# ผลกระทบของการใช้เชื้อแบคทีเรียที่ส่งเสริมการเจริญเติบโตของพืช (PGPR) ร่วมกับ *Bradyrhizobium japonicum* ต่อ การเข้าปม การเจริญเติบโต และชุมชนจุลินทรีย์บริเวณรอบรากถั่วเหลือง

นางสาวตี ตี อ่อง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษา ตามหลักสูตรปริญญา วิทยาศาสตร ดุษฎีบัณฑิต
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# CO-INOCULATION EFFECTS OF PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR) AND *Bradyrhizobium japonicum* ON SOYBEAN NODULATION, GROWTH AND RHIZOSPHERE SOIL MICROBIAL COMMUNITY STRUCTURES

**Thi Thi Aung**

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

**Suranaree University of Technology**

**Academic Year 2012**

**CO-INOCULATION EFFECTS OF PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR) AND *Bradyrhizobium japonicum* ON SOYBEAN NODULATION, GROWTH AND RHIZOSPHERE SOIL MICROBIAL COMMUNITY STRUCTURES**

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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ตี ตี อ่อง : ผลกระทบของการใช้เชื้อแบคทีเรียที่ส่งเสริมการเจริญเติบโตของพืช (PGPR) ร่วมกับ *Bradyrhizobium japonicum* ต่อการเข้าปม การเจริญเติบโต และชุมชนจุลินทรีย์บริเวณรอบรากถั่วเหลือง (CO-INOCULATION EFFECTS OF PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR) AND *Bradyrhizobium japonicum* ON SOYBEAN NODULATION, GROWTH AND RHIZOSPHERE SOIL MICROBIAL COMMUNITY STRUCTURES) อาจารย์ที่ปรึกษา : รศ.ดร.หนึ่ง เตียอำรุง, 159 หน้า

การปลูกเชื้อร่วมกันระหว่างแบคทีเรียที่สร้างปมในถั่วเหลือง ในสกุล *Bradyrhizobium* และเชื้อแบคทีเรียที่ส่งเสริมการเจริญเติบโตของพืช (PGPR) เป็นที่ได้รับความสนใจเป็นอย่างมาก โดยคาดหวังว่าการใช้เชื้อทั้งสองชนิดร่วมกันนั้นจะสามารถเพิ่มจำนวนปม และส่งเสริมการเจริญเติบโตของถั่วเหลืองได้ ในการศึกษานี้ได้ทำการตรวจสอบหาเชื้อจุลินทรีย์ในดินที่สามารถใช้ร่วมกับเชื้อ *B. japonicum* เพื่อใช้กับถั่วเหลือง จากการคัดเลือกเชื้อกลุ่ม rhizobacteria จำนวน 200 ไอโซเลต พบว่าสายพันธุ์ที่มีประสิทธิภาพในการเพิ่มจำนวนปมในรากถั่วเหลือง ได้แก่ *Azospirillum* sp. และ *Bacillus solisalsi* จากการศึกษาถึงผลกระทบของการใช้เชื้อแบบเดี่ยว และแบบใช้ร่วมกันของเชื้อ *B. japonicum* (CB 1809 และ USDA 110) กับ เชื้อ *Azospirillum* sp. หรือ *B. solisalsi* ต่อการเพิ่มจำนวนปม การเจริญเติบโต และชุมชนจุลินทรีย์บริเวณรอบรากถั่วเหลือง พบว่า การใช้เชื้อร่วมกันระหว่าง *Azospirillum* sp. กับ *B. japonicum* CB 1809 หรือ USDA 110 มีศักยภาพในการเพิ่มการสร้างปม 32.23% และ 16.85% การเพิ่มน้ำหนักปม 26.51% และ 18.83% และสามารถเพิ่มผลผลิตเมล็ดถั่วได้ 23.65% และ 34.92% ตามลำดับ เปรียบเทียบกับเมื่อใช้เชื้อ *B. japonicum* CB 1809 หรือ USDA 110 เพียงชนิดเดียวในสภาพแปลงปลูกจริง ดังนั้นจึงใช้เชื้อ *Azospirillum* sp. ร่วมกับเชื้อ *B. japonicum* CB 1809 หรือใช้ร่วมกับ USDA 110 เพื่อศึกษาถึงการแข่งขันเพื่อเข้าสร้างปมในถั่วเหลือง ผลการศึกษาการแข่งขันเพื่อเข้าสร้างปมของเชื้อดังกล่าวกับจุลินทรีย์ในดินจากประเทศพม่า และจากประเทศไทย พบว่า การ ใช้เชื้อแบบเดี่ยว หรือการใช้เชื้อร่วมกันของ *B. japonicum* USDA 110 และ *Azospirillum* sp. ที่ทำการติดฉลากด้วย *gus*-marker ให้ผลการเข้าปม 93.21-94.75% และ 74.21-100% ตามลำดับ และสามารถเพิ่มปริมาณน้ำหนักแห้งรวม 23.50-41.95% และ 50.37-73.24% ตามลำดับ เมื่อเทียบกับถั่วเหลืองที่ไม่ใช้เชื้อจุลินทรีย์ ในการทดลองใช้เชื้อ *Azospirillum* sp. ในแต่ละปริมาณเชื้อ $10^6$, $10^7$ และ $10^8$ โคโลนีต่อมิลลิตร ร่วมกับ *B. japonicum* USDA 110 พบว่าสามารถเพิ่มการเข้าสร้างปมในถั่วเหลืองได้ 73.8, 62.25 และ 95.34% และ 51.52, 62.38 และ 79.46 % ในดินจากประเทศพม่า และประเทศไทยตามลำดับ เมื่อเทียบกับการไม่ใส่เชื้อ จากการทดสอบ Denaturing Gradient Gel Electrophoresis (DGGE) และ

