified under soybean root and nodule associated stage. The early nodulation response genes including *nodD1, GmCaMK1* and *GmNIN1A* were monitored by RT-PCR on 7 DAI soybean roots. The coinoculation of *B. japonicum* and PGPR obviously enhanced the up-regulation of *nodD1* and *GmCCaMK* genes, but the

expression level of *GmNIN1A* gene was not significantly different from those of single inoculation. The relative expression levels of PGPR mode of action related genes including *iaaH* and *ipdC* were also up-regulated by coinoculation of *B. japonicum* and PGPR. Moreover, the expressions of soybean and the bacterial related genes (*GmMyb*, *otsA*, *phbC*, *dctA* and *nifH*) in nodule after single and coinoculation with *B. japonicum* and PGPR were identified using RT-PCR at different time frames. The results revealed that the related genes expression triggered discontinuously both up- and down regulations by *B. japonicum*-PGPR coinoculation. These results were in accordance with phenotyptic characters in Leonard's jar and field experiments in terms of enhancing the nodulation and N_2-fixation in soybean. The TEM micrograph of soybean nodule demonstrated densely packed poly-ß-hydroxybutyrate (PHB) granules within the bacteroids. PHBs were present in mature nodule of coinoculated treatments whilst those in single inoculated nodules were senescent. These results taken together suggest that the PGPR may facilitate the induction of trehalose accumulation and the transport of C4-dicarboxylic acids which present an increase in PHB accumulation, resulting in the enhanced nodulation and N_2-fixation in soybean. Therefore, the efficiency to enhance soybean N_2-fixation by PGPR S141 and S222 with *B. japonicum* coinoculation strategy could be developed for supreme inoculants for soybean.

School of Biotechnology

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ACKNOWLEDGMENTS

The completion of this thesis was made possible through the support and inspiration of several individuals all of whom have my gratitude.

Financial support was provided by the Strategic Scholarships Fellowships Frontier Research Networks, Office of the higher education commission, Thailand and Suranaree University of Technology, and is most gratefully acknowledged.

Special thanks to Assoc. Prof. Dr. Neung Teaumroong, my thesis advisor, for his years of encouragement, networks, and support in completing this thesis that has been a long time in the coming. I would like to warmly thank to my thesis co-advisor, Prof. Emeritus Dr. Nantakon Boonkerd and Asst. Prof. Dr. Panlada Tittabutr, for the gentle encouragement and patience.

I would like to thank the Japan Students Service Organization (JASSO) for the financial support throughout my research in Kagoshima University, Japan. I would like to record my gratitude to Prof. Dr. Mikiko Abe, Prof. Dr. Toshiki Ushiumi, Prof. Dr. Higashi Shirou and Asst. Prof. Dr. Ken-ichi Kusho for their valuable advices and continued encouragement as well as giving me kindly providing great guidance, and giving me a great hospitality.

I would also like to thank all my Ph.D. committee; Assoc. Prof. Dr. Montarop Yamabhai and Dr. Achara Nuntagij for reading and approval on the earlier version of my thesis.

1 would like to express my gratitude to Dr. Sasidhorn Innok, Department of Applied Biology, Rajamangala University of Technology Isan (RMUTI), Thailand,

for extending the research facilities of her lab to accomplish this work. Thanks so to Miss Nual-anong Narkkong, Central Instrumentation Unit, Faculty of Science, Mahasarakham University, Thailand, for her help on TEM technique. Thanks are also extended to Asst. Prof. Dr. Issra Pramoolsook for advice and comments on the language of the abstract.

I also want to thanks friends and staffs in School of Biotechnology, The Center for Scientific and Technological Equipment, Institute of Agricultural Technology, Suranaree University of Technology for their help and friendships.

Thanks also for P.M.I. lab members at Kagoshima University for friendship and great hospitality during working in Japan.

Thanks to my colleagues in NPN lab for making our lab such a pleasant and inspiring place to work, for sharing not only their scientific and technical knowledge, but also spirited prodding has kept me going for many more years than it took to complete this work.

Warmly thanks to my true friends, Rujirek Noisangiam, Aphakorn Longtonglang, Kamonluck Teamthaisong, Phrueksa Lawongsa and Tapanawat Muanjang for the encouragement, soul support and thoughtfulness.

My biggest thank goes to my family - I remember and thank them, for without their affection, love and support I would never have come this far.

Janpen Prakamhang

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LIST OF ABBREVIATIONS

°C	=	degree Celsius
µm	=	micrometer
µg	=	microgram
µl	=	microlitre
ACC	=	1-aminocyclopropane-1-carboxylic acid
ANOVA	=	Analysis of variance
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
ER	=	Endoplasmic reticulum
et al.	=	Et alia (and other)
g	=	gram
h	=	hour
ha	=	Hectare
IAA	=	Indole-3-acetic acid

LIST OF ABBREVIATIONS (Continued)

IAM	=	Indole-3-acetamide
IBA	=	Indole-3-butyric acid
ICA	=	Indoel-3-carboxylic acid
IPA	=	Indole-3-propionic acid
ISR	=	Induced Systemic Resistance
kb	=	kilobases
l	=	litre
LB	=	Luria Bertani broth
LCO	=	Lipo-Chitin Oligosaccharides
LSD	=	Least significant difference
M	=	molarity
mg	=	milligram
min	=	minute
ml	=	milliliter
mM	=	millimolar
MY	=	Marketing Year
N	=	Nitrogen
NCBI	=	National Center for Biotechnology Information
ng	=	nanogram
nm	=	nanometer
PBM	=	Peribacteroid membrane
PCR	=	polymerase chain reaction

LIST OF ABBREVIATIONS (Continued)

PGPR	=	Plant growth-promoting rhizobacteria
RNA	=	ribonucleic acid
rRNA	=	ribosomal ribonucleic acid
RT-PCR	=	Reverse Transcription Polymerase Chain Reaction
SD	=	Standard Deviation
SUT	=	Suranaree University of Technology
TEM	=	Transmission Electron Microscopy
USDA	=	United States Department of Agriculture
WAI	=	Weeks after inoculation
YEM	=	Yeast Mannitol Medium

CHAPTER I

INTRODUCTION

1.1 Significances of this study

Bradyrhizobium plays a special role in the nitrogen cycle of agroecosystems by infecting the roots of soybean (*Glycine max*) and forming dinitrogen-fixing nodules. In this way, significant amounts of nitrogen are fixed and transferred to the plant, reducing the need for nitrogen fertilizer. However, there are number of factors which impede the nodulation on soybean root. Several abiotic and biotic factors can inhibit the formation of the N_2-fixing symbiosis between rhizobia and leguminous plants. The lack of nodules or formation of ineffective ones on soybean roots may be including host micro-symbiont compatibility, and presence of both known and unknown bio-molecules such as flavonoides, polysaccharides and hormones (Daayf et al., 2012). Furthermore, the inoculation dose is an important factor in agricultural application of microbial inoculants. Many countries have the dose standards for rhizobial inoculants (Smith and Hume, 1987). In a field experiment, soybean nodule number and mass including grain yield were all related to *B. japonicum* inoculants with bacterial density ranged from 10^3 to 10^6 cells/seed. However, the nodule number does not increase as much as the density of inoculants cell (Duzan et al., 2004).

Enhancement of legume nitrogen fixation by coinoculation of rhizobia with some Plant Growth Promoting Rhizobacteria (PGPR) is an alternative approach to improve the nitrogen availability in sustainable agriculture production systems. Some

PGPR strains, from a range of genera, enhance legume growth, nodulation and nitrogen fixation when coinoculated with rhizobia. Examples of these are *Azospirillum* (Aung et al., 2013; 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. Compared to single Rhizobium inoculation, coinoculation 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. The beneficial influence of PGPR on nodulation of legumes by *Rhizobium* has been variously attributed to their ability to produce phytohormones (Drogue et al., 2013; Prakamhang et al., 2009; Remans et al., 2007; Taghavi and van der Lelie, 2013) as well as by other unidentified mechanisms (Dudeja et al., 2012). 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 (Lugtenberg et al., 2013), but these hypotheses have not been well verified. 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. It is widely accepted that plant growth promotion by PGPR does not rely on a unique mechanism, but rather is the result of produce many effects.

1.2 Hypothesis

The overall nitrogen fixing efficiency and efficacy of the soybean-bradyrhizobial symbiosis could be improved via coinoculation with PGPR strain. The PGPR coinoculations will improve the efficacy to the *B. japonicum* inoculant, resulting in more nodulation, nitrogen fixation, nodule morphology, growth and yield. The PGPR coinoculation may affect the expression level of related genes of both bacteria and soybean during nodulation and symbiosis.

1.3 Objectives

1.3.1 General objectives

The general objectives of this research were to select the appropriate PGPR starins for coinoculation with *B. japonicum* and evaluate the influence of PGPR in promoting nodulation and N_2-fixing efficiency of soybean (*Glycine max*) compared to single *B. japonicum* inoculation as well as identify the mechanism of *Bradyhizobium*-soybean-PGPR interactions.

1.3.2 Specific objectives

1. To screen and select of the most effective PGPR strains as well as identification and characterization of the selected PGPR strains in term of soybean growth promotion when coinoculated with *B. japonicum* on soybean.
2. To evaluate the effects of coinoculation with PGPR and *B. japonicum* on soybean plants growth/yield under Leonard's jar and field conditions.

3. To analyze the mode of action of PGPR upon *B. japonicum*-Soybean symbiosis using analysis of related genes expression level of *B. japonicum*, PGPR and soybean during coinoculation trait.

CHAPTER II

LITERATURE REVIEW

2.1 Soybean

Soybean [*Glycine max* (L.) Merrill] belongs to the family Leguminosae, subfamily Papilionideae. Because of its tropical to subtropical origins, the optimal temperature range for soybean growth is 25 - 30°C (Zhang et al., 1996). Soybean is one of the world's most economically important legume crops.

Global 2012/13 soybean production is reduced mostly due to the smaller U.S. crop as a result of excessive heat and drought conditions. Global trade is down significantly as a large drop in U.S. exports is partially offset by increases in Argentina and Brazil. Global import demand for soybean meal and oil in both 2011/12 and 2012/13 is partially reduced in response to higher prices. The United States Department of Agriculture (USDA) (2013) reported that soybean production in Thailand continues to decline as more soybean growers switch to growing more profitable crops like corn and sugarcane. Despite increasing soybean prices in recent years, the profitability of growing soybeans is far less than competing crops. The Office of Agricultural Economics (OAE) reported that the average return for soybeans during 2009-2011 was $160.81/hectare as compared to $282.81/hectare for corn and $505.41/hectare for sugarcane. On the other hand, domestic soybean consumption will continue to grow in Marketing Year (MY) 2012/13 and MY 2013/14 in line with anticipated growth in the animal feed and food-based industries. Soybeans delivered

to soybean crushers are likely to increase to 1.70 million metric tons (MMT) in MY 2012/13 from 1.65 MMT in MY 2011/12. Total soybean domestic consumption is anticipated to increase another 5-6 percent in MY 2013/14. Moreover, several studies have shown that global crop production needs to double by 2050 to meet the projected demands from rising population, diet shifts, and increasing biofuels consumption (Ray et al., 2013). Boosting crop yields to meet these rising demands, rather than clearing more land for agriculture has been highlighted as a preferred solution to meet this goal.

Soybean cultivars are also known to influence nodulation competition among *B. japonicum* 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), Chaing Mai 60 (CM60) (Boonkerd, 2002). These Thai soybean cultivars may have different selective influences on the soil bradyrhizobia and therefore the nodulation competition of introduced *B. japonicum* strains may be affected by the identified of these cultivars. For example, *B. japonicum* USDA110 showed higher nodulation competitiveness than the other strains on three of the five cultivars. The Thai strain THA6 appeared to be more competitive than USDA110 on cultivar SJ5 (Payakapong et al., 2004).

Soybean and *B. japonicum* are two such specific partners that can form an effective nitrogen fixing symbiosis (Long, 1989). When living in an endosymbiotic state, as bacteroids in root nodules, *B. japonicum* can fix nitrogen and supply this element to the plant (Stacey et al., 1992). Soybean plants are able to fix 100-200 kg

$ha^{-1}yr^{-1}$ of atmospheric nitrogen (Smith and Hume, 1987). Numerous studies have demonstrated significant contributions of atmospheric N_2 fixation to soybean nutrition and growth (Bai et al., 2003; Keyser and Cregan, 1987; Morel et al., 2012; Stephens and Rask, 2000). Most estimates show that soybean derives between 25 and 75% of its N from fixation (McNeil, 2010).

2.2 Biological Nitrogen Fixation

Biological nitrogen fixation was firstly discovered by the German agronomist Hermann Hellriegel and Dutch microbiologist Martinus Beijerinck (Paracer and Ahmadjian, 2000). Biological nitrogen fixation (BNF) occurs when atmospheric nitrogen is converted to ammonia by an enzyme called nitrogenase (Postgate, 1998). Nitrogen fixation is catalyzed by the complex enzyme nitrogenase, composed of two component proteins (dinitrogenase and dinitrogenase reductase). 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 MgATP-dependent reduction of nitrogen gas to

yield two molecules of ammonia. The equation for the whole reaction can be depicted as follows :

$$N_2 + 8H^+ + 16ATP + 8e^- \rightarrow 2NH_3 + H_2 + 16ADP + 16\ Pi$$

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).

2.3 *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 6–8 hours to double in population size. They tend to grow best with pentoses as a carbon source (Somasegaran and Hoben, 1994).

This 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. 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). 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. 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).

B. japonicum USDA110 was isolated in 1957 from soybean nodules in Florida, USA. In 2002 the complete nucleotide sequence of the genome of a symbiotic bacterium *B. japonicum* USDA110 was determined (Kaneko et al., 2002). The genome of *B. japonicum* was a single circular chromosome 9,105,828 bp in length with an average GC content of 64.1% (Figure 1). No mega plasmid was detected. The chromosome comprises 8,317 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 percent of the *B. japonicum* 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 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.

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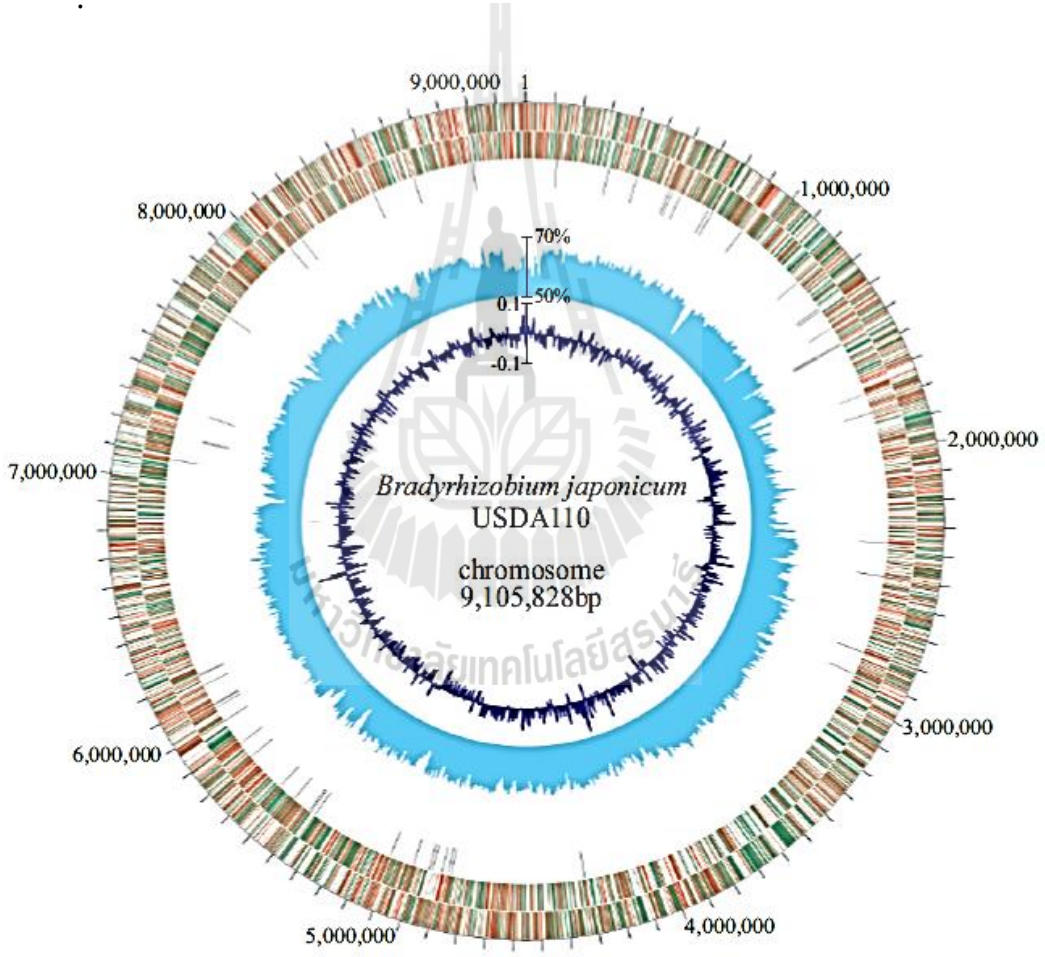


Figure 1. Circular representation of the chromosome of *B. japonicum* USDA110 (Kaneko et al., 2002).

B. japonicum THA6 was isolated from the mash of surface-sterilized nodules of soybean grown in the fields of Thailand. Strains THA6 indicated doubling times 67 min, could grow well at pH 6-7 (Nuntagij et al., 1997). The influence of Thai soybean cultivars on nodulation competitiveness of four *B. japonicum* THA6 strain was investigated. The Thai strain THA6 appeared to be more competitive than USDA110 on Thai soybean cultivar SJ5. (Payakapong et al., 2004).

At present, *B. japonicum* USDA110 and THA6 inoculant are produces for commercial purpose by the Department of Agricultural (DOA) of Thailand and recommended for Thai soybean cultivation (Boonkerd, 2002).

2.4 Molecular basis of rhizobium-legume symbiosis

2.4.1 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.

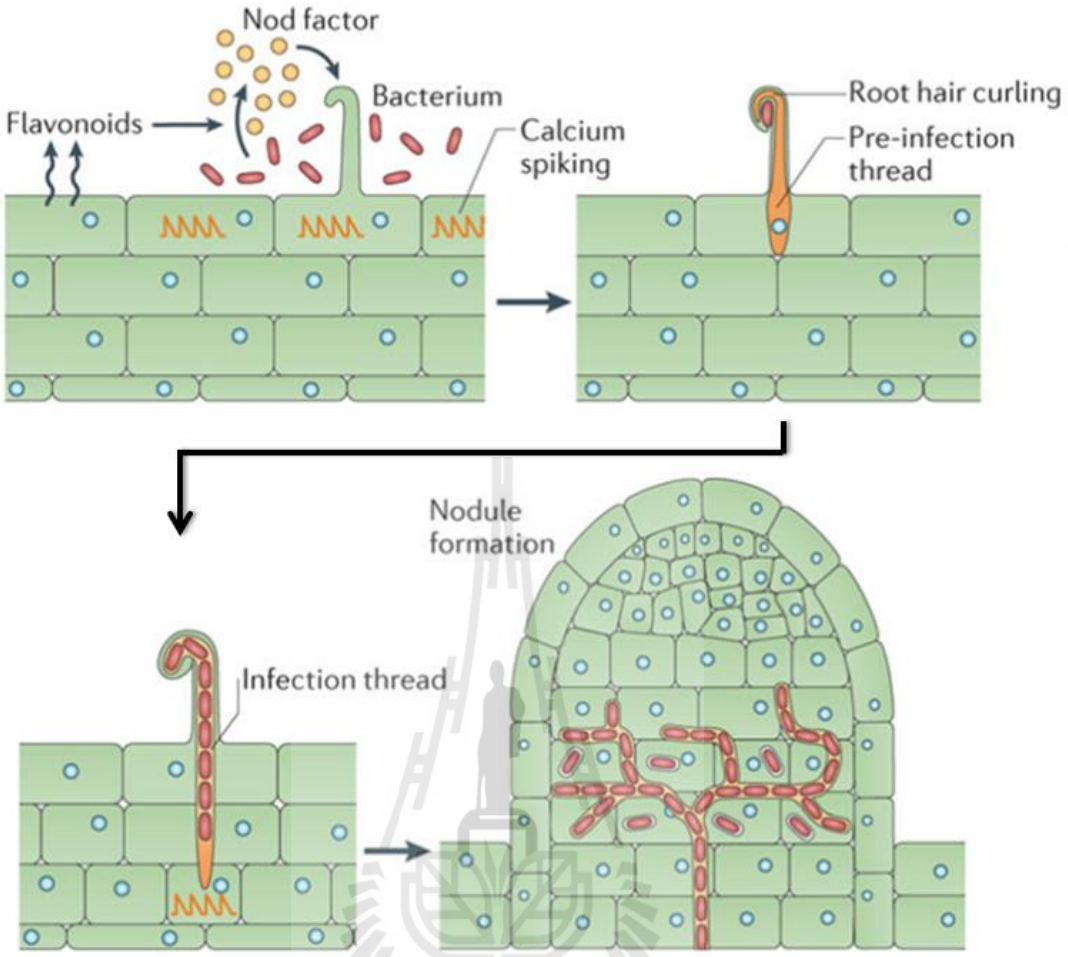


Figure 2. Rhizobial colonization (Adapted from Oldroyd (2013)).

Rhizobial colonization starts with the releasing of flavonoids by the plant root signal to rhizobia in the rhizosphere, which in turn produce nodulation factors (Nod factors) that are recognized by the plant. Nod factor perception activates the symbiosis signaling pathway, leading to calcium oscillations, initially in epidermal cells but later also in cortical cells preceding their colonization. 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 form 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.

2.4.2 Symbiotic signaling

Arbuscular mycorrhiza (AM) fungi produce lipo-chitooligosaccharides 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).

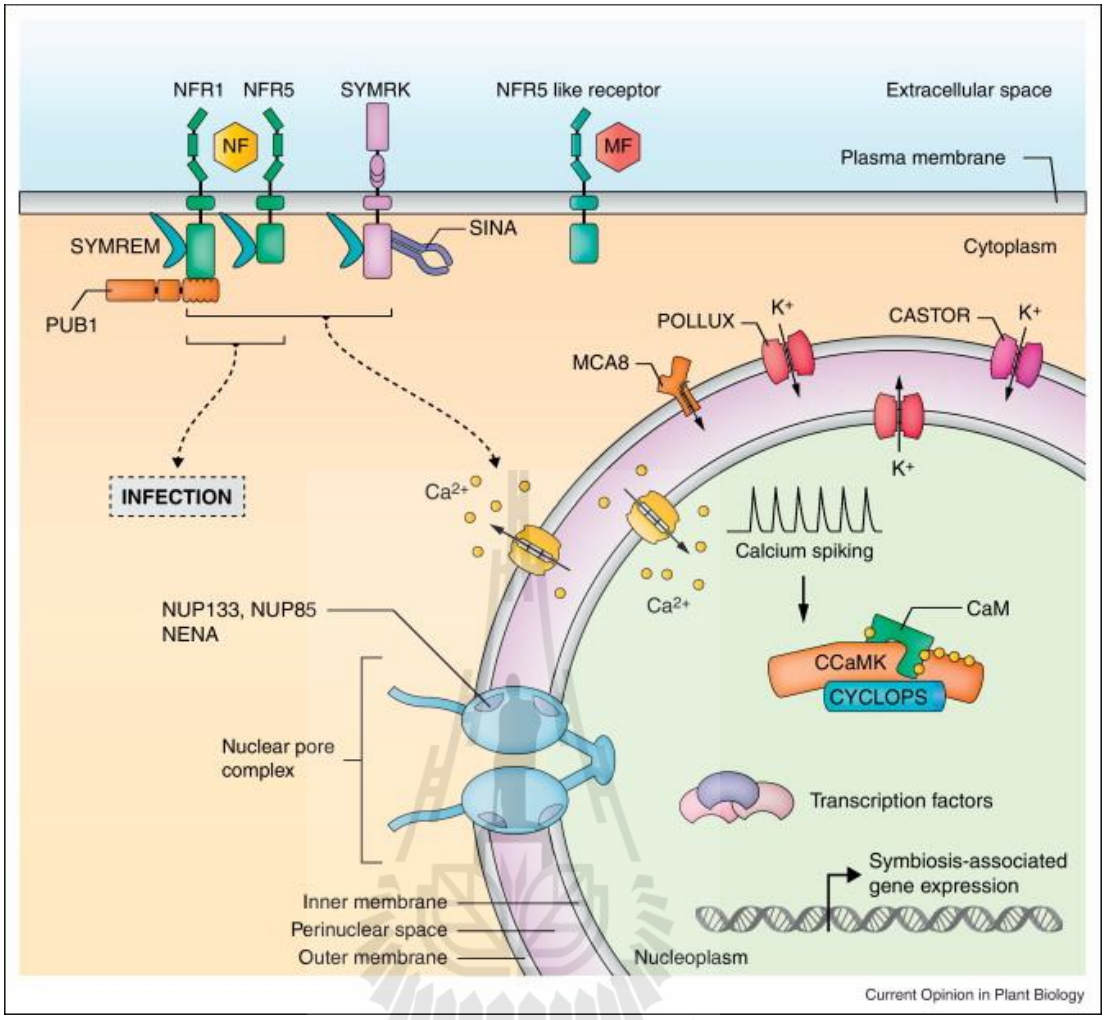


Figure 3. Symbiotic signal transduction in plant root cells (Singh and Parniske, 2012).

Symbiotic signal transduction starts with the percepting of rhizobial Nod factors (NFs) at the plasma membrane (PM) (Haney and Long, 2010) is mediated by LysM-receptor-like kinases (LYKs) including *L. japonicus* Nod factor receptor 1 (NFR1) and NFR5 and in *Medicago truncatula* LYK3 and NFP (Antolín-Llovera et al., 2012; Madsen et al., 2003). 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, LCO perception at the PM leads to a sustained nuclear Ca^{2+}-spiking response, 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).

2.4.3 Nodule organogenesis to senescence

In plants that form ‘determinate’ nodules such as soybean the succeeding development stages are demonstrated in Figure 4.

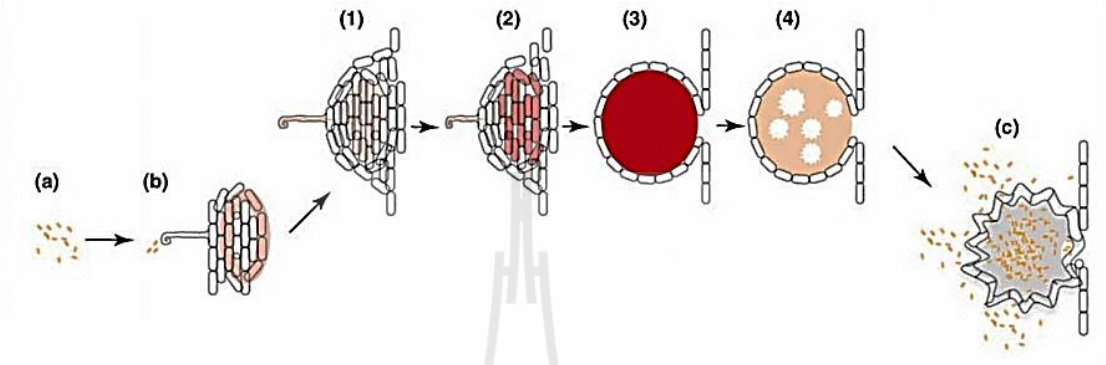


Figure 4. Nodule organogenesis - senescence (Modified from Schumpp and Deakin (2010)).

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 (the infection stage 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).

2.4.4 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 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) suggest 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).

In many types of symbioses, bacteroids appear to take up more carbon than can be immediately utilized, and under these circumstances, they may form poly-b-hydroxybutyrate (PHB). Malate and succinate are the main carbon sources used by the bacteroid (Prell and Poole, 2006), and are transported by plant and bacterial (*dctA*) dicarboxylic acid transporters across the PBM and the bacteroid plasma membrane, respectively (Den Herder and Parniske, 2009). 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). There can be a conflict of interest between rhizobia and legumes over the rate of PHB accumulation, due to a metabolic tradeoff between N_2-fixation and PHB accumulation. It has been reported that, during the first 21 days of nodule growth,

undifferentiated *S. meliloti* within alfalfa nodules accumulated enough PHB to support significant increases in reproduction and survival during starvation (Ratcliff et al., 2008). However, bacteroids in some symbioses do not accumulate PHB (Trainer and Charles, 2006).

It is interesting to consider the role of sugars and in particular trehalose in this respect. Trehalose is a non-reducing disaccharide consisting of two glucose units linked with an α-α linkage (alpha-D-glucopyranosyl-1, 1-alpha-D-glucopyranoside). It is regularly accumulated in symbiotic organs of many plants and it is thought to be derived from the microsymbiont. The most common route of trehalose synthesis is the OtsA/B pathway that forms trehalose-6-phophate from UDP-glucose and glucose-6-phophate with subsequent dephosphorylation, yielding free trehalose (Mellor, 1988). In rhizobia, Salminen and Streeter (1986) reported the synthesis of trehalose from UDP-glucose and glucose-6-P in *B. japonicum* and *B.elkanii* indicating trehalose synthesis via the OtsA/B pathway. Recently, Sugawara et al. (2010) reported that the trehalose biosynthetic genes (*otsA, treS,* and *treY*) in *B. japonicum* were induced by salinity and desiccation stresses and trehalose accumulation enhances survival and plays a role in the development of symbiotic nitrogen-fixing root nodules on soybean plants.

Analysis of the genomic organization of PHB biosynthesis genes in the α-proteobacteria revealed that the genes encoding PHB synthases are typically not colocalized with other genes in the PHB biosynthesis pathway (Rehm, 2003), a pattern that appears to be consistent amongst the rhizobial genomes (Kaneko et al., 2011; Kaneko et al., 2002). A summary of genes that were shown to elicit an effect on the PHB cycle is listed in Table 1.

Table 1. Summary of genes that were shown to elicit an effect upon PHB and/or bacteroid biosynthesis and/or degradation in rhizobia

Genes	Function	Organism studied	Null mutant phenotype	Reference
phbC	PHB synthase	*S. meliloti*, *R. leguminosarum*, *R. etli*. *B. japonicum*	No PHB synthesized	Paganelli et al. (2011) Madison and Huisman (1999)
phbB	NADP-acetoacetyl-CoA Reductase	*S. meliloti*	No PHB synthesized	Aneja et al. (2004)
aniA	Global carbon flux regulator	*R. etli*, *S. meliloti*	Reduced glycogen production and organic acid secretion, increased EPS production, global changes in protein expression	Encarnación et al. (2002)
nifDK	Nitrogenase structure	*B. japonicum*	Fix-, massive PHB accumulation	Hahn et al. (1984)
nifH	Nitrogenase structure	*B. japonicum*	Fix-, massive PHB accumulation	Hahn et al. (1984)
treS	Trehalose biosynthesis	*B. japonicum*	Less tolerant of desiccation stress	Sugawara et al. (2010)
otsAB	Trehalose biosynthesis	*B. japonicum*	Produced fewer mature nodules and a greater number of immature nodules	Sugawara et al. (2010)
treYZ	Trehalose biosynthesis	*B. japonicum*	Produced fewer mature nodules and a greater number of immature nodules	Sugawara et al. (2010)

2.5 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). The ineffectiveness of PGPR in the field has often been attributed to their inability to colonize plant roots (Benizri et al., 2001; Bloemberg and Lugtenberg, 2001). A variety of bacterial traits and specific genes contribute to this process, but only a few have been identified. These include motility, chemotaxis to seed and root exudates, production of pili or fimbriae, production of specific cell surface components, ability to use specific components of root exudates, protein secretion and quorum sensing. The generation of mutants altered in expression of these traits is aiding our understanding of the precise role each one plays in the colonization process (Lugtenberg et al., 2001; Persello-Cartieaux et al., 2003). 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.*

However, PGPR inoculated crops represent only a small fraction of current worldwide agricultural practice (Glick, 2012).

Generally, PGPR promote plant growth directly by either facilitating resource acquisition or modulating plant hormone levels, or indirectly (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, 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 5.

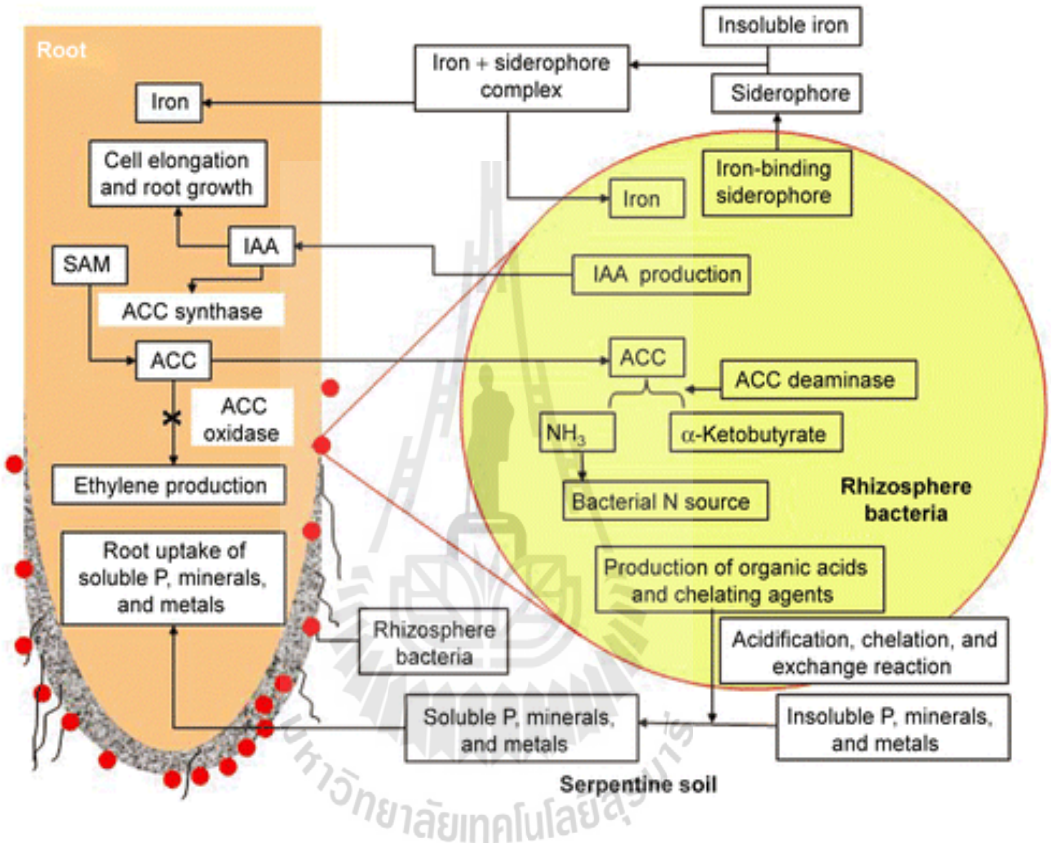


Figure 5. Plant growth-promoting mechanisms from rhizobacteria (Rajkumar et al., 2009).

2.5.1 Increase supply of nutrients

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). 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). Some PGPR can convert phosphorous into a more plant attainable form, such as to orthophosphate (Vessey, 2003). Iron is also another essential nutrient, but it is scarce in soil. PGPR, can produce compounds called siderophores, which acquire ferric iron (Fe3+), root cells can then take this up by active transport mechanisms (Ashraf et al., 2013).

2.5.2 Phytohormones

Plant growth promotion can also be regulated by the production of hormones and other compounds related to plant development. Auxin is a class of plant hormones important in the promotion of lateral root formation. Increased lateral root formation leads to an enhanced ability to take up nutrients for the plant (Saharan and Nehra, 2011). Other classes of plant hormones includes gibberellins and cytokinins, which both stimulate shoot development, however their effects on root growth are less well characterized (Zahir et al., 2003).

2.5.3 Ethylene levels

Ethylene in low levels has been observed to promote plant growth, but at moderate to high levels it may inhibit root elongation. 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, which cleaves and sequesters the plant ethylene precursor ACC and 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.

2.5.4 Nitrogen fixation

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). There are a number of PGPR, which are able to fix atmosphere nitrogen (N_2) and make it more accessible to plants. 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).

2.5.5 Induced systemic resistance

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 and colleagues (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 anti-microbial phytoalexins and the synthesis of pathogen related proteins (Van Loon, 2007). Components of bacteria that can activate ISR includes lipopolysaccharides

(LPS), flagella, salicylic acid, and sideophores (Lugtenberg and Kamilova, 2009). During root colonization, fluorescent pseudomonads produce antifungal antibiotics, elicit induced systemic resistance in the host plant or interfere specifically with fungal pathogenicity factors (Figure 6).

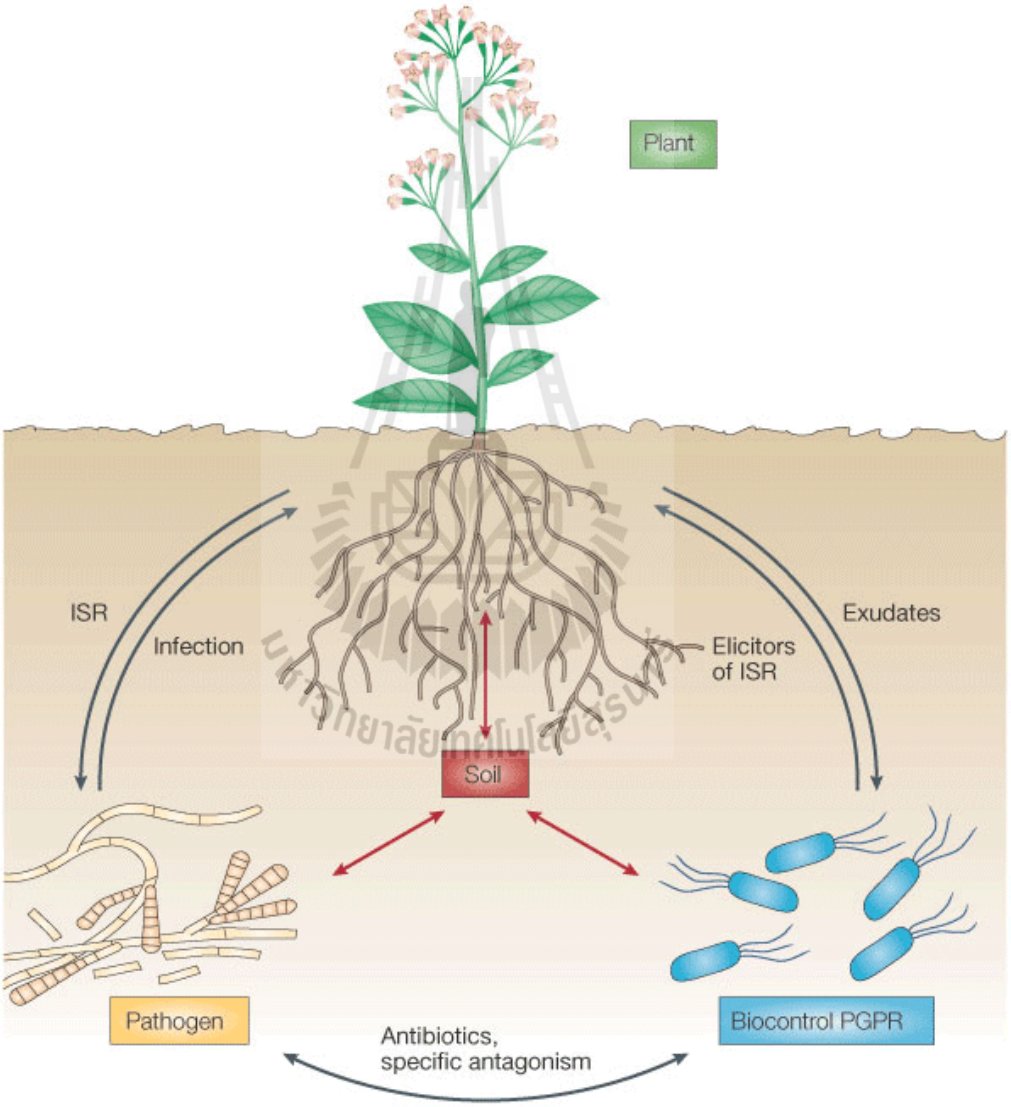


Figure 6. Interactions between biocontrol plant growth-promoting rhizobacteria (PGPR), plants, pathogens and soil (Haas and Défago, 2005).

2.5.6 Application of PGPR as biofertilizer

Agriculturally, the beneficial bacteria can be used as inoculants for crops and plants (Vessey, 2003). The term biofertilizer, which is a substance that contains living microorganisms and when applied to seeds, plant surfaces, or soil, it promotes growth by increasing the supply or availability of primary nutrients to the host plant (Vessey, 2003). 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 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.

2.6 Coinoculation 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 symbiont competitive ability and host fitness. 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 nonadditive interactions between strains (Friesen and Heath, 2013). 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). 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 coinoculation of rhizobia with some PGPR is a way to improve nitrogen availability in sustainable agriculture production systems. Some PGPR strains, from a range of genera, enhance legume growth, nodulation and nitrogen fixation when coinoculated with rhizobia. Examples of these are *Azospirillum* (Askary et al., 2009; Aung et al., 2013; Cassán et al., 2011; Remans et al., 2008), *Azotobacter* (Wu et al., 2012), *Bacillus* (Atieno et al., 2012; Bai et al., 2003; Mishra et al., 2009a), *Pseudomonas* (Argaw, 2011; Li and Alexander, 1988; Yuttavanichakul et al., 2012), *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. Compared to single Rhizobium inoculation, coinoculation 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*. The coinoculation and the application of *Bradyrhizobium* and Arbuscular Mycorrhizal Fungi (AMF) were increased soybean growth and yield in winter season (Corbera and Nápoles, 2010), under low P and/or low N conditions (Wang et al., 2011), and directly inhibited soybean red crown rot in acid soils (Gao et al., 2012). Moreover, complex interactions and competition between the three microorganisms (*B. japonicum* and *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 coinoculation with *Bradyrhizobium* and PGPR on soybean response was summarized in Table 2.

Table 2. Coinoculation with *Bradyrhizobium* and PGPR on soybean response

Bradyrhizobium	Coinoculating PGPR	Soybean response to the coinoculation	References
Bradyrhizobium spp.	*B. subtilis.* *P.fluorescens*	Increasing the nodulation, shoot dry matter and N-content. Salient superiority in suppressive disease.	Shehata et al. (2012)
Bradyrhizobium sp.	*Bacillus* sp. *Phyllobacterium* sp.	Increasing the number of nodules, nodule dry weight, plant dry weight, total N and P contents.	Xue et al. (2011)
Bradyrhizobium sp.	*Pseudomonas* sp.	Increasing grain yield in pot and field experiment and increasing survival efficiency of *Bradyrhizobium*.	Afzal et al. (2010)
B. japonicum	*Aspergillus niger*	Increasing the nitrogen fixation and induces the plant growth and inhibiting the pathogenic fungi.	Sharma and Kumawat (2011)
B. japonicum	*Azomonas agilis,* *Azospirillum lipoferum* *P. fluorescens*	Enhancing nodulation and ARA but *P. fluorescens* WCS365 decreased the nodule number and ARA.	Chebotar et al. (2001)
B. japonicum	*Azospirillum* sp.	Co-inoculation effects on competitive nodulation and rhizosphere eubacterial community structures of soybean under rhizobia-established soil conditions.	Aung et al. (2013)
B. japonicum	*Azospirillum brasilense*	Increasing number of the most active nodules, therefore, to a greater nitrogen fixation and assimilation.	Groppa et al. (1998)
B. japonicum	*Azospirillum brasilense*	Increasing soybean yield and improved nodulation under field experiment.	Hungria et al. (2013)
B. japonicum	*Azotobacter chroococcum*	Increasing the number and fresh weight of nodules and seedling dry weight under drought stress.	Hadi et al. (2010)
B. japonicum	*B. subtilis*	Increasing the yield and nodule occupancy using the liquid and granule-based formulation type.	Atieno et al. (2012)

Table 2. (Continued)

Bradyrhizobium	Coinoculating PGPR	Soybean response to the coinoculation	References
B. japonicum	*B. subtilis* *B. thuringiensis*	Enhanced soybean plant growth, soybean production systems in short growing season regions.	Bai et al. (2003)
B. japonicum	*B.thuringiensis*	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. *Streptomyces griseus*	Enhancing nodule number, pod formation, yield, and seed weight.	Li and Alexander (1988)
B. japonicum	*Serratia proteamaculans* *S.liquefaciens*	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.	Increasing in nodule occupancy varied from 0% to 18.3% and shoot nitrogen composition.	Gregor et al. (2003)

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. In addition, an increase in soil enzymatic activities (phosphatase, b-glucosidase, 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). It has been also shown that presence of ACC deaminase enzyme in PGPR enhanced the nodulation of mungbean up on coinoculation with *Bradyrhizobium* (Ahmad et al., 2011). 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 addition, 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.

CHAPTER III

MATERIALS AND METHODS

3.1 Bacterial strains

Bradyrhizobium japonicum strains USDA110 and THA6 those commercially used in rhizobial inoculants production for soybean in Thailand were obtained from the Department of Agricultural, Bangkok, Thailand. They were cultured in YEM broth (Somasegaran and Hoben, 1994). The PGPR isolates were originally isolated from soybean rhizosphere soil as described by Piromyou et. al. (2011) from 30 soybean fields in 11 Provinces of Northern and North-Eastern parts of Thailand (Figure 7) including;

- Chiang Mai (18° 34' 33.47"N/98° 52' 52.91"E)
- Chiang Rai (20° 20' 36.02"N/99° 54' 38.97"E)
- Lampang (18° 13' 44.49"N/99° 35' 30.20"E)
- Lamphun (18° 36' 21.83"N/99° 2' 42.91"E)
- Lopburi (15° 6' 18.97"N/101° 11' 49.45"E)
- Mae Hong Sorn (19° 21' 14.06"N/98° 25' 54.45"E)
- Nakhon Ratchasima (15° 1' 22.99"N/101° 36' 27.48"E)
- Nakhon Sawan (15° 39' 45.75"N/100° 34' 12.02"E)
- Phitsanulok (16° 52' 28.31"N/100° 21' 19.60"E)
- Phrae (17° 58' 25.80"N/99° 59' 52.40"E)
- Uttaradit (17° 41' 13.63"N/100° 8' 27.17"E)

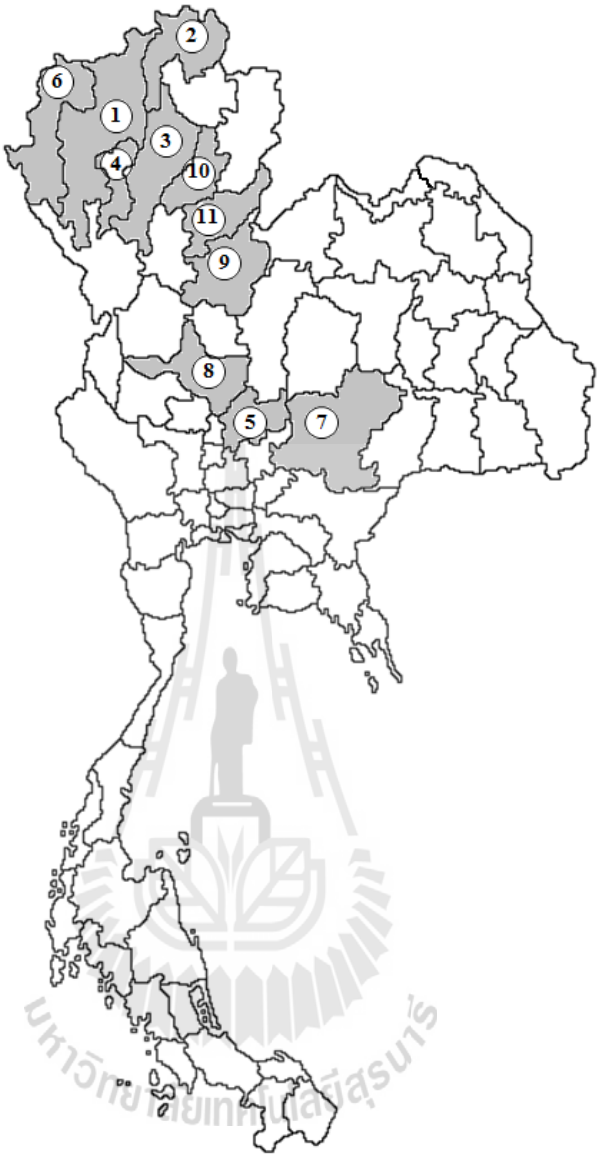


Figure 7. Site locations of soybean fields across Northern and North-Eastern part of Thailand sampled for soybean rhizpsphers soil. Number represented the name of province; 1. Chiang Mai, 2. Chiang Rai, 3. Lampang, 4. Lamphun, 5. Lopburi, 6. Mae Hong Sorn, 7. Nakhon Ratchasima, 8. Nakhon Sawan, 9. Phitsanulok, 10. Phrae and 11. Uttaradit.