Principle Component Analysis (PCA) เพื่อหาความสัมพันธ์ระหว่างการใช้เชื้อจุลินทรีย์ที่คัดเลือกแล้วกับชุมชนจุลินทรีย์บริเวณรากพืช ทั้งการทดสอบในระดับกระถาง และระดับแปลง พบว่าช่วงการเจริญเติบโตของพืชมีผลต่อการเปลี่ยนแปลงของจุลินทรีย์ในกลุ่มยูแบคทีเรียรอบรากพืช แต่ไม่เกี่ยวข้องกับอิทธิผลของเชื้อแบคทีเรียที่ปลูกร่วม ในทางกลับกัน การใส่เชื้อและช่วงการเจริญเติบโตของพืช ต่างก็ไม่มีผลต่อก ารเปลี่ยนแปลงของชุมชนเชื้อราบริเวณรอบรากพืช ดังนั้น เชื้อ *Azospirillum* sp. สามารถนำมาใช้ร่วมกับ *B. japonicum* สำหรับการปลูกถั่วเหลืองได้เป็นอย่างดี



สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2555

ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

THI THI AUNG : CO-INOCULATION EFFECTS OF PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR) AND *Bradyrhizobium japonicum* ON SOYBEAN NODULATION, GROWTH AND RHIZOSPHERE SOIL MICROBIAL COMMUNITY STRUCTURES. THESIS ADVISOR : ASSOC. PROF. NEUNG TEAUMROONG, Dr. rer. nat, 159 PP.

*Bradyrhizobium japonicum*/PLANT GROWTH PROMOTING RIZOBACTERIA/ SOYBEAN/CO-NOCULATION/COMPETITION/MICROBIAL COMMUNITY STRUCTURES

Co-inoculation of nodulated bradyrhizobia and plant growth promoting rhizobacteria has received great attention because co-inoculation can be expected to enhance the nodulation and plant growth of soybean (*Glycine max*). In this study, screening of rhizobacteria for co-inoculation with *Bradyrhizobium japonicum* on soybean was conducted. Among the 200 isolates of rhizobacteria tested, *Azospirillum* sp. and *Bacillus solisalsi* were selected as nodulation enhancers. Single and co-inoculation effects of *B. japonicum* (CB 1809 and USDA 110) and either *Azospirillum* sp. or *B. solisalsi* were studied to access the co-inoculation potential on nodulation, plant growth and rhizosphere soil community structures of soybean. *Azospirillum* sp. co-inoculated with either *B. japonicum* CB 1809 or USDA 110 under field conditions gave 32.23% and 16.85% of nodulation, 26.51% and 18.83% of nodule dry weight, and 23.65% and 34.92% seed yield over single inoculation of CB 1809 and USDA 110, respectively. *Azospirillum* sp. was selected for co-inoculation with either *B. japonicum* CB1809 or USDA 110 for competitive nodulation study.

The results from the competition study for nodulation under rhizobia-established Myanmar and Thailand soils revealed that single or co-inoculation of *gus*-marked *B. japonicum* USDA 110 and three different inoculum levels of *Azospirillum* sp. gave 93.21-94.75% and 74.21-100% in nodule occupancy, and 23.50-41.95% and 50.37-73.24% enhanced in biomass dry weight over non-inoculated control, respectively. Each of the tested inoculum levels, i.e., $10^6$, $10^7$ and $10^8$ cfu $ml^{-1}$ of *Azospirillum* sp. enhanced nodulation in combination with USDA 110 with a corresponding increase of 73.8%, 62.25% and 95.34%; and 51.52%, 62.38% and 79.46% over non-inoculated control in Myanmar and Thailand soil, respectively.

Denaturing Gradient Gel Electrophoresis (DGGE) and Principle Component Analysis (PCA) results demonstrated that soybean rhizosphere eubacterial community structures in both pot and field experiments in this study were shifted by plant growth stages not by bacterial inoculation. In contrast, neither inoculation of tested bacteria nor plant growth stages shifted the rhizosphere soil fungal community structures. Therefore, *Azospirillum* sp. could be used in co-inoculant production with *B. japonicum* for soybean.



School of Biotechnology

Academic Year 2012

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# LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| ACC | = | 1-aminocyclopropane-1-carboxylic acid |
| ANOVA | = | Analysis of Variance |
| ARA | = | Acetylene Reduction Assay |
| ARDRA | = | Amplified Ribosomal DNA Restriction Analysis |
| BNF | = | Biological Nitrogen Fixation |
| cfu | = | Colony Forming Unit |
| CLPP | = | Community-Level Physiological Profile |
| CRD | = | Completely Randomized Design |
| DAI | = | Day after Inoculation |
| DAP | = | Department of Agricultural Planning |
| DAPG | = | 2,4-diacetyl- phloroglucinol |
| DAR | = | Department of Agricultural Research |
| DGGE | = | Denaturing Gradient Gel Electrophoresis |
| DMRT | = | Duncan's Multiple Range Test |
| DOA | = | Department of Agriculture |
| DOAE | = | Department of Agricultural Extension |
| FAO | = | Food and Agriculture Organization |
| GAs | = | Gibberellins |
| GC | = | Gas Chromatograph |
| IAA | = | Indole-3-acetic acid |