The PGPR on LG plates (N-free) were incubated for 2 days at 30^{o}C and bacteria representative of the predominant morphologically distinct colonies present

on the plates were selected for further analysis. All 285 bacterial isolates were screened against both *B. japonicum* USDA110 and THA6 by antimicrobial spot test. Each *B. japonicum* culture was swab over YEM agar plates and after the plates were dried, 20 µl of each strains of PGPR suspension was spotted onto *B. japonicum* agar plate. After 4 days cultivation at 30°C, the plates were examined for growth inhibition by observing the clear zones. Only PGPR isolates which not shown any clear zone on both strains of *B. japonicum* USDA110 and THA6 agar plate were selected for further coinoculation assay. All bacterial cultures were maintained by periodic transferred and stored in the refrigerator for further studies.

3.2 Coinoculation effect of *B. japonicum* and PGPR on soybean

3.2.1 Leonard's jar experiment

The soybean cultivar tested is recommended lines for using under Thai field condition. Seeds of soybean (*Glycine max* (L.) Merrill) cv. Chiang Mai 60 were cultivated in growth chambers using modified Leonard's jar assemblies (Blauenfeldt et al., 1994). Seeds were surface sterilized and gnotobiotically germinated on wet tissue paper (Somasegaran and Hoben, 1994). The early stationary phase of PGPR and *B. japonicum* cultures were centrifuged (4,000 x g 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. A preliminary Leonard' jar experiment was conducted to evaluate the coinoculation effects of *B. japonicum* USDA 110 and THA6 with 45 PGPR isolates on soybean. For the single inoculation, the seedlings were inoculated separately with 1 ml of 10^6 CFU ml^{-1} of PGPR or *B. japonicum* and mixed in a ratio of 1 : 1 for coinoculation treatment. In the control treatment, the cell

suspensions were replaced by distilled water. Plants were grown in growth chamber at 27/20°C light room under 16/8 h light/dark photoperiod. 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 sampled at 45 days after inoculation (DAI) and the nodule number, nodule and plant dry weight were recorded. The top 12 PGPR that can promote soybean production were selected for further studies on PGPR characterization and identification. Only 2 isolates which showed the highest capable to promoting soybean production were selected for further experiments.

3.2.2 Field experiment

Single and coinoculation inocula were prepared as in Leonard's jar experiment by 9 different treatments following (1) Control; Non Inoculated, (2) USDA110; single inoculated with *B. japonicum* USDA110, (3) THA6; single inoculated with *B. japonicum* THA6, (4) S141; single inoculated with *Bacillus subtilis* strain S141, (5) S222; single inoculated with *Staphylococcus* sp. strain S222, (6) U110+S141; coinoculated with *B. japonicum* USDA110 and *B. subtilis* strain S141, (7) U110+S222; coinoculated with *B. japonicum* USDA110 and *Staphylococcus* sp. strain S222, (8) THA+S141; coinoculated with *B. japonicum* THA6 and *B. subtilis* strain S141 and (9) THA+S222; coinoculated with *B. japonicum* THA6 and *Staphylococcus* sp. strain S222. The experiment was performed in two sites; site 1 was Organic Farm in Suranaree University of Technology (SUT) campus at Nakhon Ratchasima Province, and site 2 was rice paddy field at Buriram Province (Table 3). Each field site was conducted as a completely randomized design (CRD) designed by divided in to nine treatments and each treatment consist 3 replicates making a total of

27 plots according to 9 different treatments as mentioned above. Four-row plots were used with 50 cm between rows and plots were kept 0.5 m spacing between blocks. Each plot of site 1 size was 2 x 5 m (10 m^2) and site 2 was 2 × 2 m (4 m^2). Inoculum containing 10^6 CFU ml^{-1} bacteria was poured over the seeds in each row prior to covering the seeds with soil. The regular agricultural practices were done except chemical fertilizer application and pesticide spraying. Seed yield was taken from the two middle rows of each plot. Pods were removed and threshed by hand and seed yield was determined.

Table 3. Geographic coordinates and the chemical characteristics of the soil at the experimental sites.

Properties	Field site	
	SUT Organic Farm	**Buriram site**
Location (Province)	Nakhon Ratchasima	Buriram
Latitude/longitude	14° 52' 16.11"N/ 102° 1' 31.95" E	14° 34' 3.63" N/ 102° 32' 38.93" E.
History of planting	has a history of legume cultivations but no history of chemical fertilizer application	has a history of chemical fertilizer application but no history of legume cultivations
Period of cultivation	January - April 2012	August - November 2012
Average rainfall (nm)	31.07	152.03
Average temperature (^{o}C)	24.4-30.6	26.4-29.1
Average humidity range (%)	63.1-69.2	71.1-83.5
Soil characteristic		
Texture	clay	clay loam
pH	6.41	5.79
EC (ms/cm)	0.031	0.420
Organic matter content (%)	2%	1.92
Available P (ppm)	11	19.80
Exchangeable K (ppm)	330	43.57

3.3 Effect of inoculation dose of PGPR

Soybean seedlings coinoculated with *B. japonicum* USDA110 or THA6 at 10^6 cells/seed (as recommended used for soybean cultivation in Thailand) were mixed in a ratio of 1 : 1 with PGPR isolate S141 or S222 at five inoculum doses; 10^3, 10^4, 10^5, 10^6 and 10^7 CFU ml^{-1}. *B. japonicum* and PGPR culture were used 1 ml/seed in a full dose for single inoculation or in a half dose in coinoculation 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).

3.4 Bacterial Plant growth promoting characteristics and identification

3.4.1 IAA production Assay

The IAA production of *B. japonicum* and PGPR isolates were grown in YEM and LB medium respectively, were colorimetrically determined as described by (Costacurta et al., 1998). Pure indole-3-acetic acid (Sigma, USA) was used as standard.

3.4.2 ACC deaminase activity Assay

The ACC deaminase activity of *B. japonicum* and PGPR isolates were grown in YEM and LB medium respectively, were collected according to Penrose and Glick (2003) and measured the ACC deaminase activity following to a protocol of Tittabutr et al. (2008).

3.4.3 Siderophores production assay

Bacterial isolates were assayed for siderophores production on the Chrome azurol S (CAS) agar medium (Sigma, Ltd.) described by (Schwyn and Neilands, 1987) CAS agar plates were spot inoculated with *B. japonicum* and PGPR and incubated at 30°C for up to 7 days. Development of a yellow–orange halo around the colony was considered as a positive result.

3.4.4 16S rRNA gene analysis

The selected PGPR were identified at species level based on the full length sequence analysis of 16S rRNA gene. The genomic DNA was extracted according to the method of Prakamhang et. al. (2009). The 16S rRNA universal primers 27F and 1492R were used to amplify approximately 1.5-kb internal region of the 16S rRNA gene (Weisburg et al., 1991). The nucleotide sequence of purified PCR products was analyzed at the Macrogen Service Center (Seoul, Korea). The DNA sequences were generated and the most closely related sequences were obtained from the GeneBank database.

3.5 Transmission electron microscopy (TEM) studies of bacteroids in soybean nodule sections

The fresh soybean nodules from Leonard's jar experiment were harvested at 2, 3, 4, 5 and 6 week after inoculation (WAI) and used for further investigation. The procedures for preparation of nodule were basically performed according to a method reported by Fuentes et al. (2002). Nodules that had been fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.0) overnight at 4 °C were dehydrated in an ethanol series, and embedded in Spurr's resin (Agar Scientific). Semithin sections (1 μm)

were taken for light microscopy and ultrathin (70 nm) sections were taken for TEM using an ultra-cut microtome (Ultracut RMC Boeckeler ®, Boeckeler Instruments Inc., USA) with a Diatome diamond knife. The semithin sections were collected on glass slides and stained with 0.1% toluidine blue, whereas the ultrathin sections were collected on copper grids coated with Formvar and a layer of carbon. Grids with ultrathin sections were then put on 5% uranyl acetate droplet on a piece of parafilm in the dark for 15 min. Then washed in sterilized distilled water by dipping for several times and put on drop of 0.4% lead citrate which surrounding with NaOH pellets for 15 min. Grids were washed again in new clean water and placed in a grid box when completely dry. The sections were viewed and digitally photographed using a TEM (JEOL JEM-1230, JEOL, Japan). The TEM study was conducted at Science Equipment Center, Mahasarakham University, Thailand.

3.6 Gene expression analysis

3.6.1 Sample preparation

Soybean samples were prepared and conducted in Leonard's Jar experiment as mentioned above. The fresh soybean roots were harvested at 7 days after single- and coinoculation. Whole soybean roots were washed carefully with sterilized water and flash-frozen in liquid nitrogen and then stored at –80°C for further total RNA extraction and RT-PCR analysis.

The fresh and upper most soybean nodules from main root were harvested weekly from 2-7 WAI. Nodule samples were sterilized with 95% ethanol for 10 sec and washed 5-6 times with sterilized water and then immediately frozen in

liquid nitrogen and stored at –80°C for further total RNA extraction. The experiment was laid out with CRD with three biological replications.

3.6.2 Total RNA extraction and RT-PCR analysis

The frozen plant tissues (root and nodule) were ground in TissueLyser (QIAGEN, USA) to a fine powder. Total RNA were directly isolated from plant sample using RNeasy Plant Mini Kit (QIAGEN, USA) according to the manufacturers protocol. RNAs were treated with DNaseI to prevent contamination of genomic DNA, and then cleaned by using RNeasy MinElute Cleanup Kit (QIAGEN, USA). Transcription levels were determined by Reverse-transcription polymerase chain reaction (RT–PCR). Primers for transcript amplification were listed in Table 4.

The geometric mean of relative expression ratios for three biological repetitions and corresponding upper and lower 95% confidence intervals were calculated. All RT-PCR were performed in Techne® TC-512 Thermal Cycler (TECHNE, UK) and products were visualized using 1% agarose gel electrophoresis and stained with 0.5 µg/ml of SYBR®Green, then documented on Gel documentation and analysis (Fire Reader, Uvitecv Cambridge).

3.7 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).

Table 4. Primer used in this study

Gene name	Gene description	Forward 5'-3' Reverse 5'-3'	References
PGPR mode of action			
iaaH	IAA hydrolase	CAATTTGGAACCAGTTTGGA GGGATCCATGAGAGAGGATG	Kochar et al. (2013)
ipdC	Indole-3-pyruvic acid decarboxylase	GAAGGATCCCTGTTATGCGAACC CTGGGGATCCGACAAGTAATCAGGC	Patten and Glick (2002)
Early symbiotic signaling			
nodD1	Transcriptional regulatory protein LysR family	ACGGCCTGAGGAGACGAATTGA TTGGATGATCCGCAACGGCAT	Lee et al. (2012)
GmCaMK1	Calmodulin-binding receptor-like kinase	CCTAGTTCTGTTGTCTCGCAGAA CGGGCAAGAAAGGTAACTTTCTA	DeFalco et al. (2010)
GmNIN1A	Nodulation factor receptor kinase 1	TGGCGCACCATGCTAACAT GGGTGTCATGGCAATCCTTT	Indrasumunar et al. (2011)
Nodule organogenesis and N_2 fixing			
otsA	trehalose 6-phosphate synthase	GGGGACGACCTGTGAACTTA CTTCGTAATAGCCGCCGTAA	Cytryn et al. (2007)
phbC	PHB polymerase	AAGGTGATCGCCCAGAACG ATCTGCCCGCCCTGGAT	Paganelli et al. (2011)
GmMyb	Control of nodule development	AGAGCCGGAGTAGCAGATGA ATGGCTTCAGGGTTTGATTG	Libault et al. (2009)

Table 4. (continued)

Gene name	Gene description	Forward 5'-3' Reverse 5'-3'	References
Nodule organogenesis and N_2 fixing			
nifH	Structural of nitrogenase reductase	GMRCCIGGIGTIGGYTGYGC TTGTTGGCIGCRTASAKIGCCAT	Fedorov et al. (2008)
dctA	Transport of C4-dicarboxylates	CCTGGTACCGTGTCCGCAACAATCACTG CCTGAATTCTTAGCCCGTCGTTACGGC	Trainer and Charles (2006)
Housekeeping			
27f-1492r[a]	16s rRNA	AGAGTTTGATC(A/C) TGGCTCAG GGTTAC(G/C)TTGTACCTGCCGGA	Weisburg et al. (1991)
fD1-rP2[b]	16s rRNA	AGAGTTTGATCCTGGCTCAG ACGGCTACCTTGTTACGACTT	Weisburg et al. (1991)
β-tubulin[b]	soybean *β-tubulin* gene	GACAGCATCAGCCATGTTCA AACCTCCTCCTCATCGTACT	Wang et al. (2004)

a : a housekeeping gene used for full length sequencing

b : a housekeeping gene used for normalization in this experiment

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Screening of PGPR

In this study, the PGPR isolates were preliminary isolated from soybean rhizosphere soil 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). 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.

Before any improvements to soybean coinoculant are made, it is useful to know the compatibility of *B. japonicum* and PGPR because of the possibility of antagonistic interaction among them. In this study, out of 285 soybean rhizosphere soil bacterial isolates, only 45 PGPR single isolates did not show antimicrobial activity or clear zone on *B. japonicum* strain USDA110 and/or THA6 agar plates. All 45 isolates were selected as *B. japonicum* non-inhibitors for further coinoculation experiment. The example of antimicrobial spot test was show in Figure 8.

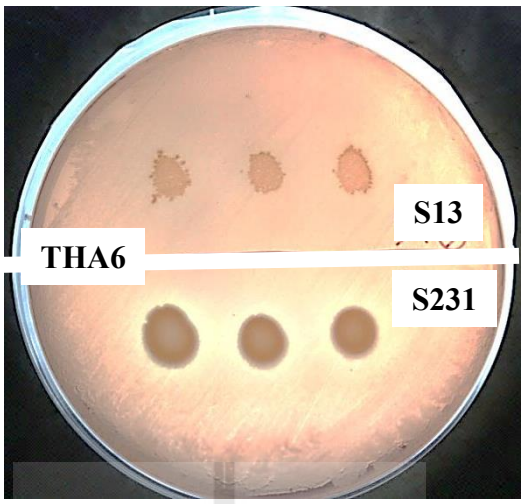


Figure 8. Example of bioassay plate showing antibacterial activity of rhizosphere soil bacterial isolates S13 and S231 on *B. japonicum* THA6 agar plate. Isolate S23 did not show clear zone on THA6 agar plate was selected for further assay whilst isolate S231 show growth inhibition of *B. japonicum* was excluded.

All 45 PGPR isolates were tested for their plant growth promotion using grown soybean for Leonard's jar trails (data not show). Only 12 PGPR showed significantly capable of promoting one or more plant parameters including nodule number, nodule and plant dry weight when coinoculated with both strains of *B. japonicum* THA6 and USDA110 over the single inoculation of *B. japonicum* on soybean at 45 DAI ($P<0.05$). Variations in nodule number at 45 DAI as consequent of different microbial inoculation and coinoculation is presented in Figure 9. The highest mean number of nodules per plant was recorded in USDA110+S222 treatment (49 nodules/plant) followed by THA6+S222 (46.75 nodules/plant) when compared with all other treatments and control. Significantly lowest nodule number

per plant was recorded in USDA110+MC3 (10.25 nodules/plant), which lower than single inoculated USDA110. Enhancement in number of soybean nodule obtained from coinoculated with USDA110 and S141 or S222 than those of single coinoculation with USDA110 by 63.1 and 76.6%, respectively. Similar trend was also found in case of THA6 and its both coinoculation of S141 and S222 by 90.8 and 54.1%, respectively.

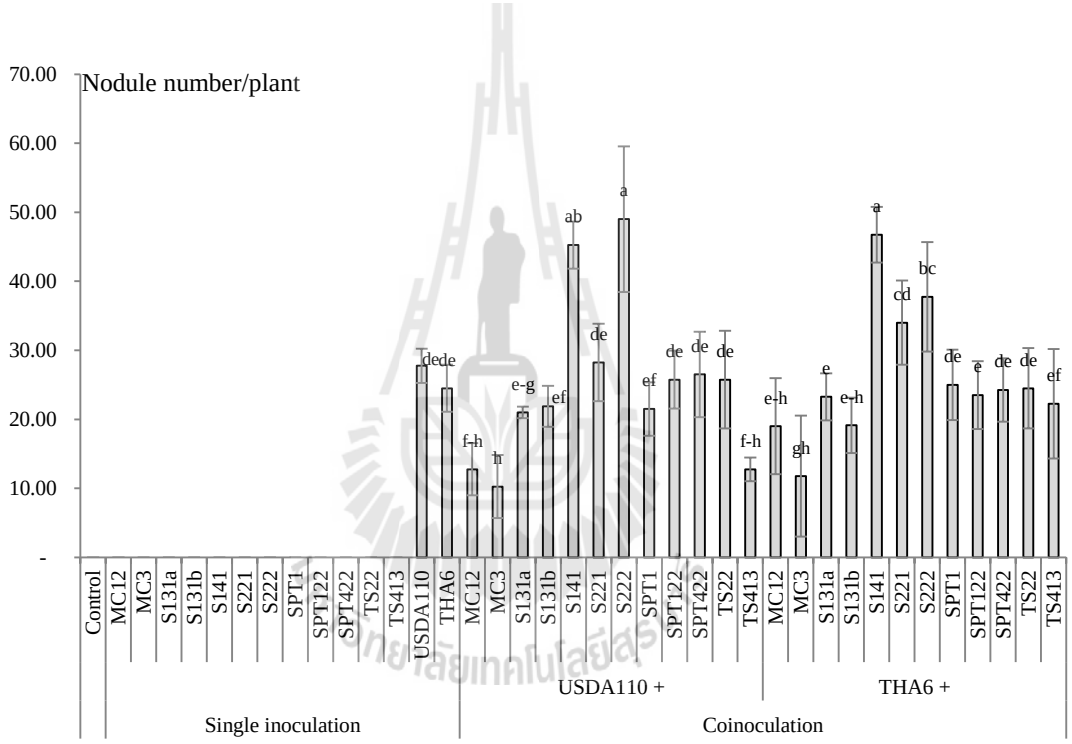


Figure 9. Nodule number of soybean after single and coinoculated between *B. japonicum* USDA110 or THA6 with 12 selected PGPR isolates at 45 DAI. Treatments are represented by Control (non-inoculated); USDA110 (*B. japonicum* USDA110); THA6 (*B. japonicum* THA6); + (coinoculation treatment). Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=5).

Significant variation in the nodule dry weight was observed due to seed treatment with single *B. japonicum* and coinoculated *B. japonicum* with PGPR. The data on the nodule dry weight was presented in Figure 10.

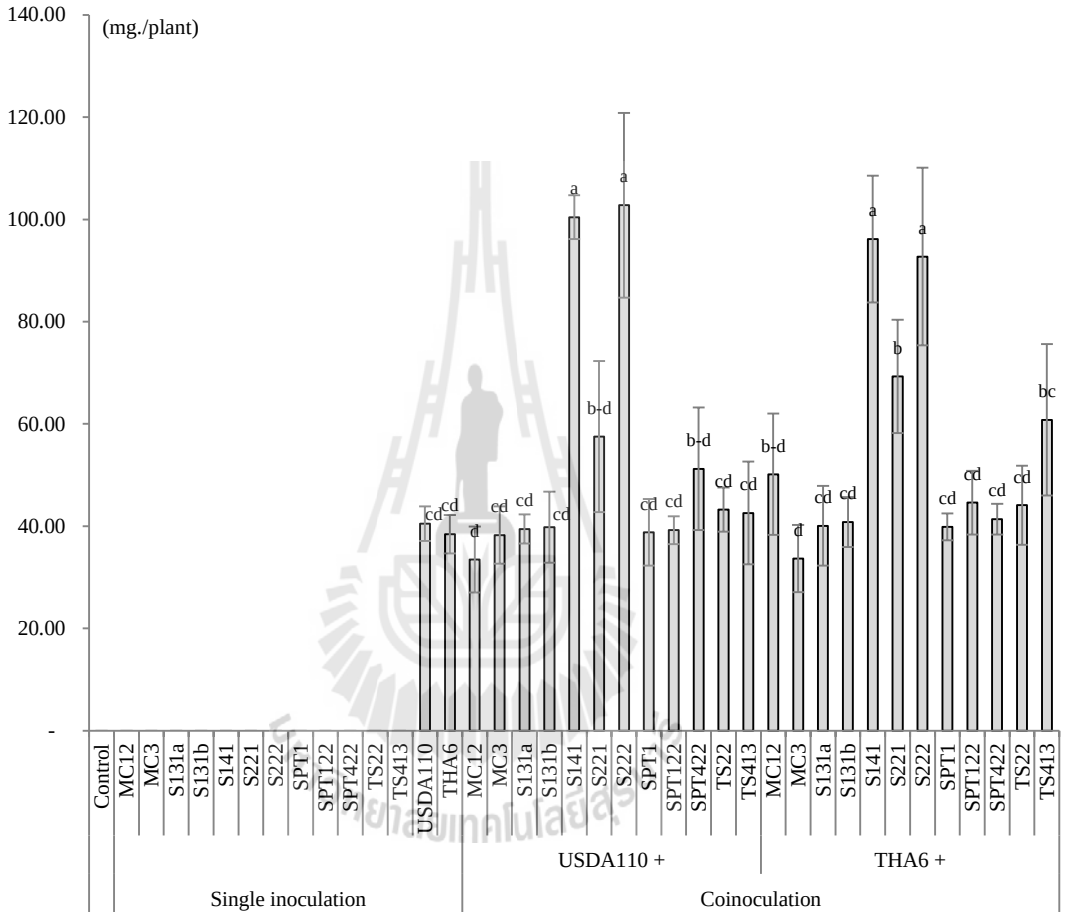


Figure 10. Nodule dry weight (mg/plant) of soybean after single and coinoculated between *B. japonicum* USDA110 or THA6 with 12 selected PGPR isolates at 45 DAI. Treatments are represented by Control (non-inoculated); USDA110 (*B. japonicum* USDA110); THA6 (*B. japonicum* THA6); + (coinoculation treatment). Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=5).

At 45 DAI, significantly higher nodule dry weight of 102.8 mg/plant was recorded in USAD110+S222 treatment followed by USDA110+S111 (100.43 mg/plant). The lower nodule dry weight of 38.45 mg/plant was recorded in coinoculation of THA6+MC3. Coinoculation of USDA110 with PGPR strains S141 and S222 enhanced nodule dry weight of soybean compared with single inoculated with USDA110 by 148.1 and 102.8%, respectively. Soybean nodule dry weight was significantly improved when coinoculated between THA6 and S141 and S222 by 150.1 and 141.2%, respectively.

Soybean plant dry weight was significantly differed at 45 DAI due to various inoculation treatments (Figure 11).

Coinoculation USDA110+S141 treatment showed significantly maximum plant dry weight (1,400.5 mg/plant) followed by USDA110+S222 treatment (1,377.9 mg/plant). Minimum plant dry weight (401.0 mg/plant) was recorded in single inoculation TS22 which was on par with uninoculation control and all PGPR single inoculation treatments. Coinoculation of USDA110 with PGPR S141 and S222 enhanced soybean plant dry weight compared with single inoculated with USDA110 by 39.3 and 37.1%, respectively. Similar results also found in case of THA6, soybean plant dry weight was increased when coinoculated with THA6 and PGPR isolates S141 (40.4%) and S222 (50.1%) when compared with single inoculated of THA6.