# LISTS OF ABBREVIATIONS (Continued)

| | | |
|---|---|---|
| ISR | = | Induced Systemic Resistance |
| LSD | = | Least Significant Different |
| MAS | = | Myanmar Agricultural Service |
| MPN | = | Most Probable Number |
| NA | = | Nutrient Agar |
| PCA | = | Principle Component Analysis |
| PCR | = | Polymeric Chain Reaction |
| PGPR | = | Plant Growth Promoting Rhizobacteria |
| PLFA | = | Phospholipid Fatty Acid |
| PS | = | Private Sector |
| QA | = | Quality Assurance |
| RAPD | = | Random Amplified Polymorphic DNA |
| RCBD | = | Randomized Complete Block Design |
| RISA | = | Ribosomal Intergenic Spacer Analysis |
| RISA | = | rRNA Intergenic Spacer Analysis |
| SSCP | = | Single Strand Conformation Polymorphism |
| SUT | = | Suranaree University of Technology |
| TGGE | = | Temperature Gradient Gel Electrophoresis |
| TRFLP | = | Terminal Resistriction Length Polymorphism |
| UPGMA | = | Unweighted Pair Group Method with Arithmetic Means |
| YIB | = | Yield Increasing Bacteria |
| YMA | = | Yeast Extract Mannitol Agar |

# CHAPTER I

# INTRODUCTION

## 1.1 Significance of this study

Soybean (*Glycine max* L. Merrill) is considered one of the oldest crops in the world and a major source of plant protein, oil and fat. It is an important legume because of its nutritive and economic values and varieties of by-products which are used in many industries and animal husbandry across the world. This crop alone contributes to about 20% of the world's oil and fat supply (Singh et al., 1989). The increase in soybean productivity has contributed to a greater use of agrochemicals, which cause major problems, such as soil and water pollution and reduction of biodiversity and have a negative impact on non-target species (Correa et al., 2009). Increasing and extending the role of biofertilizers may reduce the need for chemical fertilizers and thereby decrease adverse environmental effects. Recent advancements in the field of biofertilizers (including inoculation with microorganisms) create a growing level of interest in environmental friendly sustainable agricultural practices (O' Connell, 1992).

In legumes, symbiotic nitrogen fixation (Biological Nitrogen Fixation-BNF) is a well-known process exclusively driven by *Rhizobium* bacteria, which specifically reduces atmospheric $N_2$ to ammonia in the symbiotic root nodules, a key input of N for plant productivity. In soybean, *Bradyrhizobium japonicum* forms a symbiotic relationship and inoculation with those bacteria has been successful in increasing

soybean nodulation with increases in plant fresh weight, seed protein and seed yield in soils with a low or absent native population (Cladwell and Vest, 1970).

Zhang et al. (2002) and Kazemi et al. (2005) reported that *B. japonicum* bacteria increased number of pods per plant, number of seeds per plant, hundred seed weight, grain protein, total protein and development of plant leaves in tested soybean cultivars. Maximum benefit of $N_2$-fixation by soybean often requires the inclusion of selected strains of bradyrhizobia as seed inoculants especially in soils with low population of rhizobia. The inoculated strain must be effective in its ability to fix $N_2$ with the cultivar concerned and processes the ability to compete for nodulation of the plant with other strains of rhizobia that might be present in the soil. Therefore, the rhizobia used in inoculants should not only have high $N_2$-fixation ability in that crop but also have the competitive ability for nodulation against the indigenous rhizobia.

In addition to rhizobia, heterogenous group of bacteria can be found in the rhizosphere, at root surfaces and association with roots. Some of these bacteria show beneficial effects on plant growth when used as seed or soil inoculants and hence they are called Plant Growth Promoting Rhizobacteria (PGPR) (Glick, 1995). Those bacteria identified as PGPR have diverse taxonomy and include strains of the genera *Azospirillum*, *Azotobacter*, *Bacillus*, *Enterobacter*, *Gordonia*, *Klebsiella*, *Paenibacillus*, *Pseudomonas*, *Serratia*, among others and they can improve the extent or quality of plant growth directly and/or indirectly (Glick, 1995; Hong et al., 2011). Several mechanisms have been suggested by which PGPR can promote plant growth including phytohormone production, stimulation of nutrients uptake, and biocontrol of deleterious soil bacteria and phytopathogenic fungi (Lifshitz et al., 1987). Therefore, PGPR play in important role in sustainable agriculture.

There were several reports that combined use of two or three beneficial microorganisms as inoculation has been found to perform better than single inoculations (Alagawadi and Gaur, 1988; Jisha and Alagawadi, 1996; Prathibha et al., 1995). Combined inoculations are said to work better than single inoculation based on the principle that greater the diversity and number of inhabitants, the higher the order of interaction and more stable the ecosystem (Higa, 1991). The use of mixed cultures of beneficial microorganisms as soil inoculants is based on the principles of natural ecosystems which are sustained by their constituents; i.e., by the quality and quantity of their inhabitants and specific ecological parameters, i.e., the greater the diversity and number of the inhabitants, the higher the order of their interaction and the more stable the ecosystem. The mixed culture approach is simply an effort to apply these principles to natural systems such as agricultural soils, and to shift the microbiological equilibrium in favor of increased plant growth, production and protection (Higa, 1994; Parr et al., 1994).