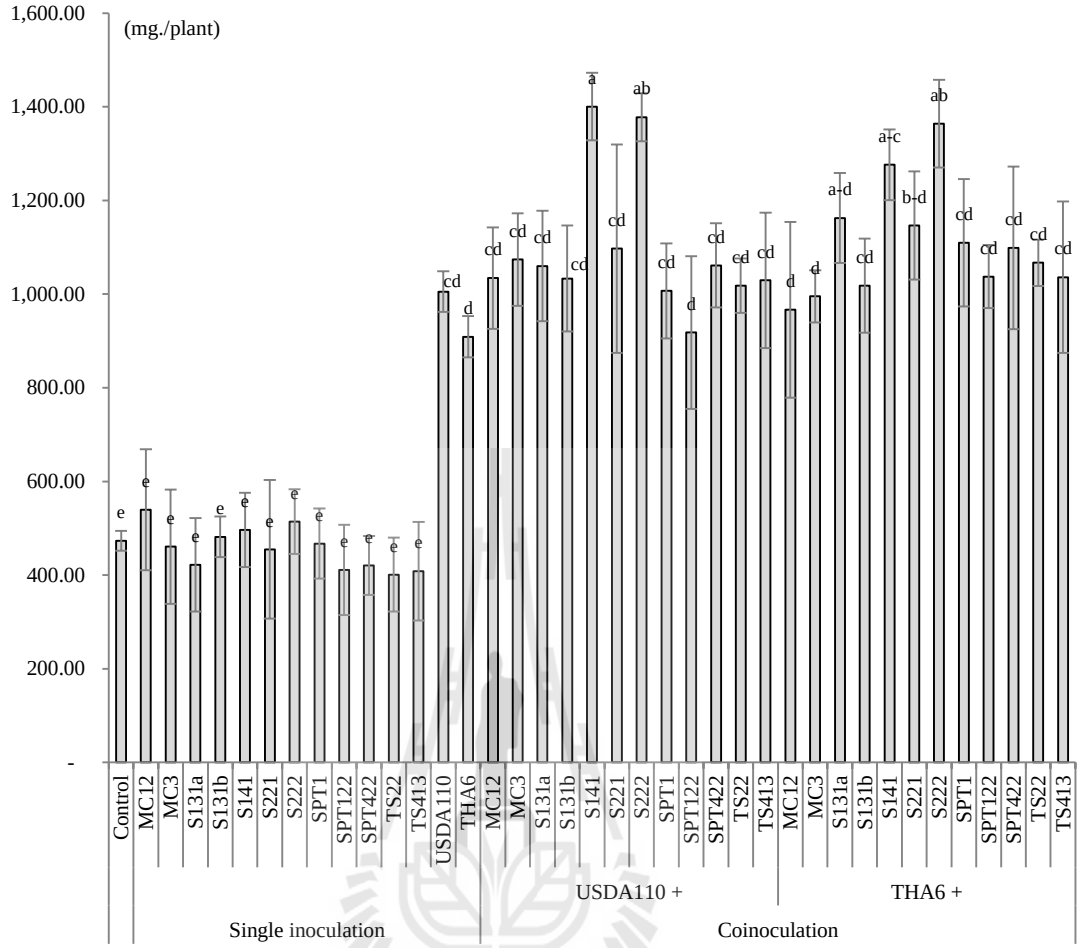


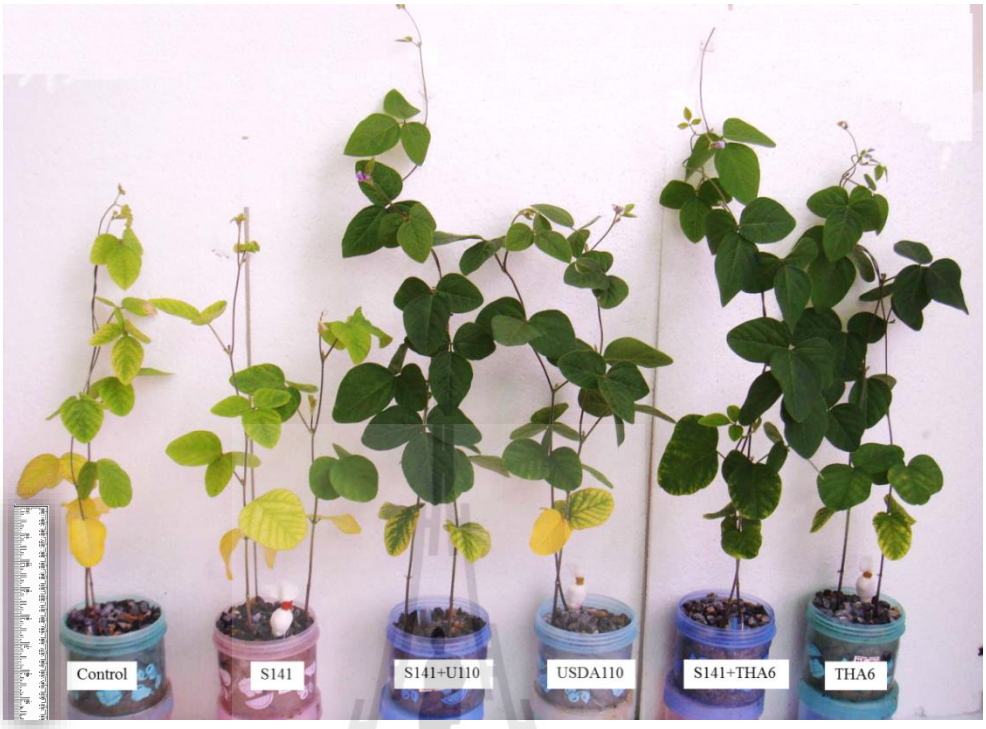
Figure 11. Plant dry weight (mg/plant) of soybean after single and coinoculated between *B. japonicum* USDA110 or THA6 with 12 selected PGPR isolates at 45 DAI. Treatments are represented by Control (non-inoculated); USDA110 (*B. japonicum* USDA110); THA6 (*B. japonicum* THA6); + (coinoculation treatment). Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=5).

All the coinoculation treatments were capable of induction one or more plant growth parameters, nodule number, nodule dry weight and/or plant dry weight. Among the coinoculation treatments, S141 and S222 were performed significantly

highest in all plant growth parameter when coinoculated with USDA110 or THA6. Therefore, isolates S141 and S222 were selected for further experiments. Soybean plant growth under Leonard's jar experiments by coinoculation between isolates S141 (Figure 12A) and S222 (Figure 12B) with *B. japonicum* USDA110 and THA6 were compared with those of single inoculation. Those of significant increase in nodule number, nodule dry weight, and plant dry weight of soybean due to coinoculation with *Bradyrhizobium* and PGPR could be attributed to a greater nitrogen fixation and nodulation property.

The IAA production and strain identification using 16S rRNA gene sequences of 12 PGPR strains including 2 strains of *B. japonicum* were assessed (Table 5).

A.



B.

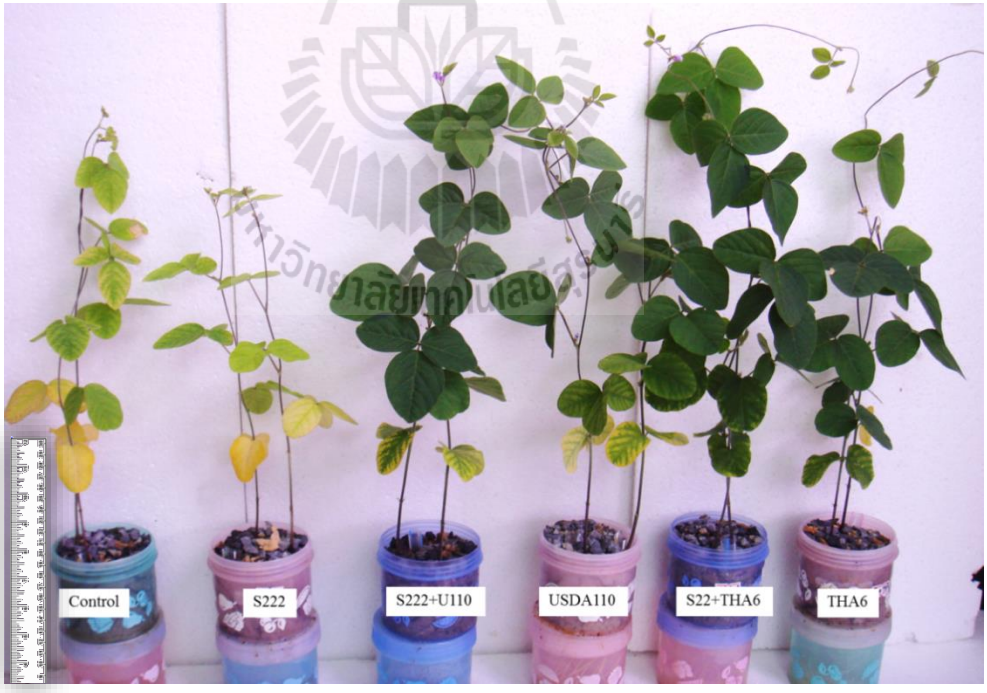


Figure 12. Soybean plant growth under Leonard's jar experiments by coinoculation with S141 (A) or S222 (B) and *B. japonicum* at 45 DAI.

Table 5. IAA production and 16S rRNA gene sequence analysis of selected PGPR isolates[a]

Isolates	IAA production (ug/ml)	% Identity
USDA110	0.01 ^{g} ± 0.00	100% *Bradyrhizobium japonicum*
THA6	0.01 ^{g} ± 0.00	100% *Bradyrhizobium japonicum*
MC12	2.00 ^{fg} ± 1.00	100% *Serratia marcescens* strain d4
MC3	11.33 ^{cd} ± 1.53	100% *Bacillus* sp. C-24
S131a	2.33 ^{fg} ± 1.53	99% *Bacillus cereus* DZ4
S131b	13.67 ^{c} ± 2.08	100% *Arthrobacter* sp. RSBA1
S141	**19.33 ^{b} ± 3.21**	**100% *Bacillus subtilis* GB03**
S221	5.33 ^{ef} ± 2.08	99% *Pseudomonas putida*
S222	**5.00 ^{ef} ± 2.00**	**99% *Staphylococcus* sp. JMP-C**
SPT1	5.33 ^{ef} ± 2.31	99% *Bacillus megaterium* XA7-7-1
SPT122	23.00 ^{a} ± 3.00	99% *Staphylococcus sciuri*
SPT422	8.33 ^{de} ± 2.89	100% *Bacillus* sp. C-1
TS22	7.00 ^{e} ± 1.00	98% *Bacillus megaterium* TOBCMDU-1
TS413	1.33 ^{g} ± 1.15	96% Unculturable *Staphylococcus* sp.

[a] Means (n=3) from a same column followed by different letters are significantly different ($p \leq 0.05$, Duncan's test), ± Standard Deviation.

Isolate SPT122 produced highest amounts of IAA followed by S141 and S131b (23.00, 19.33 and 13.67 ug/ml, respectively). However, the IAA production was neither detected in *B. japonicum* USDA110 nor THA6. All the PGPR used for inoculation were assessed for their ACC-deaminase activity and siderophore but none of PGPR produced ACC-deaminase or siderophore.

The effective culturable PGPR isolates were found to be closely related to *Bacillus subtilis*, *Staphylococcus* sp. *Serratia marcescens, B.cereus, Arthrobacter* sp., *Pseudomonas putida*, *Staphylococcus sciuri*, and *B. safensis*. Similar strains also found in co-inoculation of *B. subtilis* with two different strains of *B. japonicum* on soybean (Atieno et al., 2012; Bai et al., 2002). The co-inoculation of *Seratia* spp. with *B. japonicum* (Pan et al., 2002), *Bacillus* and *Pseudomonas* as biocontrol agent on peanut (Yuttavanichakul et al., 2012), *Staphylococcus* sp. was also isolated from root nodules of the wild legume in China (Deng et al., 2011).

The most commonly implicated mode of action of helper bacteria or PGPR are IAA production, ACC-deaminase activity and siderophore production (Drogue et al., 2013). Since, the most effective PGPR isolates in this study coinoculation with both *B. japonicum* strains were S141 and S222. The *B. subtilis* S141 and *Staphylococcus* sp. S222 were able to produce IAA in the level 19.3 and 5 ug/ml, respectively. A number of studies have reported that Bacillus species are potent IAA producer and thus may soybean plant growth (Araújo et al., 2005; Idris et al., 2007; Mishra et al., 2009b; Wahyudi et al., 2011). However, no data was found on the association between *Staphylococcus* species and the ability to increase the IAA content and growth of soybean but this found only in wheat (Ali et al., 2009). *S. sciuri* was collected from rhizosphere soil of cultivated vegetable crops and produced

IAA (Kumar et al., 2011). *Arthrobacter* sp. has identified as phosphate solubilization bacteria isolated from tomato rhizosphere soil and also demonstrated IAA production and biocontrol activities (Banerjee et al., 2010).

Interestingly, isolates SPT122 which closely relate to *S. sciuri* showed the highest IAA production (23.0 ug/ml) but did not show the highest capability of promoting plant mass when coinoculated with both strains of *B. japonicum*. It was found that, the impact of exogenous IAA on plant development ranges from positive to negative effects (Lambrecht et al., 2000). The actual concentration of bacterial IAA available to the plant is, contingent upon the physical relationship between the two organisms (Patten and Glick, 2002). However, in several studies demonstrated that *Azospirillum* IAA biosynthesis alone cannot account for the overall plant growth-promoting effect observed (Fibach-Paldi et al., 2012). Therefore, it was suggested that the growth and yield promotion with *Azospirillum*, postulating that growth promotion is the result of multiple mechanisms (phytohormone biosynthesis, nitrogen fixation, among others) working together (Spaepen et al., 2007). It can be postulated that production of an IAA by a PGPR does not always stimulate the soybean-rhizobia symbiosis.

Furthermore, the ACC-deaminase activity and siderophore production could not be detected in all selected PGPR. These results implied that the ACC-deaminase activity and siderophore production may not always help to promote soybean production when coinoculated with *B. japonicum*.

Taken together with the results of preliminary coinoculation experiment and PGPR indentification, PGPR isolates S141 and S222 were selected for further experiment of determined inoculant dose experiments.

4.2 Determination for dose of Bradyrhizobium and PGPR

The inoculation dose of Bradyrhizobium and PGPR coinoculation inoculant is important factor in the application of microbial inoculants to soybean. To determine how the proportion of the two strains in the inoculum mixtures might affect soybean production, the four possible paired combinations involving two *B. japonicum* and 2 PGPR strains were used to coinoculate soybean in five different ratios under Leonard's jar condition.

There were differences among PGPR doses for all soybean parameters including plant dry weight (Figure 13). The results of the controls and single inoculation of each PGPR were not different from each other on soybean plant dry weight. All inoculant doses tested (10^3 - 10^7 CFU ml^{-1}) of single inoculation of *B. japonicum* or coinoculation treatments produced higher plant dry weight than those obtained in uninoculated control or single inoculated PGPR treatments.

Soybean nodule number (Figure 14) and nodule dry weight (Figure 15) of single inoculation of *B. japonicum* and all coinoculation treatments were significantly different ($P < 0.05$) among the bacterial concentrations tested. In case of USDA110 with S141 and S222, the inoculum dose at 10^6 : 10^6 CFU ml^{-1} increased plant biomass by 115.5 and 65.8%, nodule number by 64.85 and 113.3%, nodule dry weight by 127.0 and 133.9% and ARA activity by 98.5 and 114.8%, respectively. In case of THA6, the optimum coinoculation dose was also 10^6 CFU ml^{-1} for both PGPR isolates S141 and S222. Since this dose increased plant biomass by 78.6 and 48.7%, nodule number by 38.0 and 23.4%, nodule dry weight by 67.0 and 142.5%, and ARA activity by 69.9 and 128.8%, respectively (Figure 16). Therefore, the optimum

coinoculation dose was at 10^6 CFU ml^{-1} for *B. japonicum* USDA110 and THA6 with 10^6 CFU ml^{-1} for both PGPR isolates S141 and S222.

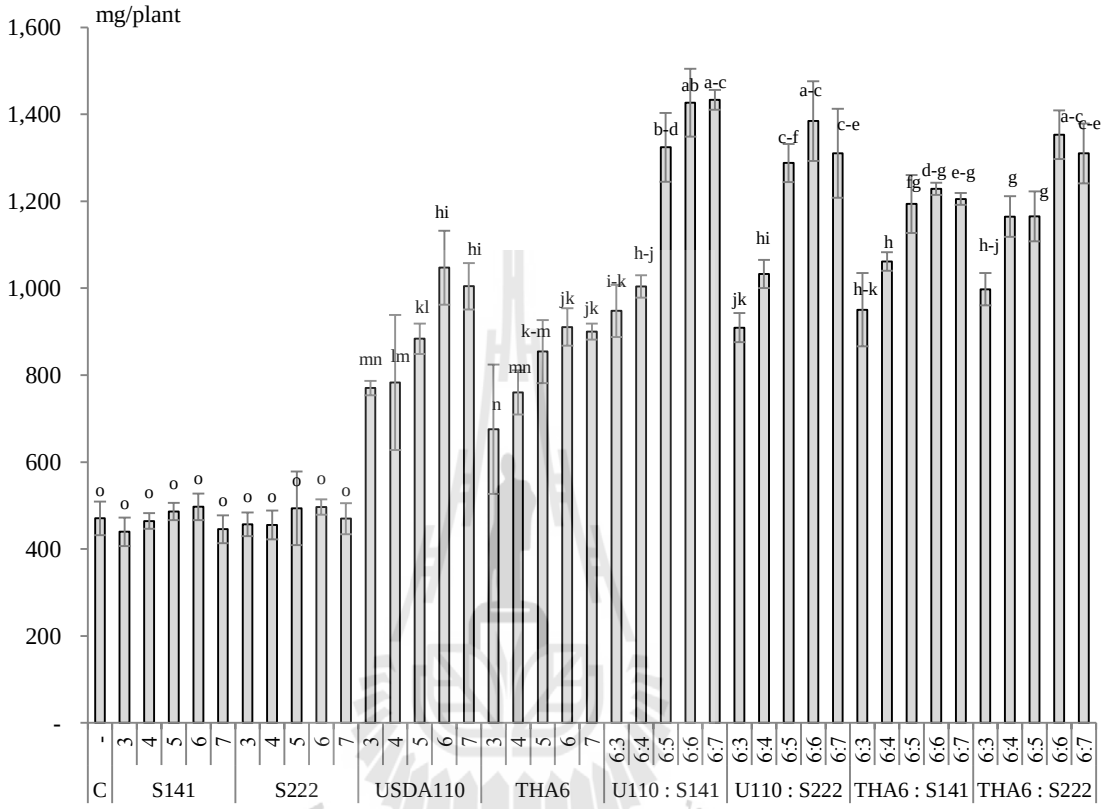


Figure 13. Effects of inoculation dose between *B. japonicum* USDA110 or THA6 at 10^6 cells/seed with various dose of PGPR isolates S141 and S222 on soybean plant dry weight (mg/plant). The number at the x-axis symbolized the varied inoculation doses of PGPR are 10^3, 10^4, 10^5, 10^6 and 10^7 CFU $ml^{-1}$and the coinoculation ratio between *B. japonicum* at 10^6 CFU ml^{-1} and varies inoculation dose of PGPR from 10^3 – 10^7 CFU ml^{-1}. Treatments are represented by Control (non-inoculated); U110 (*B. japonicum* USDA110); THA6 (*B. japonicum* THA6); + (coinoculation treatment). Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=5).

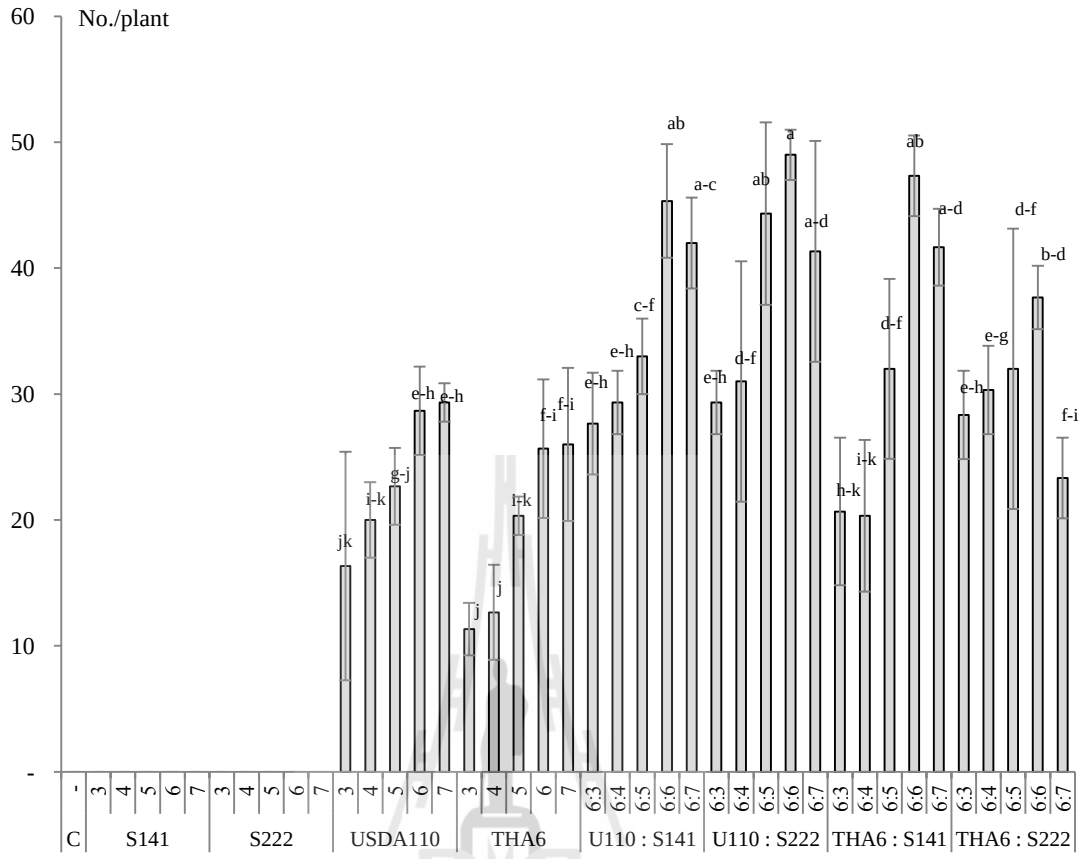


Figure 14. Effects of inoculation dose between *B. japonicum* USDA110 or THA6 at 10^6 cells/seed with various dose of PGPR isolates S141 and S222 on nodule number per plant. The number at the x-axis symbolized the varied inoculation doses of PGPR are 10^3, 10^4, 10^5, 10^6 and 10^7 CFU $ml^{-1}$and the coinoculation ratio between *B. japonicum* at 10^6 CFU ml^{-1} and varies inoculation dose of PGPR from 10^3 – 10^7 CFU ml^{-1}. Treatments are represented by Control (non-inoculated); U110 (*B. japonicum* USDA110); THA6 (*B. japonicum* THA6); + (coinoculation treatment). Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=5).

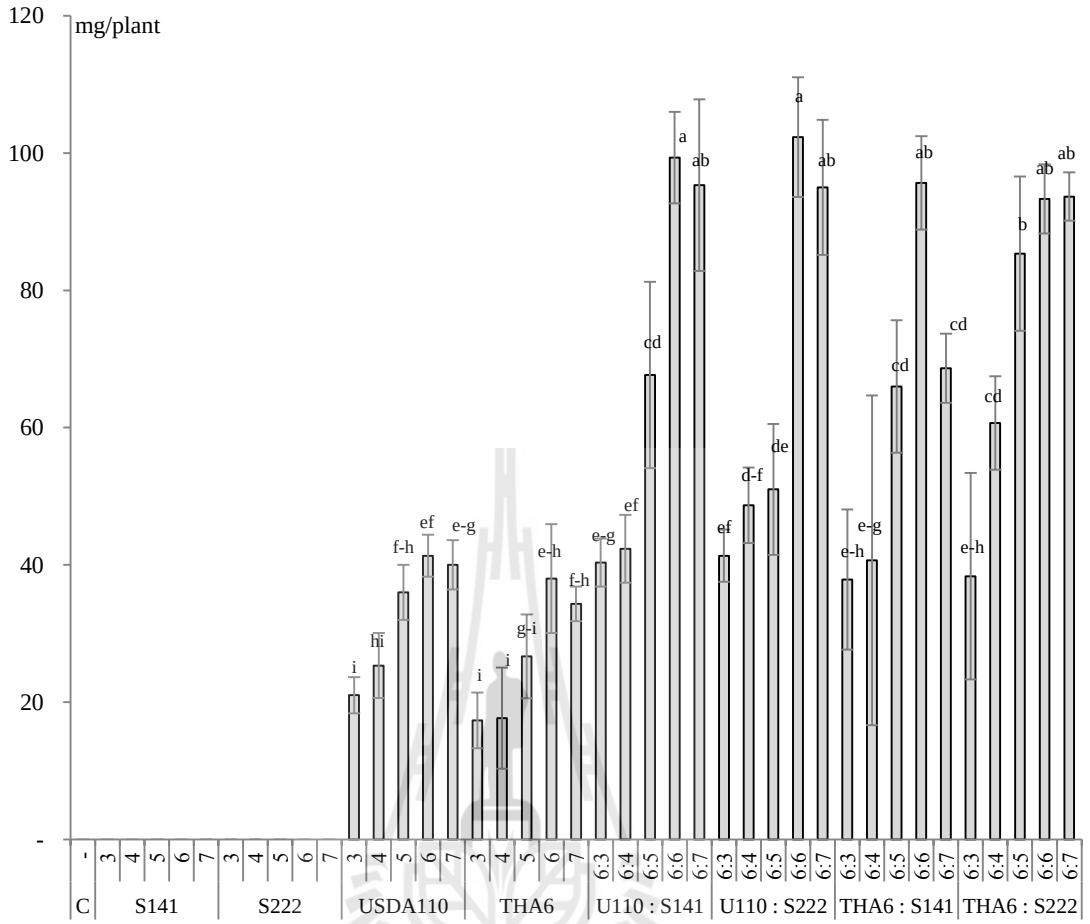


Figure 15. Effects of inoculation dose between *B. japonicum* USDA110 or THA6 at 10^6 cells/seed with various dose of PGPR isolates S141 and S222 on nodule dry weight (mg/plant). The number at the x-axis symbolized the varied inoculation doses of PGPR are 10^3, 10^4, 10^5, 10^6 and 10^7 CFU $ml^{-1}$and the coinoculation ratio between *B. japonicum* at 10^6 CFU ml^{-1} and varies inoculation dose of PGPR from 10^3 – 10^7 CFU ml^{-1}. Treatments are represented by Control (non-inoculated); U110 (*B. japonicum* USDA110); THA6 (*B. japonicum* THA6); + (coinoculation treatment). Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=5).