Some of the nodulation promoting rhizobacteria increase nodulation leading to increase plant growth (Zhang et al., 1997). Therefore, co-inoculation of legume with symbiotic rhizobia and free-living microbes like PGPR has received great attention in many studies. Co-inoculation studies with PGPR and *Bradyrhizobium* have shown the increasement of plant nodulation and $N_2$-fixation under normal growth conditions (Verma et al., 1986; Li and Alexander, 1988). For instance, Dashti et al. (1998) reported that co-inoculation of soybean with *B. japonicum* and *Azospirillum* or PGPR increases nodulation, nitrogenase activity and plant growth. Co-inoculation with *Pseudomonas* spp. and *Rhizobium* spp. has been shown to increase the degree of colonization of the legume rhizosphere by rhizobia resulting in

enhanced plant nodulation (Cook and Baker, 1983). Field tests with some pseudomonad strains have demonstrated the yield increases (Bashan and Holguin, 1997), delayed the leaf senescence at the later stages of growth (Sarig et al., 1990) and promotion of legume nodulation by nitrogen-fixing rhizobia (Zhang et al., 1996).

Although they are beneficial for agriculture, exploitation of PGPR as biocontrol or biofertilizer inoculants has been hampered by inconsistent results at the field scale (Mark et al., 2006; Morrissey et al., 2004). Soil is considered to be the richest environment, with a high diversity of microorganisms belonging to the three domains of life, Bacteria, Archaea and Eukarya (Fierer and Jackson, 2006). PGPR that have been added to soil or seeds to improve plant growth and/or health will also modify the composition of the resident bacterial community of the rhizosphere. Microbes residing in the rhizosphere can be beneficial or detrimental for the plant and therefore can influence crop yields significantly (Sturz and Christie, 2003).

Genetic fingerprinting techniques are able to provide a profile representing the genetic diversity of a microbial community from a specific environment. PCR-DGGE of ribosomal DNA was introduced into microbial ecology by Muyzer et al. (1993). It was originally developed to detect specific mutations within genomic genes due to one base mismatch and it is based on the separation of Polymerase Chain Reaction (PCR) amplicons of the same size but different sequences. This method enables to sequence data to be obtained on the DNA of dominant species from individual bands; therefore, it perhaps the most commonly used among the culture-independent fingerprinting techniques (Muyzer et al., 1993).

Currently, the biofertilizers are being produced including different microorganisms and widely available around the world. The presence of mix

microorganisms in biofertilizers gave low quality and less effectiveness in some cases. As mention above, PGPR have potential for agriculture because under certain conditions, they can improve plant growth. Therefore, pure cultures of PGPR in inoculants such as *Azotobacter* and *Azospirillum* tend to be more science based products for many crops. Moreover, "Rhizobial Biofertilizer Inoculants" are also being produced for different leguminous crops. Although number of studies showed the potential of co-inoculants as well as beneficial effects of co-inoculation on leguminous plants, as far as we know, there has been no report about the co-inoculant production and usage of this biofertilizer in legume growing area in Myanmar and Thailand. Therefore, to develop this potential to co-inoculating of PGPR with *B. japonicum* and to evaluate whether it can be possible to select PGPR adapted to the conditions in Myanmar and Thailand soils. Thus, this effort was focused on the selection of effective PGPR for co-inoculation purpose.

## 1.2 Research questions

The overall aims of this study were to obtain the PGPR isolate which is nodule formation enhancer when co-inoculated with bradyrhizobia and to identify the microbial community structure of soybean rhizosphere shifted by this co-inoculation. The following research questions were addressed:

1. Whether PGPR have antagonistic effect on *B. japonicum* when they were used as co-inoculant
2. Whether the selected rhizobacteria can promote the bradyrhizobial ability in terms of nodulation and plant growth of soybean as the best one under

pot and field conditions with soybean nodulating-bradyrhizobia-non-established soils

3. Whether the selected PGPR can enhance the competition for nodulation (nodulation occupancy) of co-inoculated *B. japonicum* USDA 110 inoculum against indigenous soil bradyrhizobia of Myanmar and Thailand soybean growing field
4. Whether co-inoculation of *B. japonicum* and related PGPR affects co-inoculation shift the rhizosphere soil microbial community structure or not.

## 1.3 Structure of dissertation

This dissertation contains seven chapters.

**Chapter 1** provides a short introduction to bradyrhizobia and PGPR and their potential contributions on biological $N_2$-fixation, and how to focus the effects of inoculation on rhizosphere soil microbial community structures. This chapter also highlights the research questions addressed in this study.

**Chapter 2** provides a literature review outlining the importance of soybean, bradyrhizobia, PGPR and the contributions to their co-inoculation effects attributed to agriculture. It also explains the soil microbial community structures, factors that affects those communities, and how to approach to detect those changes.

**Chapter 3** presents the results of screening study to select the effective rhizobacteria for combined inoculation with bradyrhizobia. Moreover, this selected rhizobacteria were continued to select under pot condition where I evaluated the co-inoculation effects on nodulation, plant growth of soybean and rhizosphere soil microbial

community structures. The resulted best rhizobacterium was selected as PGPR and further field study was undertaken to evaluate their co-inoculation effects on nodulation, plant growth, $N_2$-fixation, seed yield and rhizosphere soil bacterial community structures. Both pot and field studies were carried out under soybean-nodulating bradyrhizobia-non-established soil conditions.

**Chapter 4** compares the competition for nodulation of inoculated bradyrhizobial strain and soil bradyrhizobia under soybean-nodulating bradyrhizobia-established soil conditions. Here, we insert *gus*-reporter gene into *B. japonicum* USDA 110 to detect its single and co-inoculation effects on nodule occupancy.

**Chapter 5** synthesizes the main results of Chapter 3 and 4, and presents the main conclusions along with the implications with respect to the general findings from the research.

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# CHAPTER II

# LITERATURE REVIEW

## 2.1 Importance and situation of soybean production in the world

Soybean (*Glycine max* L. Merrill) is one of the most important grain legume crops and it has occupied third place in oil seed crop of the world. It is offering high-quality protein (40-42%) and increasing the input of combined $N_2$ into the soil. It can be grown in tropical, subtropical and temperate climates. World production of soybeans production was 251.5 million tones in 2011 (FAO, 2011) and predicted to increase by 2.2% annually to 371.3 million tones by 2030 using an exponential smoothing model with a damped trend (Masuda and Goldsmith, 2009). The top five countries including United States, Brazil, Argentina, China and India produce more than 92% of the world's soybeans, and USA, Brazil and Argentina produced soybean in a total of 80.70, 57 and 32 million tones by cultivation of 29, 23 and 14 million ha, respectively in 2011-2012 (FAO, 2011).

## 2.2 Situations of soybean production in Myanmar and Thailand

Myanmar is one of the world's major pulses (food legume) producing countries (DAP, 2010) and soybean is one of the important cash crops to the increasing demand for domestic consumption and export. It covers about 153,000 ha, mainly grown about 42%, 21%, 13% and 7% in the Shan State, Mandalay, Sagaing

and Ayeyarwaddy Division, respectively with an average yield of 1.17 tones per ha (DAP, 2006).

In Thailand, soybean is grown in a variety of locations, cropping patterns, land types and seasons, and dominant production area is located in the Northern region which produces 74% of total production. Jierwiriyapant and Hadi (1992) reported that the total soybean cultivation areas in Thailand were approximately 2.67 million Rais and total production was 568,000 tons or 213 kg $rai^{-1}$ in 1989-90. According to the Food and Agriculture Organization (FAO) data of 2011, increasing soybean production in Thailand is an important policy of the government because about 1.8 million tons of soybeans were imported in 2010 as soybean production is not sufficient to meet human and animal needs in Thailand (Jaidee et al., 2013).

## 2.3 Utilization of chemical nitrogen fertilizer in the world

Plants have ability to take up several chemical form of nitrogen. The most common are ammonium ($NH_4^+$), nitrate ($NO_3^-$) and Urea ($(NH_4)_2$ CO). Among them, Urea is increasingly farmers' high-analysis nitrogen fertilizer of preference. Between 150 and 200 million tones of mineral N are required each year by plants in agricultural systems to produce the world's food, animal feed and industrial products (Unkovich et al., 2008). Global ammonia capacity is projected to increase from 150 million tones N in 2008 to 173 million tones N in 2012. The forecast for world nitrogen fertilizer demand showed increasing at an annual rate of about 1.4% until 2011-2012, which is an overall increase of 7.3 million tones (FAO, 2008).

## 2.4 Importance of biological nitrogen fixation in the world

In the current agriculture, nitrogen is a limited nutrient for growth and consequently to the yield of cultivars. The extensive uses of chemical fertilizers are costly and may create environmental problems (Esitken et al., 2005). Even though 78.1% of the earth's atmosphere consists of the chemically inert nitrogen gas ($N_2$), nitrogen availability is limited in many soils, and $N_2$ is inaccessible for most of the living beings (Ferguson et al., 2010). Hence, nitrogen must be converted either chemically or biologically to a usable form that life on earth can profit from. Before its incorporation into a living system, $N_2$ must first be combined with the element hydrogen. This process commonly referred to as "nitrogen fixation" ($N_2$-fixation) and which may be accomplished by chemically or biologically (Hubbell and Kidder, 1978).

Biological Nitrogen Fixation (BNF) is the process whereby a number of species of bacteria such as *Rhizobium*, *Azotobacter*, *Azospirillum*, etc., use the enzyme nitrogenase to convert atmospheric $N_2$ into ammonia ($NH_3$), a form of nitrogen (N) that can then be incorporated into organic components, e.g., protein and nucleic acids, of the bacteria and associated plants while the plant partner supplies the carbon (C) sources that provide the energy required for the $N_2$ reduction reaction. In this way, unreactive $N_2$ enters the biologically active part of the global N cycle (Unkovich et al., 2008). Globally, symbiotic $N_2$-fixation has been estimated to amount to at least 70 million metric tones N $year^{-1}$ (Brockwell et al., 1995). Since atmospheric $N_2$ is an unlimited source of N, the process of $N_2$-fixation is of great potential for sustainable agriculture, and in the special case of legumes.

## 2.5 Utilization of rhizobial inoculants for soybean cultivation in the world and its benefits

Soybean plants can use nitrogen released from different sources, i.e. mineralized N, soil N, fertilizer N, or atmospheric $N_2$ through a symbiotic relationship (Abaidoo et al., 2007). $N_2$-fixing bacteria in legume nodules collectively designated as rhizobia have been known since 1888 (Quispel, 1988). Members of the genus - *Bradyrhizobium* are slow-growing, gram-negative soil bacteria which invade and form nitrogen fixing nodules on the root of specific leguminous plants. The major soybean-nodulating rhizobia are *Bradyrhizobium japonicum*, *B. elkanii*, and *Sinorhizobium/Ensifer fredii* (Jordan, 1982; Scholla and Elkan, 1984; Kuykendall et al., 1992; Young, 2003). Generally, nodulation of soybean requires specific *Bradyrhizobium* species and *B. japonicum* is the best example of successful symbiotic fixation under very large scale field conditions (Penna et al., 2011). When inoculated with compatible rhizobia, the formation of effective (functional) nodules in soybean leads to fixation of atmospheric nitrogen ($N_2$) making nitrogenous fertilization of the soybean unnecessary (Gwata et al., 2003). Therefore, *B. japonicum* has been successfully incorporated as the active principle of soybean inoculants in Argentina, Brazil, Paraguay, USA, Canada and other soybean producing countries worldwide in the last 30 years (Penna et al., 2011). However, in soils where the soybean crop has not been grown previously, compatible populations of bradyrhizobia are seldom available (Abaidoo et al., 2007).