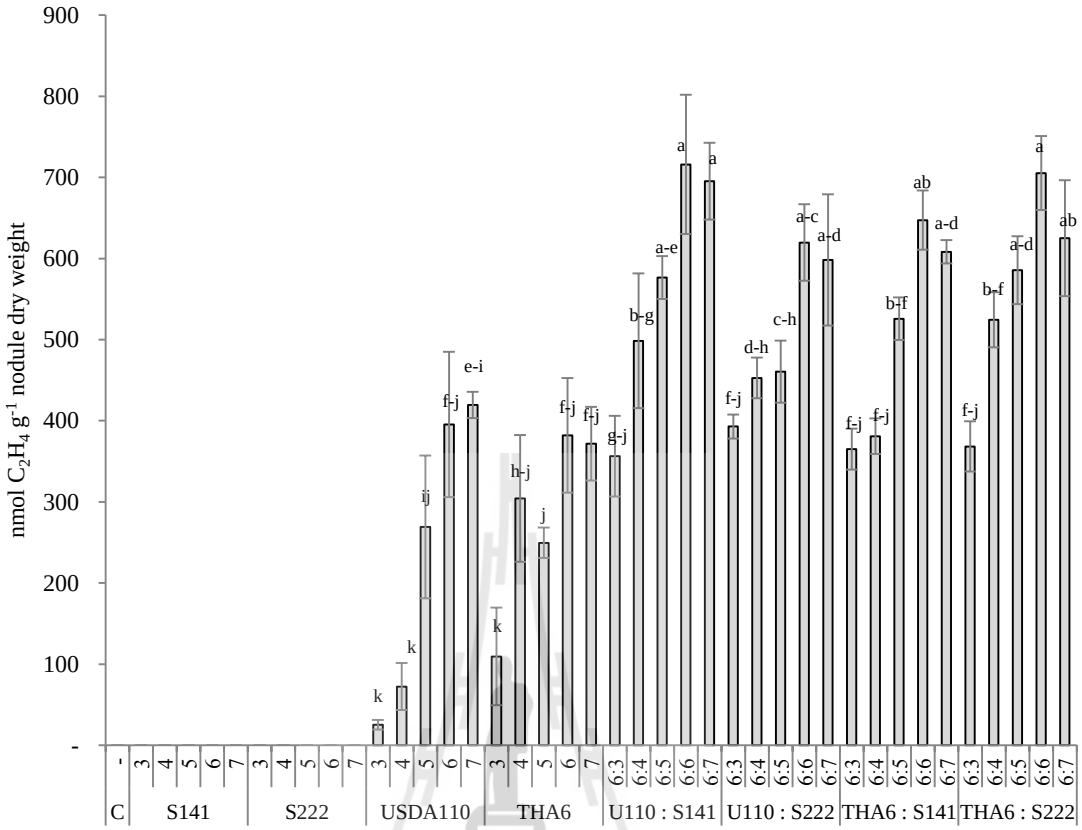


Figure 16. Effects of inoculation dose between *B. japonicum* USDA110 or THA6 at 10^6 cells/seed with various dose of PGPR isolates S141 and S222 on N_2-fixing activity (nmol C_2H_4 g^{-1} nodule dry weight) (D). The number at the x- axis symbolized the varied inoculation doses of PGPR are 10^3, 10^4, 10^5, 10^6 and 10^7 CFU $ml^{-1}$and the coinoculation ratio between *B. japonicum* at 10^6 CFU ml^{-1} and varies inoculation dose of PGPR from 10^3 – 10^7 CFU ml^{-1}. Treatments are represented by Control (non-inoculated); U110 (*B. japonicum* USDA110); THA6 (*B. japonicum* THA6); + (coinoculation treatment). Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=5).

The influence of cell number on competition has been reported in several studies. It has been reported that numbers of soybean rhizobia per seed required to ensure maximum soybean seed yields are 10^5–10^6 rhizobia/seed (Catroux et al., 2001)**.** Result of this study revealed that the more increase in inoculum proportion, the more soybean production achieved. The soybean parameters were not statistically different when the inoculum rate reached 10^7 CFU ml^{-1}. This may be symbiotic nodulation of soybeans was regulated by the plant, and is suppressed in response to a high inoculum dose of *B. japonicum* (Takats, 1986). Therefore, the inoculation ratio (*B. japonicum* : PGPR) of only 1 : 1 at inoculation dose 10^6 : 10^6 CFU ml^{-1} can provide reasonable estimation of soybean production in term of plant dry weight, nodule number, nodule dry weight as well as N_2-fixing activity.

4.3 Effect of coinoculation on soybean yield under field condition

In order to evaluate the effects of coinoculation on nodule number, nodule dry weight and soybean seed yield under natural field, the experiments were carried out at two different locations, Buriram Province and SUT organic farm field (Figure 17).

A.

B.

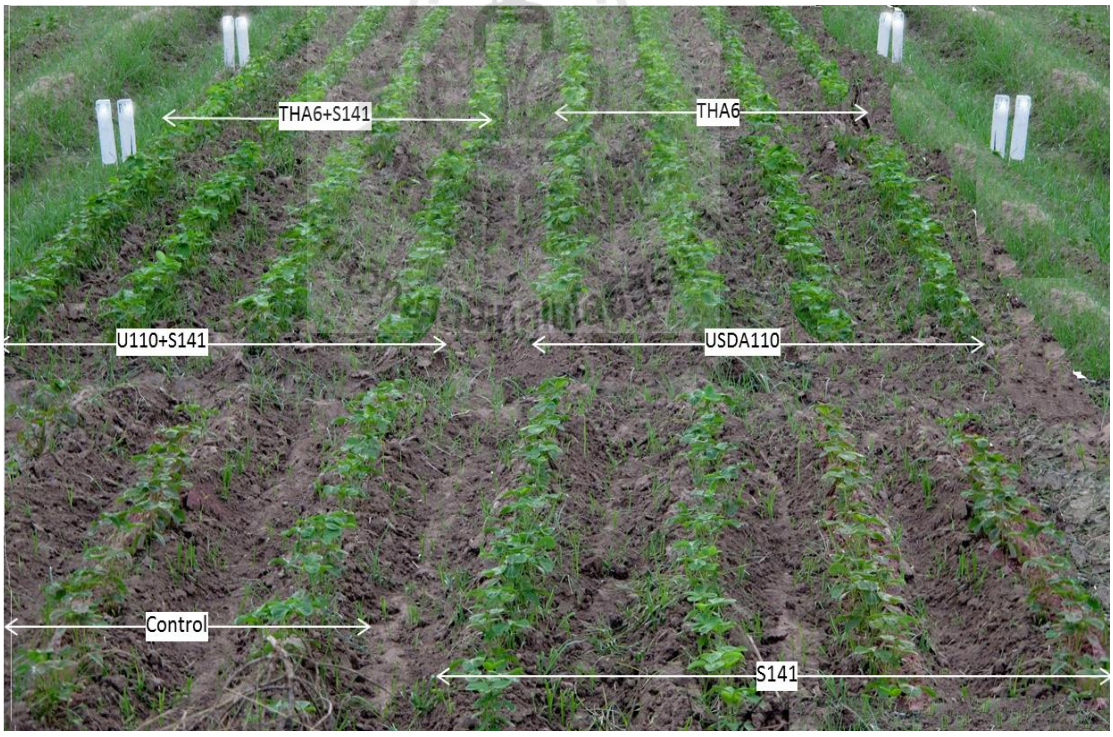


Figure 17. Soybean growth under field condition at SUT Organic Farm (A) and Buriram site (B).

4.3.1 Nodule number

Significant differences in the nodule number of soybean were observed at 45 DAI due to various inoculation treatments at Buriram site (Figure 18) and SUT Organic Farm site (Figure 19).

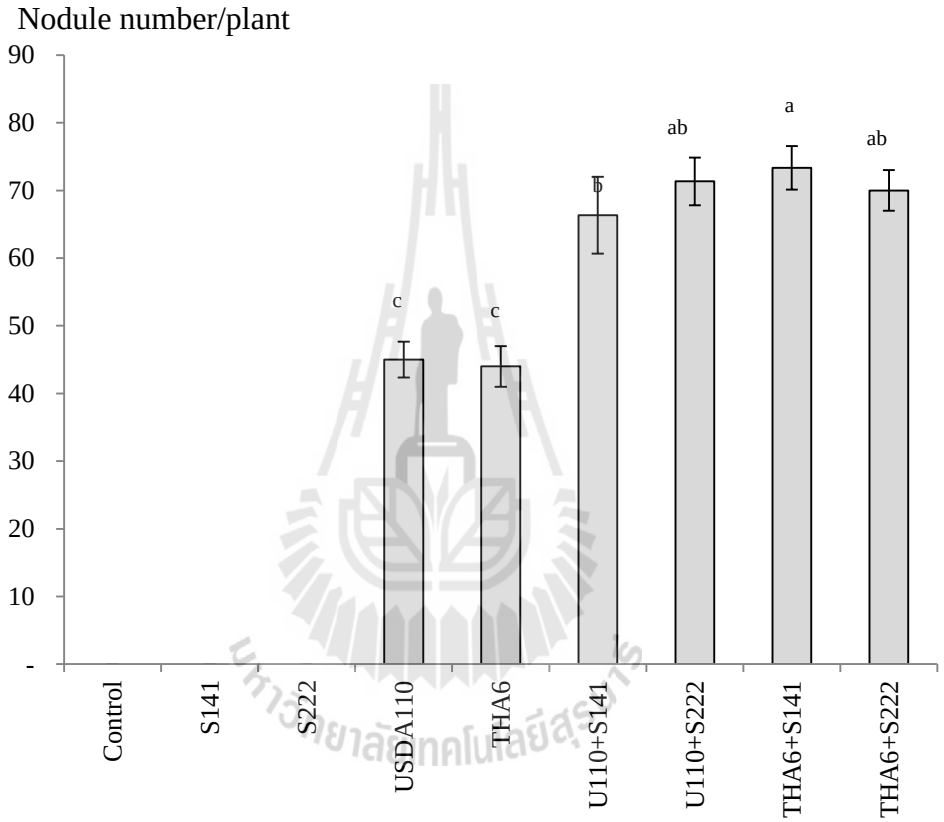


Figure 18. Effects of coinoculation between *B. japonicum* USDA110 or THA6 with PGPR isolates S141 and S222 at 10^6 : 10^6 cells/seed on nodule number per plant performed at Buriram site. Data represent the means of nine experiments, each with three replicates. Values represents mean ± SD (n=5). Within treatment, means labeled with different letters are statistically different at $P<0.05$.

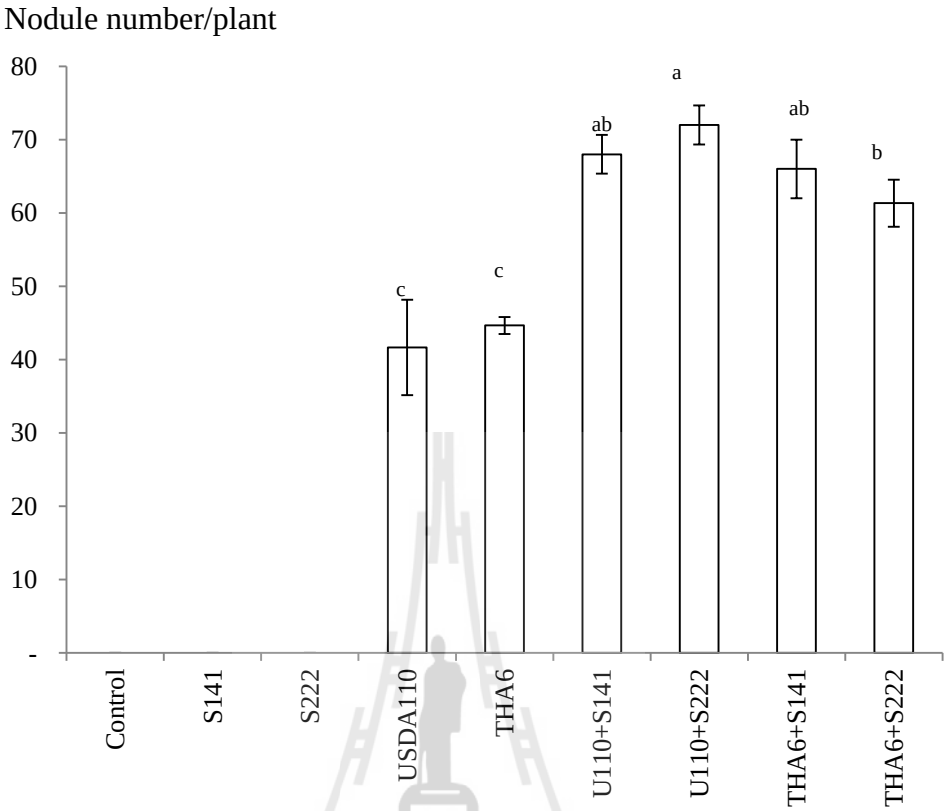


Figure 19. Effects of coinoculation between *B. japonicum* USDA110 or THA6 with PGPR isolates S141 and S222 at 10^6 : 10^6 cells/seed on nodule number per plant performed at SUT Organic Farm site (B). Data represent the means of nine experiments, each with three replicates. Values represents mean ± SD (n=5). Within treatment, means labeled with different letters are statistically different at *P<0.05*.

At Buriram site, significantly highest number of nodules per plant was recorded in THA6+S141 (73.33 nodules/plant) followed by USDA110+S222 (71.33 nodules/plant) when compared with all other treatments and significantly lowest nodule number per plant was recorded in THA6 (44.00 nodules/plant). Enhancement

in number of soybean nodule obtained coinoculated with USDA110 and S141 and S222 than those of single coinoculation with USDA110 by 47.4 and 58.5%, respectively. Similar trend was also found in case of THA6 and its both coinoculats S141 and S222 by 66.7 and 59.1%, respectively.

At SUT Organic Farm site, among single and coinoculation treatments USDA110+S222 recorded maximum nodule number (70.00 nodules/plant) and lowest nodule number per plant (41.67 nodules/plant) was recorded in *B. japonicum* USDA110. Significantly increase in nodule number obtained coinoculated with USDA110 and S141 and S222 than those of single coinoculation with USDA110 by 63.1 and 72.7%, respectively. Parallel trend was also found in case of THA6 and coinoculation with both S141 and S222 by 47.7 and 37.2 %, respectively.

4.3.2 Nodule dry weight

Significant differences in the of soybean nodule dry weight observed at 45 DAI due to various inoculation treatment at Buriram site (Figure 20) and SUT Organic Farm site (Figure 21).

At Buriram site, among coinoculation treatments USDA110+S141 has recorded significantly higher nodule dry weight (203.67 mg) followed by THA6+S141 (202.33 mg) compared to other treatments. The lowest nodule dry weight (137.33 mg) was recorded in single inoculated with *B. japonicum* USDA110. The coinoculated between USDA110 with S141 and S222 induced the nodule dry weight compared to the single coinoculation with USDA110 by 48.3 and 40.1%, respectively. In case of THA6, the coinoculated with S141 and S222 induce the nodule dry weight by 40.8 and 37.5%, respectively.

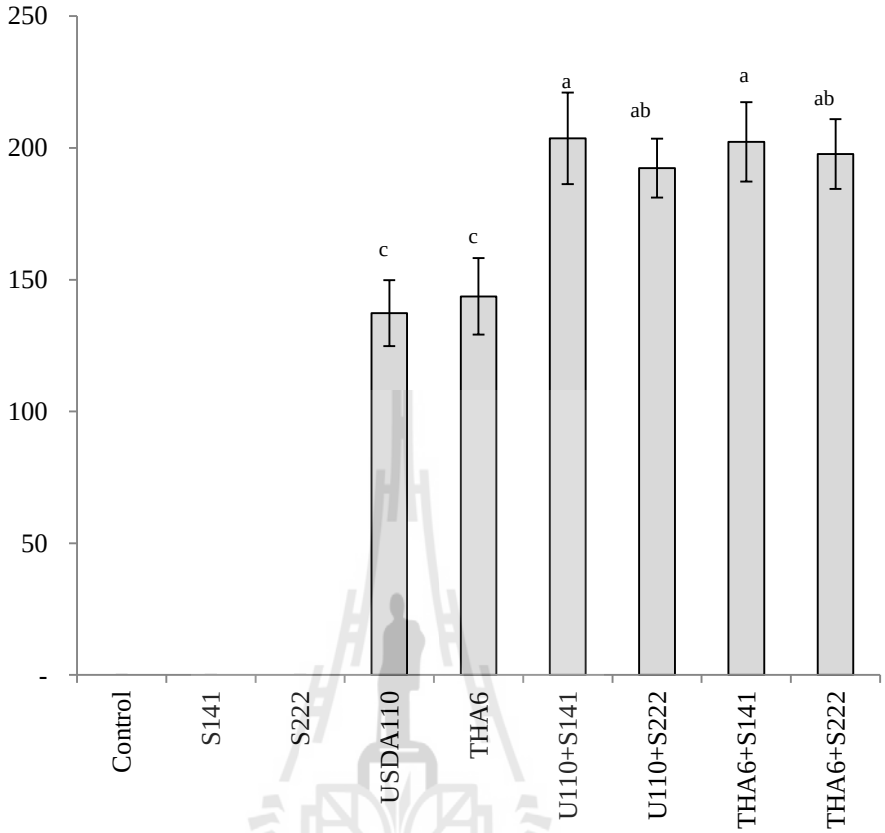


Figure 20. Effects of coinoculation between *B. japonicum* USDA110 or THA6 with PGPR isolates S141 and S222 at 10^6 : 10^6 cells/seed on nodule dry weight (mg/plant) performed at Buriram site. Data represent the means of nine experiments, each with three replicates. Values represents mean ± SD (n=5). Within treatment, means labeled with different letters are statistically different at $P<0.05$.

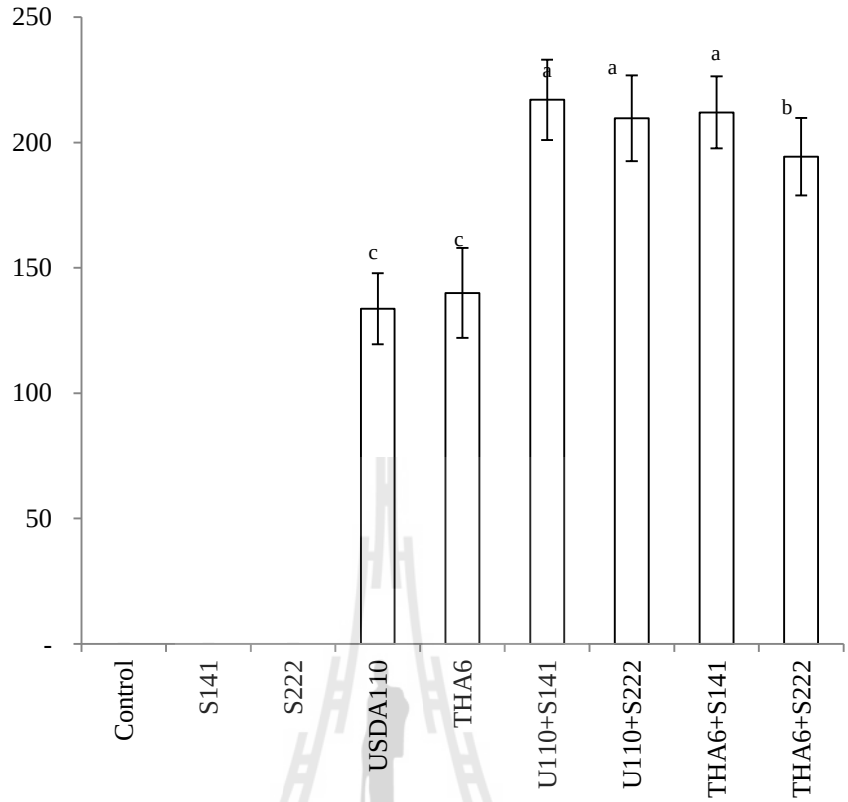


Figure 21. Effects of coinoculation between *B. japonicum* USDA110 or THA6 with PGPR isolates S141 and S222 at 10^6 : 10^6 cells/seed on nodule dry weight (mg/plant) performed at SUT Organic Farm site (B). Data represent the means of nine experiments, each with three replicates. Values represents mean ± SD (n=5). Within treatment, means labeled with different letters are statistically different at $P<0.05$.

At SUT Organic Farm site, treatment USDA110+S141 recorded maximum nodule dry weight (217.00 mg) followed by THA6+S141 (212.00 mg) and lowest nodule dry weight (133.67 mg) was recorded in *B. japonicum* USDA110. Among single and coinoculation treatments, the coinoculation treatments performed higher

nodule dry weight compared to single inoculation with *B. japonicum* treatments. The nodule dry weights of coinoculation between USDA110 with S141 and S222 were higher than those of single coinoculation with USDA110 by 48.3 and 40.1%, respectively. In case of THA6, the coinoculation with S141 and S222 induced the nodule dry weight by 40.8 and 37.5%, respectively.

4.3.3 Seed yield

Significant variations in seed yield of soybean plant due to inoculation of different microbial treatments at Buriram site (Figure 22) and SUT Organic Farm site (Figure 23).

At Buriram site, the highest seed yield was noticed in USDA110+S141 (2,811.70 kg/ha) followed by THA6+S222 (2,732.45 kg/ha). Lowest seed yield was recorded in uninoculated control (1070.14 kg/ha). The soybean seed yields of single inoculation with PGPR S141 (1351.52 kg/ha) and S222 (1287.93 kg/ha) treatments were not significantly different ($P<0.05$) from uninoculated control treatment. Single inoculations of *B. japonicum* USDA110 and THA6 were induced the soybean seed yield by 104.6 and 100.6% compared to those of uninoculated control treatment. Moreover, when those *B. japonicum* USDA110 and THA6 were coinoculated with PGPRs, the soybean seed yields were increased when compared with those of single inoculation to *B. japonicum*. Furthermore, all coinoculation treatments were on par with each other. The seed yields in coinoculation with USDA110 and S141 and S222 treatments were increased by 28.4 and 23%, respectively. In case of THA6, the coinoculated with S141 and S222 induced the seed yield by 25.2 and 27.3%, respectively.

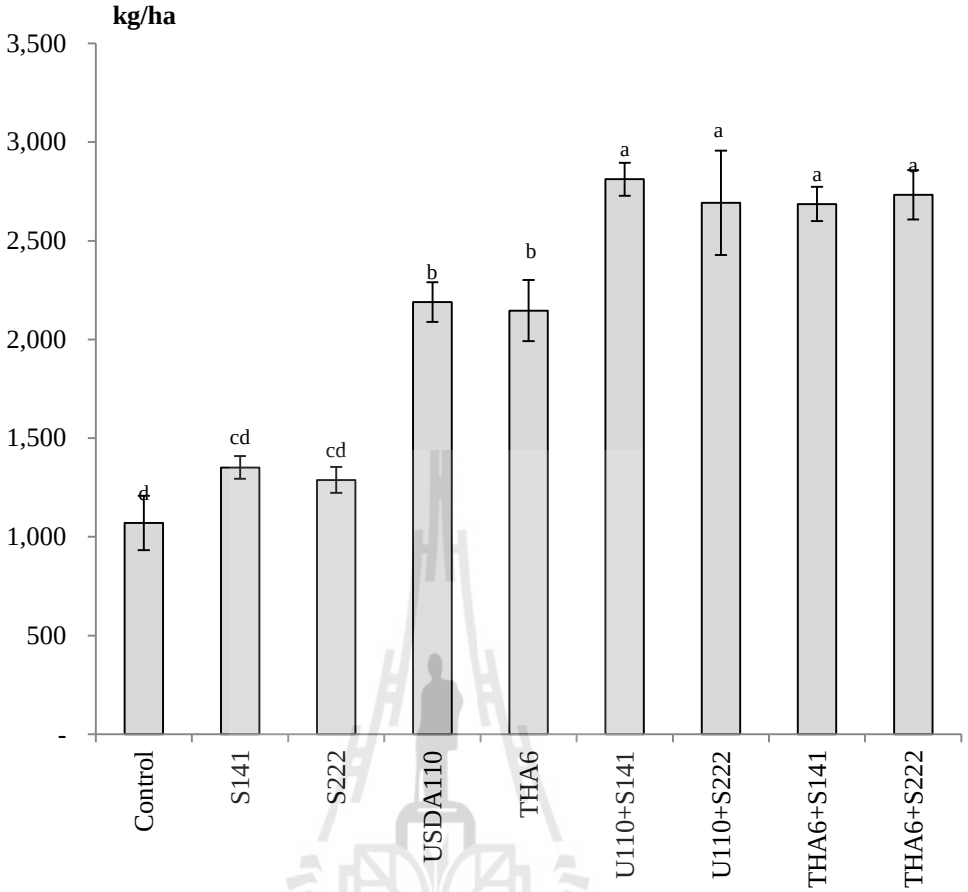


Figure 22. Effects of coinoculation between *B. japonicum* USDA110 or THA6 with PGPR isolates S141 and S222 at 10^6 : 10^6 cells/seed on soybean seed yield (kg/ha) performed at Buriram site. Data represent the means of nine experiments, each with three replicates. Values represents mean ± SD (n=5). Within treatment, means labeled with different letters are statistically different at *P<0.05*.