Generally, nitrogen fertilizers are not usually required for soybeans. Studies of nodulated soybeans showed significant yield response to frequent N additions when the $N_2$-fixation apparatus could not meet N demand (Thies et al., 1995). However,

when fertilizer N is applied, it can reduce the amount of $N_2$-fixation. The contradictory results obtained in N fertilization studies do not provide clear evidence as to whether N fertilization is required to complement the N supply from BNF to achieve soybean yields that approach yield potential levels (Salvagiotti et al., 2008). Therefore, inoculation with symbiotic $N_2$-fixing bradyrhizobia has become a simple and effective way to significantly improve soybean yield and productivity (Penna et al., 2011).

Among the legume growing areas, it is likely that only 10-15 million ha (i.e., 14-21% of the total) are inoculated annually. However, virtually all of the 11 million tones of N currently fixed by soybean results from either past or current inoculation. This is because soybean, for the most part, is grown on land that initially did not contain the soybean rhizobia (Herridge, 2002). When in symbiotic association with *B. japonicum*, soybean plants can fix up to 200 kg N $ha^{-1}$ $year^{-1}$ (Smith and Hume, 1987), reducing the need for expensive and environmentally damaging nitrogen fertilizer.

Estimated amount of nitrogen fixed by soybean-rhizobia symbiosis under field conditions varied from 60-115 kg $ha^{-1}$ $year^{-1}$ (Evens and Barbar, 1977). BNF can reduce the need for N fertilizers, resulting in an economy estimated in US$ 3 billion per crop season (Nicolás et al., 2006). Therefore, partial supplement of fixed-N to plants may reduce the use of chemical-N fertilizers, and subsequently reduce N-losses and environmental pollution (Herridge, 2002).

Although soybeans have the ability to symbiotically fix nitrogen, not all of the soybean's nitrogen needs are met through fixation (Sawyer et al., 2006). Therefore, they recommend that it is appropriate to provide approximately 50% of a soybean

crop's total nitrogen need through manure nitrogen and the plant will fix the remaining nitrogen required. Using this approach, a manure application nitrogen rate of 112 -140 kg-N $ha^{-1}$ (100-125 lbs-N $acre^{-1}$) is suggested for a soybean crop with a 3,358 kg $ha^{-1}$ yield and a total nitrogen requirement of 269-280 kg-N $ha^{-1}$ (240-250 lbs-N $acre^{-1}$).

## 2.6 Utilization of chemical nitrogen fertilizers and rhizobial inoculants for soybean production in Myanmar and Thailand

BNF from legumes offers more flexible management than fertilizer nitrogen because the pool of organic nitrogen becomes slowly available to non-legume species (Peoples et al., 1995). They were the first biofertilizers produced and allow savings of millions of dollars in chemical fertilizers. Herridge et al. (2008) estimated that about 21 million tones of nitrogen are fixed annually through the crop legume-rhizobia symbiosis.

In Myanmar, Urea is the main source of nitrogen applied to all cultivated crops but it is very expensive and not readily available (Than and Han, 1988). Myanmar farmers use nitrogenous (N) fertilizers sparingly, particularly on legume crops. Thus, low-nodulation induced N deficiencies of the legumes are not remedied by inputs of fertilizer N and the value of lost production could exceed $100 million annually. Generally, it is necessary to inoculate the seeds or soils with highly effective rhizobial cultures before sowing. Myanmar farmers have used, and continue to use, rhizobial inoculants when sowing legumes, but the practice is currently not widespread (Herridge et al., 2008).

In Myanmar, rhizobial research and inoculant production was initiated at the "*Rhizobium* Inoculant Production Unit", Plant Pathology Section of Department of Agricultural Research (DAR). About 250,000 packets of peat-based rhizobial inoculants for seven legumes crops (groundnut, chickpea, blackgram, greengram, pigeonpea, soybean and cowpea) are annually produced and distributed through Extension Division of Myanmar Agricultural Service (MAS) (Than et al., 2006). Production by DAR peaked during the 1980s at 600-700,000 packets annually. Current production is less than 100,000 packets, due to limitations in the whole supply chain from production and quality assurance (QA) to distribution to demand. Currently, exotic bradyrhizobial strains of TAL 379 (CB 1809), TAL 377 and TAL 102 (USDA 110) from NifTAL (Nitrogen fixation for Tropical Agricultural Legumes) are used in rhizobial inoculant production for soybean at DAR (DAR, 2004).

In Thailand, most rhizobial legume inoculant extension work has been conducted by Department of Agricultural Extension (DOAE) while Department of Agriculture (DOA) is responsible for inoculant production and multi-disciplinary research (Boonkerd, 2002). The Ministry of Agriculture and Cooperatives through the Department of Agriculture (DOA) and the Department of Agricultural Extension (DOAE) are responsible for introducing inoculation technology to farmers. The cooperation between DOA and DOAE is structured so that the DOA is responsible for inoculant production and multidisciplinary research, while the DOAE is responsible for the distribution and promotion of the inoculant through training and other activities. In 1990, DOA (Thailand) produced a total of 126.35 metric tons of inoculants for soybean, groundnut, mung bean, and other minor legumes including 477,333 bags of soybean inoculant which were distributed through DOAE, private

sector (PS), and Marketing Farmer Organization (MFO) (Chanaseni and Kongngoen, 1992).