At SUT Organic Farm site, the highest seed yield was noticed in THA6+S141 (2,868.87 kg/ha) followed by USDA110+S141 (2,748.23 kg/ha) as shown in Figure 20B. Lowest seed yield was recorded in single inoculated with S141 (1,415.07

kg/ha). The soybean seed yields of single inoculation with PGPR S141 and S222 treatments were not significantly different ($P<0.05$) from uninoculated control treatment. Single inoculations of *B. japonicum* USDA110 and THA6 induced the soybean seed yield by 66.5 and 32.9% compared to those of uninoculated treatment. Moreover, when those *B. japonicum* USDA110 and THA6 were coinoculated with PGPRs, the soybean seed yields were induced when compare to those of *B. japonicum* single inoculation. The seed yields in coinoculation with USDA110 and S141 and S222 treatments were increased by 9.8 and 1.4%, respectively. In case of THA6, the coinoculated with S141 and S222 induced the seed yield by 43.6 and 27.5 %, respectively.

Determination of N_2 fixation effectiveness in the process of strain selection is normally a multiple step procedure involving an initial selection under greenhouse conditions and a final testing in field trails (Lepo and Ferrenbach, 1987). The results of field experiment were correlated with the results of Leonard's jar experiment. The coinoculation of both strains of *B. japonicum* with S141 and S222 was obviously differed from single inoculation of *B. japonicum* in field experiment at both experiment field sites.

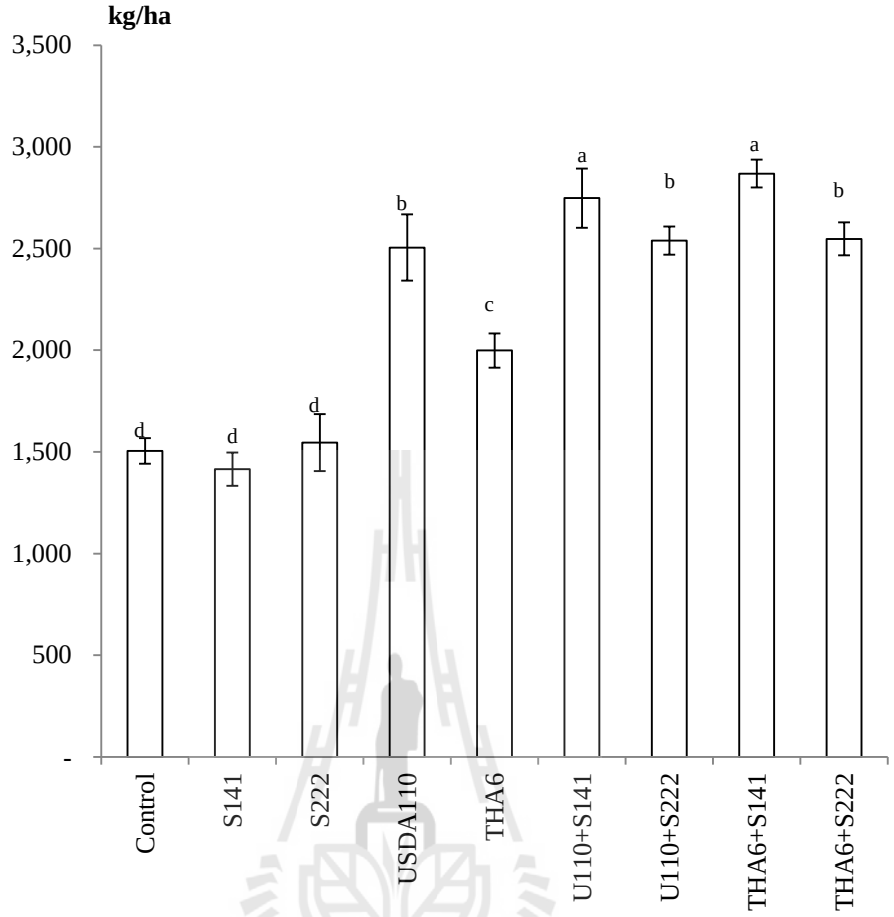


Figure 23. Effects of coinoculation between *B. japonicum* USDA110 or THA6 with PGPR isolates S141 and S222 at 10^6 : 10^6 cells/seed on soybean seed yield (kg/ha) performed at SUT Organic Farm site. Data represent the means of nine experiments, each with three replicates. Values represents mean ± SD (n=5). Within treatment, means labeled with different letters are statistically different at $P<0.05$.

By comparison, total soybean seed yield coinoculated with both *B. japonicum* and PGPR was higher than those of uninoculated control plants by 151-162% at Buriram site and 68-91% at SUT Organic Farm site. The soybean seed yield of

uninoculated control at Buriram site (1,070 kg/ha) was lower than the same treatment at SUT Organic Farm (1,504 kg/ha). It may be because of the tested soil at Buriram site has very low in organic matter content (1.02%) and no chemical fertilizer was applied which altered that plant cannot accumulate the fixed nitrogen. Moreover, the relative level of exchangeable potassium (K) at Buriram site has very low (43.57 ppm.) while at SUT Organic Farm was high (330 ppm.). It has been reported that application of both Phosphorus and Potassium individually increased soybean nodulation and pod formation with more response from Potassium than Phosphorus (Jones et al., 1977). These findings of the effect of soil properties on uninoculated soybean were also consistent supported with those of single inoculated of PGPR isolates S141 and S222.

The soybean yield trend at SUT organic farm site was obviously increased especially when coinoculated with THA6+S141. This might also depended on the soil property such as legume planting history under no circumstances chemical fertilizer application history which according to the research of Krey et al. (2011) which showed that organic matter available can promote the dispersal and activity of applied PGPR whilst the chemical fertilizer apply was disrupted. This PGPR may not only influence the inoculated rhizobia adversely through saprophytic competition, but also help them in survival through synergism resulting in an increase in their nodulation ability and N_2-fixing efficiency. The maximum nodule number of due to inoculation of two or more beneficial organisms over single inoculation and uninoculated control has been reported (Bai et al., 2003; Chebotar et al., 2001; Mishra et al., 2009a; Remans et al., 2008; Zahir et al., 2011)

The results under field experiment revealed that conoculation of PGPR isolates in combination with *B. japonicum* had significant influence on different plant growth parameters and yield of soybean and was significantly superior over the unicoculated control. The increase in the nodule number and nodule dry weight attributed to the presence of PGPR in the soybean rhizosphere influencing the soybean roots to secrete growth promoting substances, which in turn might have enhanced the growth of Bradyrhizobia and a synergistic effect may have achieved in case of coinoculation treatments. The increase in both nodulation and seed yield in co-inoculated plants represents a marked enhancement of symbiotic effectiveness of soybean and offer a significant advantage to the success of developing for inoculants for soybean.

4.4 The related genes expression level of *B. japonicum*, PGPR and soybean during coinoculation

4.4.1 The relative expression levels of PGPR related genes after single and coinoculation in soybean root

The relative expression levels of PGPR mode of action related genes were identified in 7 DAI soybean root using RT-PCR. The PGPR related genes were selected from their mode of action including *ipdC* gene (indole-3-pyruvic acid decarboxylase) and *iaaH gene (*IAA hydrolase).

The expression levels of *ipdC* gene were presented in Figure 24 and *iaaH* gene in Figure 25. The single inoculation with S141 recorded significantly highest *ipdC* gene expression level (1.56 folds) followed by single inculated with THA6 (1.34 folds). The reduction of *ipdC* expression level was observed in the single inoculation with S141 when coinoculated with both strains of *B. japonicum* USDA110 and THA6 by 27.90 and 41.38%, respectively. Moreover, the *ipdC* gene expression level of strains S222 was also significantly reduced when coinoculated with both strains of *B. japonicum* USDA110 and THA6 by 60.12 and 60.02%, respectively.

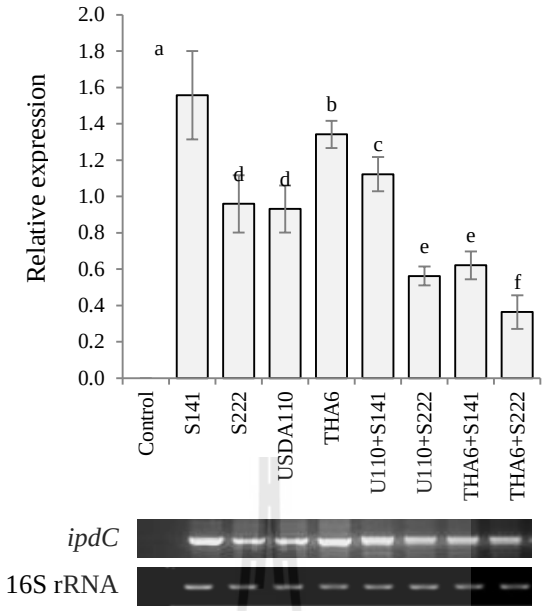


Figure 24. Relative expression of *ipdC* gene in soybean root. The housekeeping gene, 16S rRNA was used as an internal control. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=3).

The highest relative expression level of *iaaH* genes (Figure 25) showed in the single inoculation with S141 and S222 (0.867 and 0.749 folds) compared to the single inoculation with *B. japonicum* or coinoculation treatments (0.533 and 0.526 folds). However, the *iaaH* gene expression level of strain S141 was significantly reduced when coinoculated with both strains of *B. japonicum* USDA110 and THA6 by 39.92 and 48.91%, respectively. Moreover, the *iaaH* gene expression level of strain S222 was also significantly reduced when coinoculated with both strains of *B. japonicum* USDA110 and THA6 by 40.19 and 58.34%, respectively.

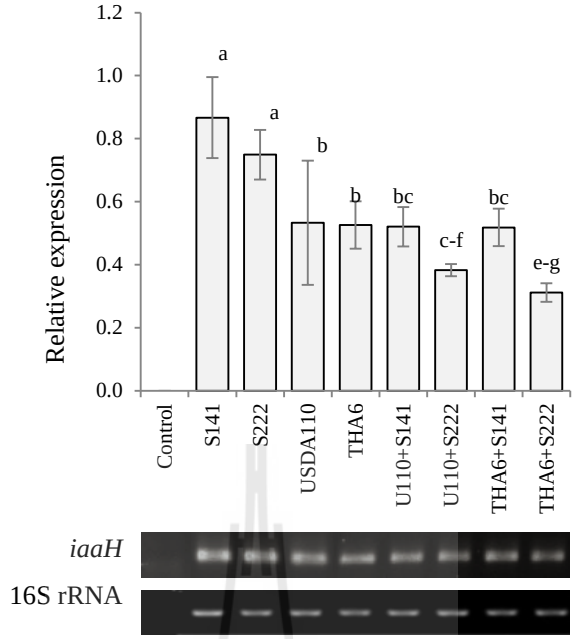


Figure 25. Relative expression of *iaaH* gene in soybean root. The housekeeping gene, 16S rRNA was used as an internal control. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=3).

In this context, bacterial biosynthesis of indole-3-acetic acid (IAA) is well established as it can positively regulate developmental processes of plant roots. The expression of two represented genes encoding indole-3-pyruvate decarboxylase (*ipdC*) and indole-3-acetaldehyde hydrolase (*iaaH*) which are involved in IAA biosynthesis were conducted. Similar to plant IAA production, microorganisms also possess several different IAA biosynthetic pathways. The metabolic routes are classified in terms of their intermediates as the indole-3-acetamide (IAM), IPyA, indole-3-acetonitrile, and tryptamine pathways (Costacurta et al., 1998). The conversion of indole-3-acetamide to IAA is catalyzed by IaaH (indole-3-acetamide hydrolase). The IPyA pathway is the major IAA biosynthetic pathway catalyzed by

IpdC, (indole-3-pyruvate decarboxylase). In many cases, a single bacterial strain may possess more than one pathway (Yang et al., 2007).

However, the *ipdC* and *iaaH* genes expression levels of single inoculation with PGPR strains were reduced when coinoculated with *B. japonicum* during the root hair infection process. These results suggested that the *B. japonicum* may be competed with PGPR strains for the utilizing of the tryptophan as precursor for IAA production in the soybean rhizoplane. Even the reduction of IAA related genes expression level in coinoculated treatments at the early stage of infection was found, but the coinoculation treatment showed positive effect on symbiotic nitrogen fixation by enhancing soybean yield in both Leonard's jar and field experiments. It has been reported that auxin transport inhibition precedes root nodule formation in white clover roots and was regulated by flavonoids and derivatives of chitin oligosaccharides (Mathesius et al., 1998). This suggested that changes in the auxin balance are a prerequisite for nodule organogenesis (Lambrecht et al., 2000). Moreover, IAA can act as a signal molecule, indicating that use of hormones as signaling molecules is not confined only to the plants but also takes part in communication between bacteria and other microorganisms (Spaepen et al., 2007).

4.4.2 The relative gene expression levels in early nodule development after single and coinoculation in soybean root

The related plant genes are sequentially activated or regulated after single and coinoculation in soybean root and their expression serves as molecular markers for the microbial signaling perception, signaling regulation and the infection process. The expressions of 3 early nodulation response genes including *nodD1*

(Figure 26), *GmCaMK1* (Figure 27) and *GmNIN1A* (Figure 28) were monitored by RT-PCR on 7 DAI soybean roots.

The *nodD1* gene expression level of USDA110 coinoculated with S141 and S222 was induced by 4.04 and 3.28-folds, respectively. In case of THA6 coinoculated with S141 and S222 was induced by 3.48 and 2.94-folds, respectively. Coinoculation of *B. japonicum* USDA110 with S141 and S222 was induced the expression levels by 31.79 and 7.15% compared to those of single inoculated USDA110 treatment. In case of THA6, significant decrease of expression levels was observed under coinoculated with S141 and S222 by 11.67 and 25.57% (Figure 26).

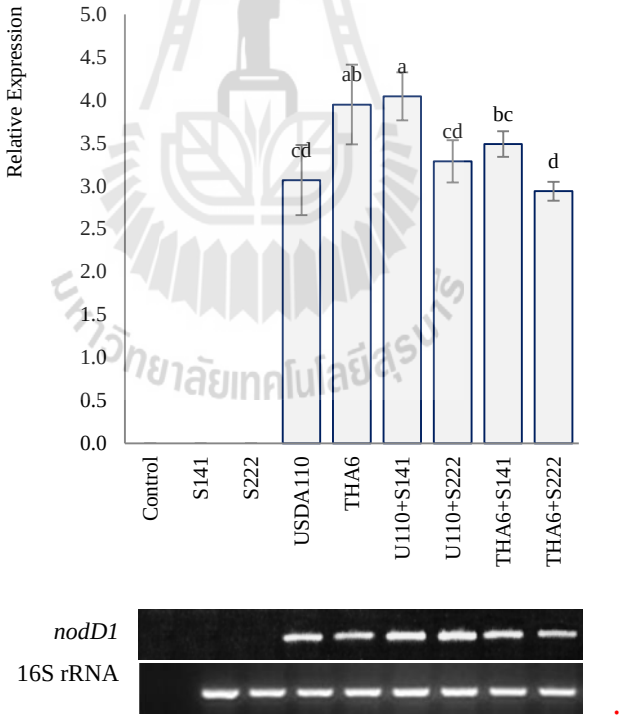


Figure 26. Expression of early nodulation gene *nodD1* in response to single and coinoculation with *B. japonicum* and PGPR in the 7 DAI main root of soybean plants.

It has been well established that the leguminous plant-released iso/flavonoid signals induce *nod* genes of rhizobia so that they can produce Nod-factors, lipo-chito oligosaccharides that specifically trigger various plant responses and initiation of cell division to form the nitrogen-fixing root nodules. The *nodDl* gene of *B. japonicum* was found to be essential for flavonoid induction of the *nod* gene. In response to Nod factors, the signal generated by Nod factor receptor (*nodD1*) flows into the gene cascades in early symbiotic signaling pathway was required for successful infection of rhizobia. This perception of Nod factor by *nodD1* gene was solely required for the generation of Ca^{2+} spiking (Oldroyd and Downie, 2004), and may resulting constitutive activated of CCaMK which was sufficient to trigger both the formation of infection structures (prepenetration apparatus) and cortical cell divisions (Venkateshwaran et al., 2013).

Among the single inoculation treatments, the maximum *GmCCaMK1* gene expression level was recorded in S222 (5.25 folds) followed by THA6 (4.80 folds). The minimum expression level was recorded in S141 (3.86 folds). Whereas among coinoculation treatments, the maximum gene expression level was recorded in USAD110+S141 (5.78 folds) followed by THA6+S222 (5.35 folds). The minimum expression level was recorded in USDA110+S141 (4.71 folds). Considering to the effect of coinoculation, USDA110 coinoculated with S141 and S222 were induced the expression levels by 34.22 and 9.24% compared to those of single inoculated USDA110 treatment. In case of THA6, significant increase of expression levels was observed under coinoculated with S141 and S222 by 8.27 and 11.32% (Figure 27).

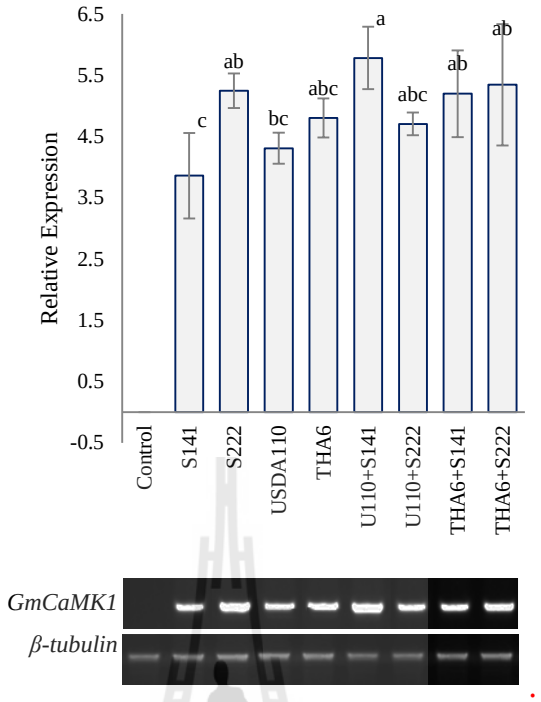


Figure 27. Expression of early nodulation gene *CCaMK1* in response to single and coinoculation with *B. japonicum* and PGPR in the 7 DAI main root of soybean plants.

The early signaling events activate a nuclear CCaMK1 are the central players in legume symbiosis signal transduction pathway (Venkateshwaran et al., 2013). Constitutive activation of CCaMK is sufficient to trigger both the formation of infection structures (prepenetration apparatus) and cortical cell divisions (Gleason et al., 2006). The results of this study revealed that not only *B. japonicum*, the *GmCCaMK* gene expression was also activated by those of PGPR especially in *B. japonicum* USDA110+S141. It has been reported that the CCaMK was also involved in legume interactions with PGPR and/or endophytic bacteria as it was shown using inoculation of *M. truncatula* by and *P. fluorescens* that *MtDMI3* gene (encoding for

CCaMK) regulates intercellular root colonization by bacteria as well as expression of some plant housekeeping genes known earlier as mycorrhizins (Hayashi et al., 2010; Singh and Parniske, 2012). The *GmCaMK1* gene was performed as a central player in the Nod factor signaling and coordinates the expression of symbiotic genes, including early nodulin genes. The Ca^{2+} flux regulated a set of transcriptional regulators and activated in infection thread progression and elongation and also invasion of nodule primordial follow by nodule organogenesis.

The expression level of infection process and nodule organogenesis involved gene (*GmNIN1A*) was compared by RT-PCR on 7 DAI soybean roots (Figure 28).

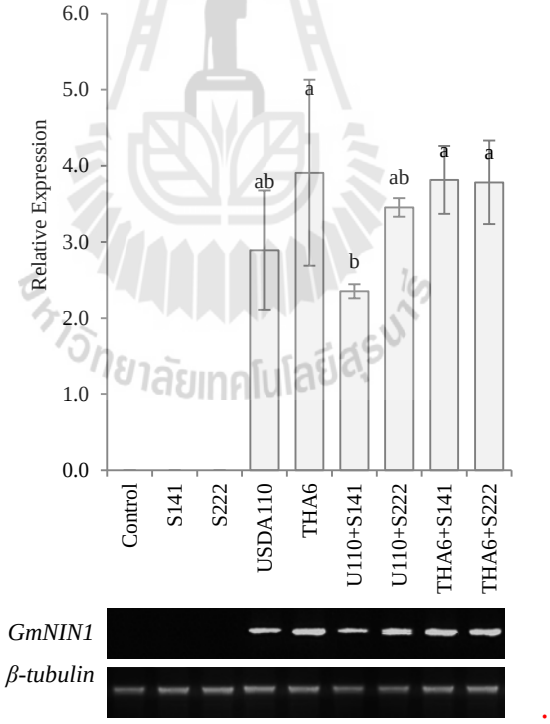


Figure 28. Expression of early nodulation gene *GmNIN1* in response to single and coinoculation with *B. japonicum* and PGPR in the 7 DAI main root of soybean plants.

The expression level of *GmNIN1A* was not detected in single inoculated with PGPR or uninoculated control treatment. The single inoculation with USDA110 induced the *GmNIN1A* expression level by 2.89 folds and was on par with USAD110+S222 (3.45 folds). However, the expression level was reduced when coinoculated with S141 by -18.67% (2.35 folds). In case of THA6, the expression levels were not significantly different among single inoculated with THA6 or coinoculated with S141 or S222 (the expression level by 3.90, 3.45 and 3.81 folds, respectively).

The coinoculation of USAD110+S141 was obviously enhanced the up-regulation of *nodD1* and *GmCCaMK* genes which accordingly related with phenotyptic characters in Leonard' jar and field experiments. Since the early infection process and nodule organogenesis as nodulation factor receptor kinase 1 encoded by *GmNIN1A* gene response to *B. japonicum* infection (Libault et al., 2010) and controls nodule organ number in soybean (Indrasumunar et al., 2011). The down-regulated in coinoculated plant suggests that the inhibition of nodule development by PGPR signaling occurs very early in nodule ontogeny, perhaps even directly following Nod factor perception. This decline may relate to an inhibition of nodule development because of the onset of Autoregulation of Nodulation (Searle et al., 2003). Alternatively, it could reflect a requirement for these genes in early nodule development, followed by a decline in need, and hence expression, as nodule primordia mature (Hayashi et al., 2012). Similar expression patterns were reported for these genes in *B. japonicum* -inoculated soybean root hairs (Libault et al., 2010). Taken together, the results demonstrated that the increasing of early symbiosis involved genes was continually increased root hairs curl and trap the rhizobia, which

then enter the root hair through infection threads that are formed by the plant. The infection threads then grow into the developing nodule tissue.

4.4.3 The relative genes expression levels in soybean nodule after single and coinoculation in the different time frames

The relative expression levels of soybean and bacterial related genes in nodule after single or coinoculating with *B. japonicum* and PGPR during 2 to 7 WAI were identified using RT-PCR. The selected genes including *GmMyp* gene which control soybean nodulation, *otsA* encoded trehalose 6-phosphate synthase, *dctA* controled transport of C4-dicarboxylates in nodule, *phbC* encoded poly-β-hydroxybutyrate (PHB) polymerase and *nifH* encoding the Nitrogenase Fe protein.

The relative expression levels of *GmMyb* gene were upregulated at the 2 WAI and almost stopped expressing at the 6 WAI (Figure 29). Among the single inoculation of *B. japonicum* treatments, *GmMyb* gene expression levels were induced 0.25- to 2.04-folds by inoculation of USDA110 and 0.01- to 2.75-folds by THA6 during 2 – 7 WAI. *GmMyb* gene in nodule inoculated with USDA110 was strongly up-regulated between 3 - 5 WAI (1.93-, 0.89- and 2.04-folds), followed by stopped the expression at 6 WAI. In case of THA6, the highest up-regulated of this gene was recorded at 4 WAI by 2.74-folds and the expression level was completely stopped at 6 WAI.

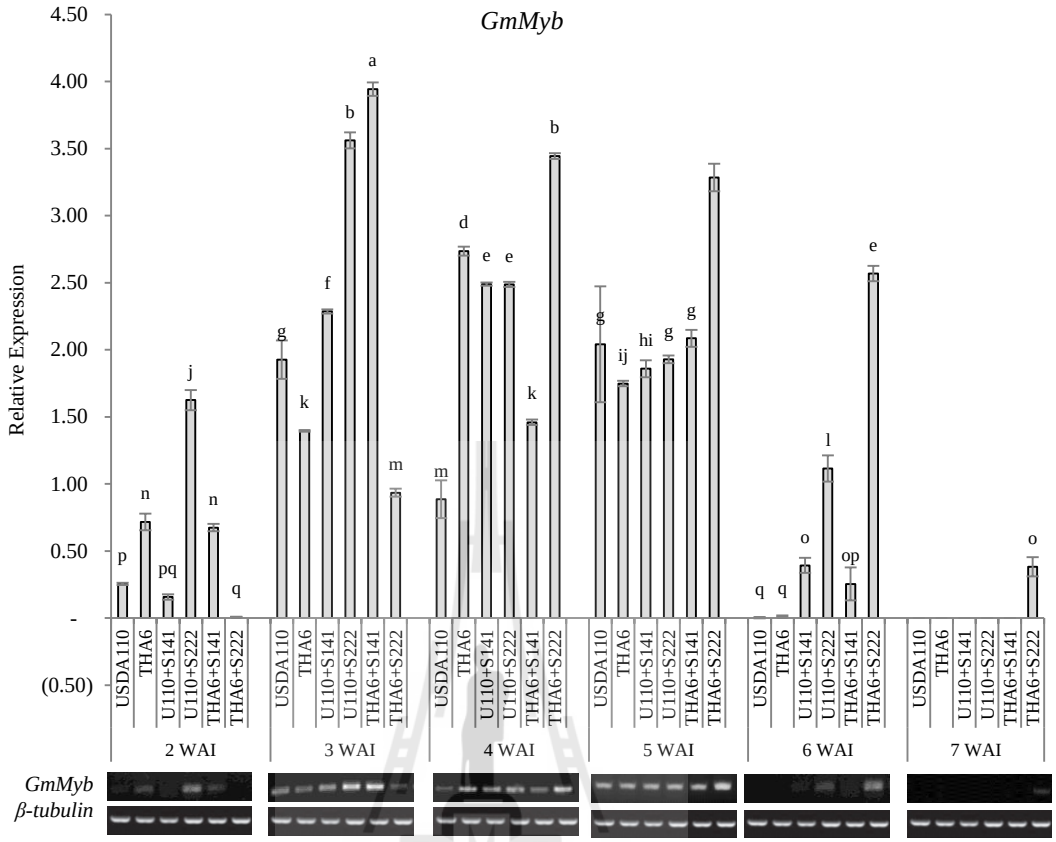


Figure 29. Relative expression level in 2-7 WAI soybean nodules of *GmMyb* gene. The transcript level was determined by RT-PCR, the electrophoresis bands shown in the lower part of the graph. Soybean *β-tubulin* was used as an internal control. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=3).

At 2 WAI, *GmMyb* gene expression level in USDA110+S141 was lower than those of single inoculation USDA110. However, the expression level of USDA110+S141 treatment was 18.66 and 84.86% higher up-regulated (2.29- and 2.49-folds) than USDA110 treatment (1.93- and 0.89-folds) at 3 and 4 WAI. Furthermore, USDA110+S141 also induce the expression level of *GmMyb* gene by

537.92, 84.86 and 180.72% compared to single USDA110 at 2, 3 and 4 WAI, respectively. The level of expression was reduced by 5.80% than USDA110 treatment at 5 WAI. However, at 4 WAI only coinoculation treatment remained induced. At 5 WAI, the expression levels of all USDA110 involved treatment were inhibited.

In case of THA6, the expression level of *GmMyb* gene at 2 WAI was induced by THA6 treatment and THA6+S141 by 0.72- and 0.67-fold, respectively but THA+S222 were not. At 3 WAI, the expression level was increased 3.94-folds (182.27%) by the coinoculation THA6+S141 but reduced 0.93-fold (-33.09%) by coinoculation THA6+S222 compared to THA6 treatment. At 4 WAI, the expression level was 1.46-folds which reduced 46.64% by the coinoculation THA6+S141 but induced 3.45-fold (25.93%) by coinoculation THA6+S222 compared to THA6 treatment. Interestingly, significantly higher expression level of *GmMyb* gene was recorded in the coinoculation of THA6+S222 treatment at the late of nodulation (4-7 WAI) especially at 7 WAI that was still expressed while the expression other treatments were stopped.

During nodule formation, a higher percentage of genes expression level were related to primary metabolism, cell-wall modifications and the antioxidant defense system (de Carvalho et al., 2013). The MYB subfamily is the largest transcription factor subfamily in plants. In soybean, the *GmMyb* gene is involved in nodule formation, named Control of Nodule Development [CND] (Chen et al., 2013). However, GmMYB transcription factor identified so far which is involved in plant secondary metabolism and may involve requirement for additional co-factors for its function in root pointing towards a cooperative and combinatory mechanism of gene regulation (Yi et al., 2010).

During nodulation, the *GmMyb* gene was expressed during the first WAI but was strongly disrupted at 7 WAI. 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 (e.g. cortical cell division). However, functional analyses of soybean MYBs found that they regulate numerous processes including responses by various abiotic stresses (Liao et al., 2008). These results suggested that the coinoculation with PGPR may regulate differentially the expressions of the downstream genes, which are related to abiotic stresses of soybean. However, the functional characteristics of the *GmMyb* in legume-specific nodulation remain to be elucidated.

Considering to *otsA* gene expression, all coinoculation treatments gave the strongly expression since the 2 to the 7 WAI but the single inoculation with *B. japonicum* showed trend of declining at the 6-7 WAI. At the 4 WAI, the relative *otsA* gene expression levels of coinoculated with USDA110 and S141 or S22 and THA6 with S222 were highest while THA6+S141 treatment was strongly expressed at the 3 WAI (Figure 30).

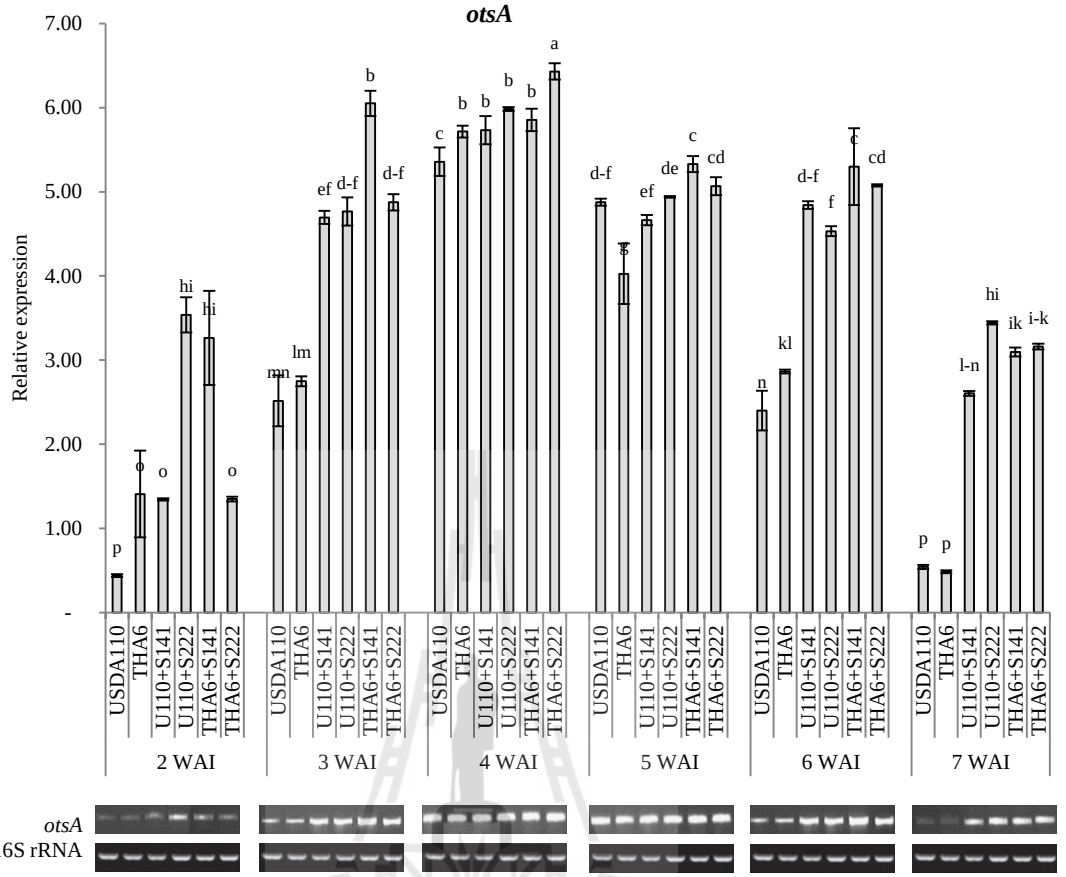


Figure 30. Relative expression level in 2-7 WAI soybean nodules of *otsA* gene. The transcript level was determined by RT-PCR, the electrophoresis bands shown in the lower part of the graph. Soybean 16S rRNA gene was used as an internal control. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=3).

The trehalose biosynthetic pathways, trehalose 6-phosphate synthase encoded by *otsA* gene plays an important role as a protectant during periods of physiological stress. In bacteria, trehalose functions as a storage carbohydrate and protects against a variety of stresses (Domínguez-Ferreras et al., 2009). In plants, this role has been largely replaced by sucrose, although trehalose does protect against desiccation in

certain specialized resurrection plants (Schluepmann et al., 2003). Absence or trace amounts of trehalose in most plants preclude a role as a reserve or stress protectant. Gene expression analysis reported here demonstrated that the trehalose biosynthesis gene *otsA* was induced by coinoculation with PGPR that caused an increasing in trehalose accumulation levels. Various studies reported that the trehalose biosynthetic genes in *B. japonicum* were induced by salinity and desiccation stresses (Sugawara et al., 2010) or when the *Bradyrhizobium* entered a symbiotic relationship with the soybean plant (Streeter and Gomez, 2006). However, there are no reports considered the effect of coinoculation with PGPR. Our results suggested that expression of the *otsA* genes may modulated by the trehalose accumulation in bacterial cells influences its symbiosis with soybeans, or perhaps could help it to resist host defense responses induced at the start of the association and occurs during the nodulation possess. Moreover, the ability to accumulate trehalose in their cells enhanced their nodulation competitiveness via increased number of infections (Streeter and Gomez, 2006). Taken together with soybean yield results, these indicated that the increasing of trehalose accumulation caused from PGPR coinoculation enhanced N_2 fixation and soybean yield. This is similar to what has been reported for the *Rhizobium etli-Phaseolus vulgaris* symbiotic interaction (Suárez et al., 2008) and *R. tropici* CIAT899 in *P. vulgaris* (Bargaz et al., 2013). The higher expression in coinoculation experiment than those of single inoculated with *B. japoncum* at the late nodulation (7 WAI) may cause by effect of their symbiotic phenotype appears to be dependent on the rhizobial species and host genotype and is not consistent across symbiotic systems (Sugawara et al., 2010). In young soybean nodule, the bulk of trehalose is located in the cytosol, and only a small proportion in the bacteroids. The

older the plants get, the more this distribution is changing to the opposite (Streeter and Gomez, 2006). The increasing retention of trehalose in bacteroids with increasing nodule age is indicating that its function is changing during nodule development or trehalose may play a role during some stage of the life cycle of the bacterium outside of the nodule (Streeter and Gomez, 2006).

Altogether these results indicated that the trehalose/trehalose-6-phosphate ratio in nodules has a profound effect on bacteroid viability, nitrogen fixation levels and the establishment of a systemic sink-and-source relationship within the nodulated plant.

The relative *dctA* gene expression levels were similar to the results of *otsA* gene expression which begin to express at the 2 WAI and remaining expressed up to the 7 WAI. The relative *dctA* expression levels in all coinoculated treatments were highest at the 6 WAI (Figure 31). The relative expression levels in coinoculated with USDA110 and S141 treatment were elevated 3.1 folds while USDA110+S222 promoted 1.9 folds. For coinoculation treatments, a 4.1 and 2.4 folds increase in THA6+S141 and THA6+S222 were highest, respectively.

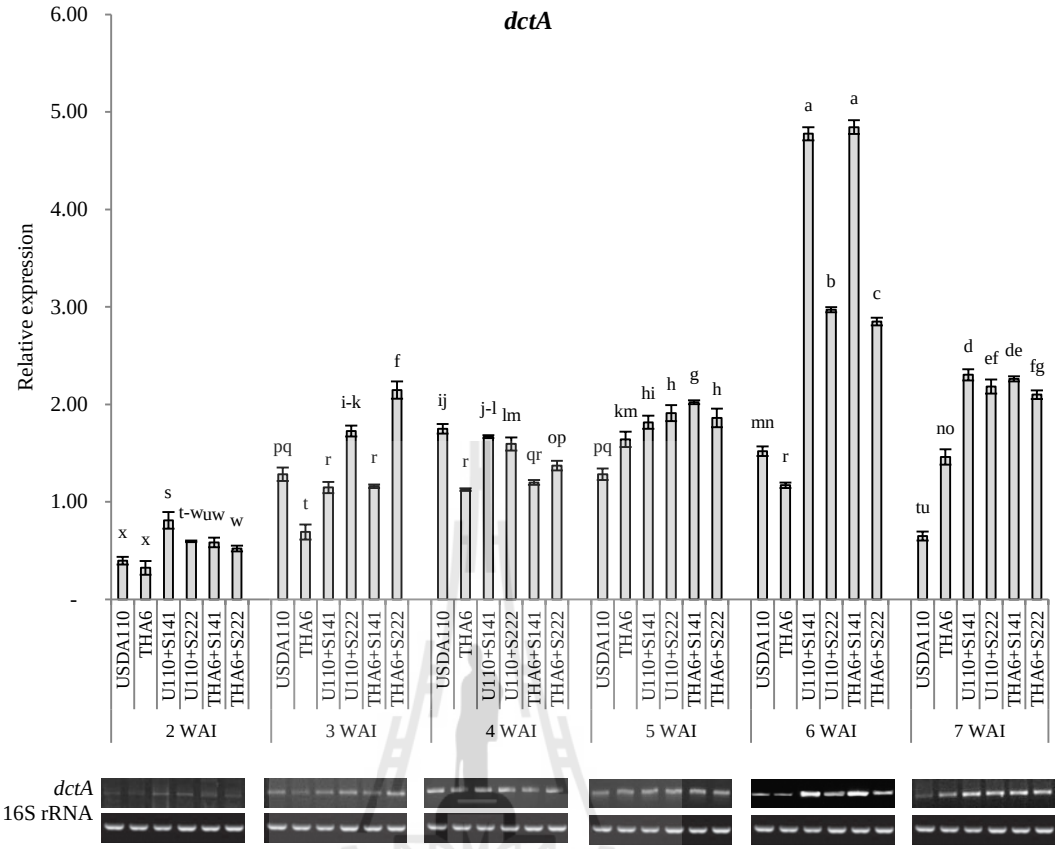


Figure 31. Relative expression level in 2-7 WAI soybean nodules of *dctA* gene. The transcript level was determined by RT-PCR, the electrophoresis bands shown in the lower part of the graph. Soybean *β-tubulin* was used as an internal control. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=3).

During symbiosis, rhizobial cells are dependent on the provision of carbon from the host plant in order to fuel cellular metabolism. This carbon is transported into the bacteroids via the dicarboxylate transport protein, DctA which encoded by *dctA* gene (Batista et al., 2009). The expression of *dctA* gene was expressed in all developmental stages from early to late symbiotic bacteroid differentiation (6-7

WAI). These findings suggested that the coinoculation seems to influence the expression of *dctA* gene especially during the bacteria become mature bacteroids and able to fix nitrogen. Concordantly results with the operation of the alternative system of symbiotic *dctA* activation (ASA) is concomitant with the onset of nitrogen fixation, which could be consistent with an increased need for transport of C4-dicarboxylic acids by the nitrogen-fixing bacteroids (Boesten et al., 1998).

The *phbC* gene of single and coinoculated treatment was expressed at 4 WAI and remaining up-regulated until the 7 WAI (Figure 32). The relative *phbC* expression levels in coinoculated with USDA110+S141, USDA110+S222, THA6+S114 and THA6+S222 were raised 2.5, 1.9, 3.3 and 2.3 folds at the 6 WAI, respectively. The RT-PCR amplifications of *phbC* and 16S rRNA genes were shown in Figure 32.

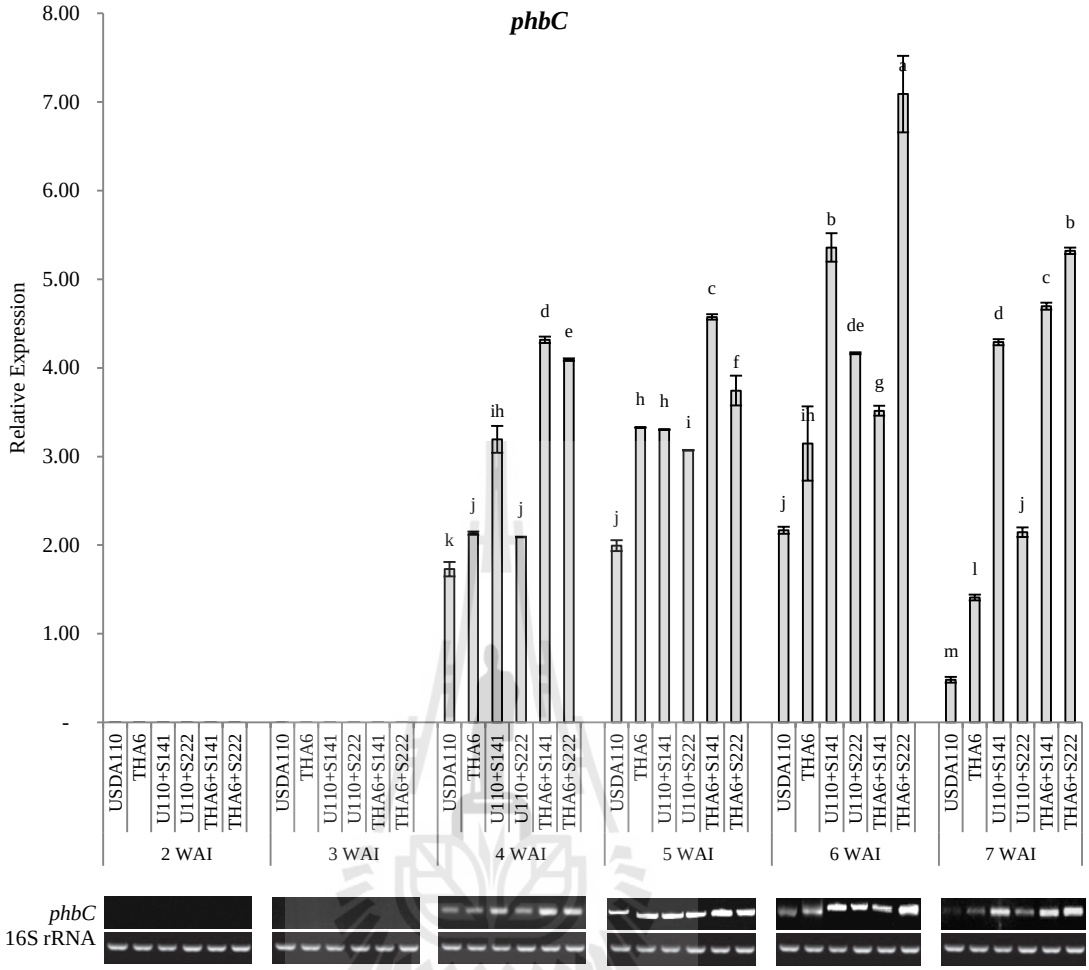


Figure 32. Relative expression level in 2-7 WAI soybean nodules of *phbC* genes. The transcript level was determined by RT-PCR, the electrophoresis bands shown in the lower part of the graph. Soybean *β-tubulin* was used as an internal control. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=3).

While most of the bacteroid carbon supplied by the plant is channeled into energy production to fuel nitrogen reduction, in certain types of nodules, some carbon is diverted by the bacteroids into the production of intracellular storage polymers

composed of either glycogen or poly-β-hydroxybutyrate (PHB) (Resendis-Antonio et al., 2011). The expression of *phbC* gene was observed at 4 WAI henceforward probably because PHB accumulation activity was taken place after the peak of nitrogenase activity had passed. However, the ability to accumulate PHB during symbiosis appears to be dependent on the fluctuate relative to nitrogenase activity by compete for the same energy and reductant sources and therefore, PHB synthesis in bacteroids must compete with nitrogen-fixation for photosynthate (Trainer and Charles, 2006). Interestingly the data presented in this study suggested that coinoculation 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. Moreover, the *phbC* was expressed under different bacterial inoculations, the growth and metabolism of different species of bacteria may be influenced and reflect the increase or decrease in PHB accumulation (Paganelli et al., 2011). However, studies of the effect coinoculation on PHB synthesis and transporters of soybean nodules are needed to analyze in more detail.

Biological nitrogen fixation in root nodules is a process of soybean, as it may provide the bulk of the plant's needs for nitrogen. Dinitrogenase reductase is a component of the enzyme nitrogenase which is used to fix atmospheric nitrogen into ammonia and genes that encode the nitrogenase structural components is *nifH*. Therefore, this study aimed at analyzing the expression of *nifH* genes in soybean nodule after single or coinoculation with *B. japonicum* and PGPR (Figure 33).

The expression level of *nifH* gene were up-regulated by all treatments, both single and coinoculation treatments. Considering on single inoculation of *B.*

japonicum, boths USDA110 and THA6 induced the expression level of *nifH* at 2 WAI by 1.83- and 1.22-folds, respectively. Then the expression levels were up-regulated at 3 WAI (2.33- and 2.30-folds) and the expression levels were reached the highest at 3 WAI (6.91- and 7.08-folds). The up-regalation of *nifH* was decreased from 5 to 7 WAI.

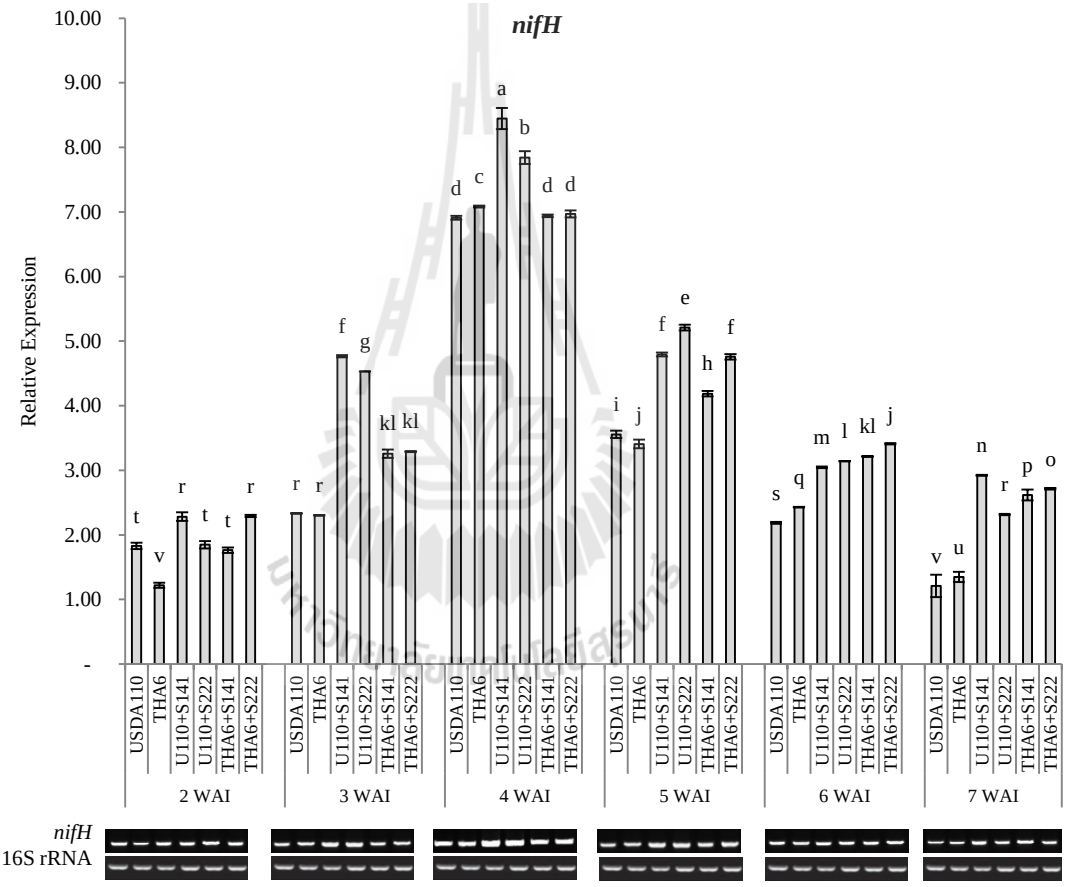


Figure 33. Relative expression level in 2-7 WAI soybean nodules of *nifH* gene. The transcript level was determined by RT-PCR, the electrophoresis bands shown in the lower part of the graph. Soybean *β-tubulin* was used as an internal control. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=3).