## 2.7 Limitation of using rhizobia as inoculants

The establishment of an effective and efficient symbiosis between rhizobia and the host legume is essential to viable legume production. To enhance the performance of the *Rhizobium*-legume symbiosis, the practice of inoculating legume seeds with carrier-based inoculants of the desired rhizobia is widely practiced. However, establishment of effective $N_2$-fixing symbioses between legumes and their $N_2$-fixing bacteria (rhizobia) is dependent upon many environmental factors, and can be greatly influenced by farm management practices (Peoples et al., 1995).

Generally, beneficial microorganisms introduced into rhizospheres are affected by a large number of abiotic and biotic factors, each potentially producing an unfavorable effect. Biotic factor includes leguminous plant host, rhizobial strain and other soil microorganisms (Sadowsky, 2000) as well as host-strain specificity. Abiotic factors are involved in several substances or environment conditions which affect the nodulation of competition. Several environment conditions such as salinity, unfavorable soil pH, soil type, soil temperature, nutrient deficiency, mineral toxicity, temperature extremes, insufficient or excessive soil moisture are severe factors affecting growth and competitive of $N_2$-fixating bacteria (Triplett and Sadowsky, 1992).

In addition to the environmental constraints, the availability of good quality soybean seed, good quality inoculants, quality storage for the seed and the inoculants, lack of good application equipment and knowledge of proper application of

inoculants to the seed or soil and access to pertinent production information are major barriers. However, survival, persistence and competitive ability of the inoculated strains limit their success in the soil (Lakshminarayana and Sharma, 1994). Use of herbicides, fungicides, and other pesticides can be lethal if they come in contact with the inoculants and have a potential hazard to the establishment and performance of the $N_2$-fixing root nodules because it can alter the morphology of root hairs and reduce nodule numbers and nitrogenase activity (Ljunggren and Martensson, 1980).

Nodule occupancy by inoculated rhizobia is also dependent on host-bacteria interactions, bacteria-bacteria interactions and many other abiotic influences (Yuhashi et al., 2000). The inoculated strains have to compete for nutrients with a rhizosphere community which is well adapted to that environment. Therefore, it is considered that inoculation with rhizobia should be performed in two different situations:

(1) in soils which are depleted or contain a low indigenous rhizobial population, and

(2) when there is an established but inefficient rhizobial population.

Very often the use of rhizobial inocula to resolve the first problem has led to the latter due to the low effectiveness of the introduced strains. The established but inefficient rhizobial population will lead to competition for nodulation of inoculated strains.

**Competitiveness of indigenous rhizobial strains for nodulation:** To achieve the $N_2$-fixation state, the rhizobia need to infect and nodulate the legume roots (Patriarca et al., 2004). However, the availability of infection sites and the total number of nodules formed are limited. Boonkerd et al. (1978) reported that inoculum strains

superior in $N_2$-fixation have been shown to fail to compete successfully with indigenous rhizobia due to the predominance of competitive, yet ineffective, indigenous soil rhizobia in nodules. Therefore, success of inoculation requires that the inoculum strain must be both highly effective in nitrogen fixation and highly competitive against the native strains in the soil (Segovia et al., 1991).

The term "competition", when used for the *Rhizobium* spp., generally implies the competition for nodule formation between the various *Rhizobium* strains from the moment these strains are present in the same environment until the moment of their presence inside the nodules (Simon et al., 1996). Triplett and Sadowsky (1992) defined competitiveness in rhizobia as the ability of a given strain which can infect a legume host and form nodules in the presence of other strains. Highly competitive indigenous strains of *Rhizobium* spp. present in agricultural soils often nodulate the plants to the exclusion of inoculated strains that are superior in $N_2$-fixation (Araujo et al., 1994).

In soybean, inoculation of soybean seed with highly effective *B. japonicum* strains does not always result in higher yields. Moawad et al. (1984) reported that a soybean rhizosphere is colonized by $10^5$-$10^7$ soybean nodulating rhizobia; however, only $10^1$-$10^2$ nodules are formed in a root, i.e., <0.01% of all the rhizobia that are close in contact with a single root can finally occupy the nodules. This situation leads to strong competition between the soil population and the inoculated rhizobia.

Triplett (1990) indicated that a high competitiveness of inoculated strains is as important as the effectiveness of symbiotic $N_2$-fixation itself. Strains that dominate nodules are considered more competitive than other strains. Therefore, the inoculants strain must be effective in its ability to fix $N_2$ with the cultivar concerned and possess

the ability to compete for nodulation of the plant with other strains of rhizobia that might be present in the soil (Triplett and Sadowsky, 1992). That strain competitiveness is influenced by the genetic diversity of both symbiotic partners (Triplett and Sadowsky, 1992) as well as the soil environment in which nodulation occurs (Streeter, 1994).

Root nodulation by an introduced rhizobial inoculant has to overcome intense competition not only from the native soil rhizobia but also other antagonistic microorganisms that colonize the rhizosphere (Boonkerd and Weaver, 1982). Moreover, a competitive and persistent rhizobial strain is not expected to express its full capacity for nitrogen fixation if limiting factors (e.g., salinity, unfavorable soil pH, nutrient deficiency, mineral toxicity, temperature extremes, insufficient or excessive soil moisture, inadequate photosynthesis, plant diseases, and grazing) impose limitations on the vigor of the host legume (Peoples et al., 1995; Thies et al., 1995).