Among coinoculation treatments, significantly highest expression level was noticed in USDA110+S141 (8.45-folds) followed by USDA110+S222 (7.08-folds) at 4 WAI. The expression level of *nifH* gene was strongly induced by the coinoculation with *B. japonicum* and PGPR. Coinoculation of USDA110 with S141 and S222 induced the expression level than those of single inoculated USDA110 by 24.77 and 1.11% at 2 WAI, 104.28 and 94.27 22.28 % at 3 WAI, 22.28 and13.52% at 4 WAI, 34.79 and 46.50% at 5 WAI, 39.17 and 43.55 % at 6 WAI and finally 142.06 and 91.57% at 7 WAI. The higher up-regulation levels of nifH gene were also found in THA6+S141 and S222 than single inoculation of THA6 by 44.53 and 87.82% at 2 WAI, 41.52 and 42.86 at 3 WAI. However, the expression levels of coinoculation of THA6 + S141 and S222 (6.94- and 6.97-folds) were on par with single inoculation THA6 (6.91-folds) at 4 WAI. The up-regulation of coinoculation THA6+S141 and S222 higher than single inoculation with THA6 by 22.89 and 39.65% at 5 WAI, 32.20 and 40.29% at 6 WAI, 94.06 and 101.19% at 7 WAI.

A significant increase in *nifH* expression level in soybean root nodule was observed after coinoculation of *B. japonicum* and PGPR. This suggested that *B. japonicum* was supported by PGPR in order enhanced nitrogen fixation and also nodulation.

4.5 Effect of coinoculation on bacteroid morphology

The transmission electron microscopy (TEM) was used to observe the changes of bacteriod morphology when single inoculation with *B. japonicum* was compared to the coinoculation with PGPR strains S141 or S222. There was no obvious difference in size, spacing or morphology of infected cell at the 2, 3, 4, 5 WAI nodules (data not show) in every treatments. The 6 WAI of single inoculated *B. japonicum* USDA110 nodules showed the widespread of cytoplasmic disruption, including cytoplasmic breakdown and lesions in the symbiosome membranes as indicated by arrow in Figure 34A-B. Moreover, the coinoculation between USDA110 and S141 had symbiosome containing few bacteroids with appendages within the symbiosome large air space. Bacteroids are enclosed in a plant-derived membrane called the symbiosome membrane. Host cell cytoplasm surrounds the symbiosomes. However, the coinoculation between USDA110 and S1222 showed the compactly of bacteroids which densely packed poly-ß-hydroxybutyrate (PHB) granules within the bacteroids (Figure 34C-F).

The single inoculation with THA6 was also perceptibly disintegrated of symbiosome membrane similar to USDA110 (Figure 35A-B). For the coinoculation of THA6 with either PGPR strains S141 or S222, the symbiosome is composed of either a single or of multiple bacterial cells surrounded with the peribacteroid space and a plant derived peribacteroid membrane. Moreover, the PHB granules within the abundantly of bacteroids in symbiosome were densely packed (Figure 35C-F).

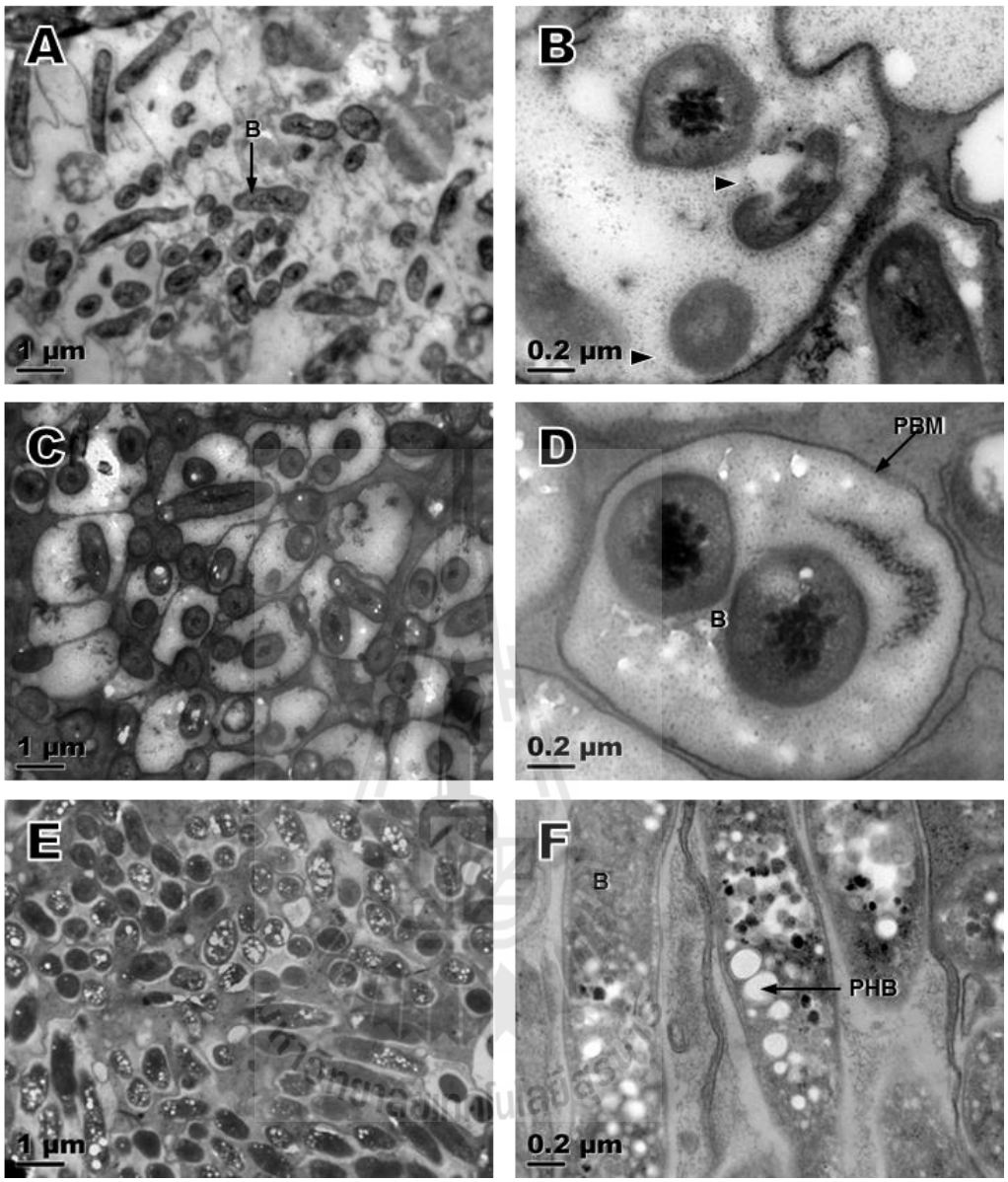


Figure 34. Transmission electron micrographs (TEM) of infected cells of 7 WAI nodules single inoculated with *B. japonicum* USDA110 (A and B), USDA110+S141 (C and D), and USDA110+S222 (E and F). The magnification is the same in panels A, C and E (bars = 1 µm) and in panels B, D and F (bars = 0.2 µm). CW; cell wall, B; bacteroid, PBM; peribacteroid membrane, PHB; poly-β-hydroxybutyrate granule**.**

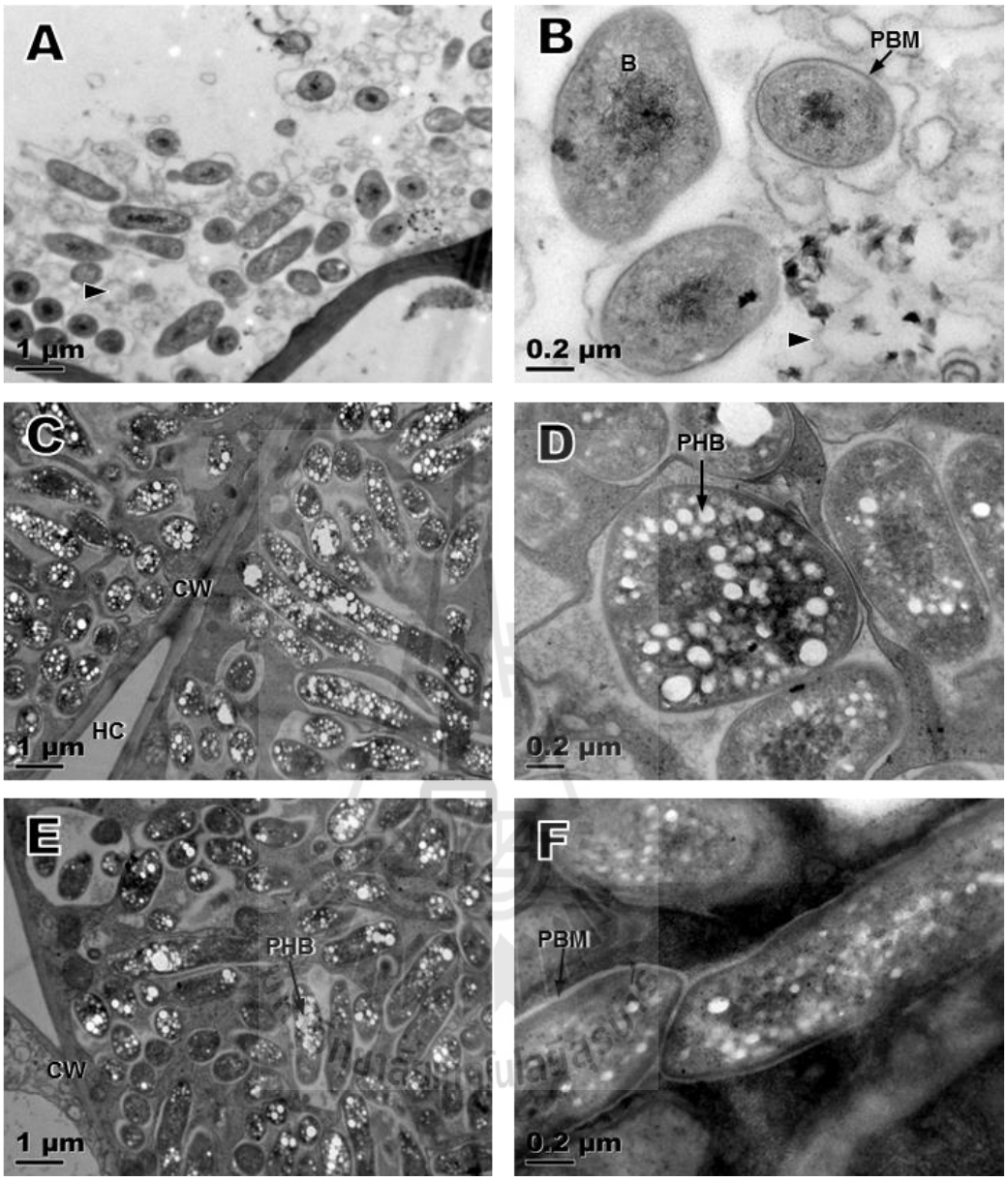


Figure 35. Transmission electron micrographs (TEM) of infected cells of 7 WAI nodules single inoculated with *B.japonicum* THA6 (A and B), THA6+S141 (C and D), and THA6+S222 (E and F). The magnification is the same in panels A, C and E (bars = 1 µm) and in panels B, D and F (bars = 0.2 µm). CW; cell wall, B; bacteroid, PBM; peribacteroid membrane, PHB; poly-β-hydroxybutyrate granule.

The TEM micrograph of 6-week-old of either USDA110 or THA6 nodules showed different responses with regard to the indicators of senescence of symbiosis. The bacteroids are a huge accumulation of soluble carbohydrates such as trehalose, glutamate, myo-inositol and homospermidine as well as Pi, nucleotide pools and intermediates of the primary carbon metabolism (Vauclare et al., 2013). Nodule senescence can be triggered by a signal or signals from the tight metabolic controled by the host plant (Puppo et al., 2005). The accumulation of the storage compound (PHB), which diverts carbon supply from nitrogen fixation, is very frequently observed in reversibly differentiated. Inhibition of PHB accumulation could be a direct or indirect consequence of the terminal differentiation. Moreover, terminally differentiated bacteroids with their weakened membranes and locked-within nodule cells might be more effectively digested during nodule senescence than reversible bacteroids. Thus, recycling their components might provide more nutrients for the plant (Kereszt et al., 2011).

4.6 The proposed model for *B. japonicum* and PGPR coinoculation interaction in soybean

The results presented here define the proposed model for the effect of single inoculation of *B. japonicum* and coinoculation of *B. japonicum* and PGPR on soybean was demonstrated in Figure 36.

The common soybean-Bradyrhizobia symbiotic interaction commences with the host perception of microbial signaling molecules called 'Nod factors' (NF) released by rhizobia (Figure 36A). In response to Nod factors, the signal generated by Nod factor receptor (*nodD1*) flows into the gene cascades in early symbiotic signaling pathway was required for successful infection of rhizobia. This perception of Nod factor by *nodD1* gene was solely required for the generation of Ca^{2+} spiking. Later these Ca^{2+} signals were perceived and decoded by calcium/calmodulin-dependent protein kinase (*GmCaMK1*), which acts as a central player in the Nod factor signaling and coordinates the expression of symbiotic genes, including early nodulin genes. The Ca^{2+} flux regulated a set of transcriptional regulators and nodule inception (*GmNIN1)*. The roles of Ca^{2+} signaling and CCaMK activated in infection thread progression and elongation and also invasion of nodule primordial follow by nodule organogenesis. The result is that root hairs curl and trap the rhizobia, which then enter the root hair through infection threads that are formed by the plant. The infection threads then grow into the developing nodule tissue. The newly formed nodule consists of bacteria that are differentiating into bacteroids enclosed in a plant cell membrane, is called a symbiosome and typically contain several bacteroids.

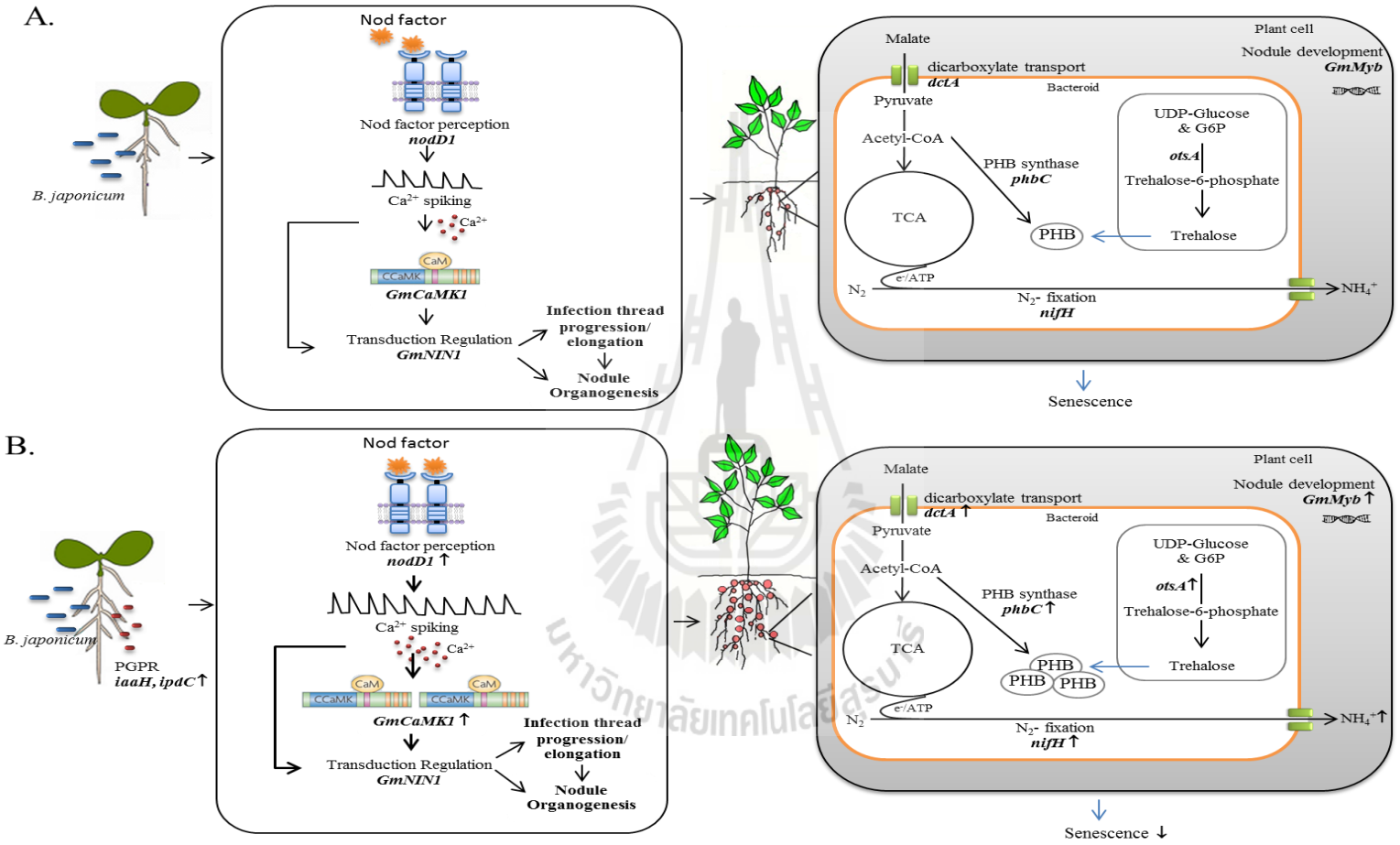


Figure 36. Proposed model for the effect of single inoculation of *B. japonicum* (A) and coinoculation of *B. japonicum* and PGPR (B) on soybean.

The metabolism of bacteroids was overwhelmingly focused on the production of fixed nitrogen, which is then transferred to the host plant. This process is fuelled by the plant host through the provision of large quantities of C_4-dicarboxylic acids such as malate via dicarboxylate uptake system (*dctA*) through the TCA cycle. While most of the carbon from the plant is channeled into energy production to fuel nitrogen-fixation (*nifH*) and some carbon is diverted by the bacteroids into the production of intracellular storage polymers composed of either glycogen or PHB. Synthesis of intracellular amorphous PHB storage granules from TCA cycle intermediates by PHB synthase (*phbC*). Moreover, the role of sugars and in particular trehalose in this respect should be considered. The route of trehalose synthesis is the OtsA/B pathway by the action of trehalose-6-phosphate synthase (*otsA*) that forms trehalose-6-phophate from UDP-glucose and glucose-6-phophate with subsequent dephosphorylation, yielding free trehalose for carbon metabolism in bacteroids as well as for PHB accumulation.

For coinoculation of *B. japonicum* with PGPR, the up-regulation of PGPR mode of action related genes, *iaaH* and *ipdC* genes were up-regulated (Figure 36B). 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 (e.g. cortical cell division). The coinoculation 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 coinoculation with PGPR that caused an increase in trehalose accumulation levels. Furthermore, the accumulation of carbon source and PHB granules were also induced. The coinoculation 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 coinoculation with *B. japonicum* and PGPR Taken together results indicated that the coinoculation approachs are able to enhance the nodulation and nitogen fixation in soybean, resulting increased soybean yield.

CHAPTER VI

CONCLUSION

Selected 12 PGPR were performed significantly capable of promoting N_2-fixation, nodule number, nodule and plant dry weight with both commercial Bradyrhizobial strains, *B. japonicum* THA6 and USDA110 (P<0.05). Furthermore, isolates S141 and S222 which are closely related to *Bacillus subtilis* and *Staphylococcus* sp. were selected for coinoculation with *B. japonicum* USDA110 and THA6. The effective coinoculation doses of PGPR-Bradyrhizobium on soybean were 10^6 : 10^6 cells/seed. The effect of coinoculation experiment under field condition could increase 9.7-43.6% of seed yield per hectare which higher than those of uninoculated or single inoculation of PGPR or *B. japonicum*.

The effect of simultaneous presence of coinoculation on the plant and bacterial response by the gene expression analyses were identified under soybean root and nodule associated stage. The relative expression levels of PGPR mode of action related genes including *iaaH* and *ipdC* were also up-regulated by coinoculation of *B. japonicum* and PGPR. The early nodulation response genes including *nodD1*, *GmCaMK1* and *GmNIN1A* were monitored by RT-PCR on 7 DAI soybean roots. The coinoculation of *B. japonicum* and PGPR was obviously enhanced the up-regulation of *nodD1* and *GmCCaMK* genes but the expression level of *GmNIN1A* gene was not significantly different from to those of single inoculation. Moreover, the expressions of soybean and the bacterial related genes (*GmMyb*, *otsA*, *phbC*, *dctA* and *nifH*) in

nodule after single and coinoculated with *B. japonicum* and PGPR were identified using RT-PCR at different time frames. The results revealed that the related genes expression was discontinuously triggered both up- and down regulation by *B. japonicum*-PGPR coinoculation. These results were accordingly related with phenotyptic characters in Leonard' jar and field experiments in term of enhancing the nodulation and N_2-fixation in soybean.

The TEM micrograph of soybean nodule demonstrated densely packed poly-ß-hydroxybutyrate (PHB) granules within the bacteroids. PHBs were presented in mature nodule of coinoculated treatments whilst single inoculated nodules were senescence. These results taken together suggested that the PGPR may facilitate the induction of trehalose accumulation and the transport of C4-dicarboxylic acids which represented an increase in PHB accumulation which rendering enhance the nodulation and N_2-fixation in soybean.

These are the first observations that demonstrate mechanism of PGPR strains *Bacillus subtilis* and *Staphylococcus* sp. on *Bradyrhizobium*-soybean symbiosis. The coinoculation with *B. japonicum* 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: infection, nodulation, and/or nitrogen fixation.

Therefore, the efficiency to enhance soybean N_2-fixation by coinoculation strategy is significantly for maximum nodulation and can be developed for supreme inoculants for soybean inoculants.

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APPENDICES

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

D-Mannitol	10.0	g
K_2HPO_4	0.5	g
$MgSO_4.7H_2O$	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, G., 1951)

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

Appendix 3. LG medium (Lipman J. G., 1904)

Glucose	10.0	g
KH_2PO_4	0.41	g
K_2HPO_4	0.52	g
$CaCl_2$	0.2	g
Na_2SO_4	0.05	g
$MgSO_4 \cdot 7H_2O$	0.1	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. N-free Nutrient Solution (Broughton and Dillworth, 1970)

Stock Solutions	Elements	Form	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

BIOGRAPHY

Miss Janpen Prakamhang was born on October 20th, 1975 in Buriram, Thailand. She graduated with the Bachelor Degree of Animal Production Technology, Suranaree University of Technology in 1998 and Master Degree enrollment in the School of Biotechnology, Institute of Agricultural Technology, Suranaree University in 2006. During her Ph.D., she presented research work in the 1st Asian Conference on Plant-Microbe Symbiosis and Nitrogen Fixation, September 20-24, 2010, Miyazaki, Japan (Poster presentation; in "Maximization nodulation in soybean (*Glycine max*) using coinoculation with PGPR and *Bradyrhizobium japonicum* (Poster presentation). The CHE-PhD-THA congress, April, 2010, Pattaya, Thailand (Poster presentation; in "Enhancement of soybean-*Bradyrhizobiun* nitrogen fixation efficiency using Plant Growth Promoting Rhizobacteria (PGPR) coinoculation") The 2nd Asian Conference on Plant-Microbe Symbiosis and Nitrogen Fixation, October 28, 2012, Phuket, Thailand (Poster presentation; in "Potential for enhancement of nodulation and nitrogen fixation of soybean coinoculated with PGPR and *Bradyrhizobium*"). She received a scholarship JASSO (Japan Student Service Organization) during August 24, 2010 – March 31, 2011 at Faculty of Science and Engineering, Kagoshima University, Kagoshima, Japan.