In order for proper nodulation to occur, effective inoculation needs to happen to maintain high numbers of viable bacteria until such time as they can nodulate the roots of legume (Belachew, 2010) because they must survive long enough after sowing to nodulate the host, and to persist between cropping seasons (Boonkerd and Weaver, 1982). The survival of rhizobia on the seed surface is usually lower than on solid carriers (Bashan et al., 2002) due to the lack of protection against desiccation, high temperature, and/or toxic compounds on the seed coat. Vriezen et al. (2006) reported that the seed storage temperature after inoculation is empirically considered the most important parameter related to rhizobial survival after seed treatment. Even if an increase of provided *B. japonicum* cells improves the nodulation process, plant

nitrogen assimilation and grain yield in laboratory conditions, bacterial physiological state and its resistance to environmental stress may also be critical for its survival on seed and field conditions (Streeter, 2007).

## 2.8 Plant growth promoting rhizobacteria (PGPR) and general roles of PGPR vs plants

Numerous species of soil bacteria which flourish in the rhizosphere of plants, but which may grow in, on, or around plant tissues, stimulate plant growth by a plethora of mechanisms (Vessey, 2003) and those were termed as rhizobacteria. Those rhizobacteria positively influence plant growth and health and often referred as plant growth promoting rhizobacteria (PGPR) (Raaijmakers et al., 2009). They include a broad spectrum of bacteria such as *Azotobacter*, *Azospirillum*, *Pseudomonas*, *Acetobacter*, *Burkholderia* and *Bacillus* (Glick, 1995) and those have several reports of beneficial effects on the host plants owing to their biological control traits, plant-growth promotion, competition for nutrients and niches and induction of systematic resistance in the host plant. The beneficial effects of PGPR are attributed to improvement of plant growth and health and can be evidenced by an increase in seedling emergence, vigor, root system development and yield. Positive effects of PGPR on diverse hosts such as bean, soybean, peanut, maize, and sugarbeet are common in literatures (Vikram et al., 2007).

## 2.9 Mechanisms of PGPR

There are various mechanisms which involved in plant growth promotion by PGPR in direct and indirect means (Glick, 1995; Kloepper, 1993). Different indirect mechanisms such as induced systemic resistance, production of antimicrobial compounds, and competition for nutrients and colonization sites with pathogens have been described (Kloepper et al., 2004) while direct effects are dependent on production of plant growth regulators such as production of plant hormones like auxins, gibberellins and cytokinins, nitrogen fixation, phosphate solubilization and uptake of essential plant nutrients (Spaepen et al., 2009; Vessey, 2003) or improvements in plant nutrient uptake (Kloepper, 1993; Glick, 1995).

**Phytohormone production**: The synthesis of phytohormones such as indole-3-acetic acid (IAA) and gibberellins (GAs) is one of the several modes of action of phytostimulatory PGPR, such as α-proteobacterium *Azospirillum* (Bashan et al*.,* 2004). Another nitrogen fixing bacterium, *Azotobacter* produces growth regulators such as IAA, gibberellin, cytokinins and vitamins. These growth regulators influence plant root proliferation, respiration rate and metabolism, improving mineral and water uptake in inoculated plants (Okon and Itzigsohn, 1995).

**ACC deaminase activity**: For many plants, a burst of ethylene is required to break seed dormancy; however, following germination, a sustained high level of ethylene would inhibit root elongation. A number of PGPR such as *Agrobacterium genomovars, Azospirillum lipoferum*, *Burkholderia, Pseudomonas* and *Ralstonia solanacearum*, *Alcaligenes*, *Bacillus*, and *Variovorax paradoxus*, *Enterobacter*,

*Rhizobium*, *Rhodococcus* and *Sinorhizobium meliloti* contain the enzyme (ACC) deaminase and this enzyme can cleave the plant ethylene precursor ACC, and thereby lower the level of ethylene in a developing or stressed plant (Saleem et al., 2007). Moreover, PGPR that contain the enzyme ACC deaminase, when bound to the seed coat of a developing seedling, act as a mechanism for ensuring that the ethylene level does not become elevated to the point where initial root growth is impaired. By facilitating the formation of longer roots, these bacteria may enhance the survival of some seedlings, especially during the first few days after the seeds are planted. In addition, plants that are treated with ACC deaminase-containing PGPR are dramatically more resistant to the deleterious effects of stress ethylene that is synthesized as a consequence of stressful conditions such as flooding, heavy metals, the presence of phytopathogens, and drought and high salt (Shaharoona et al., 2006).

**Phosphate solubilization**: Most of phosphorus in soil and a large portion of soluble inorganic phosphate applied to soil as chemical fertilizer are immobilized rapidly after application due to phosphate fixation by aluminum, calcium, iron, magnesium and soil colloids (Pradhan and Sukla, 2006). Several rhizobacteria including *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Azotobacter, Micrococcus, Aereobacter, Flavobacterium* and *Erwinia* are capable of increasing availability of phosphorus to plants either by mineralization of organic phosphate or by solubilization of inorganic phosphate by production of organic acids or phosphatases (Rodriguez and Fraga, 1999) or production of organic acids and chelating oxo acids from sugars (Peix et al., 2001). These bacteria are referred to as phosphate solubilizing bacteria (PSB). Nodule forming *Rhizobium* has been

recognized as a P-solubilizer (Halder et al., 1991). Production of organic acids results in acidification of the microbial cell and its surroundings. Consequently, Pi (phosphate) may be released from a mineral phosphate by proton substitution for $Ca^{2+}$ (Goldstein, 1994). Gluconic acid seems to be the most common acid of mineral phosphate solubilization (Maliha et al., 2004). That affects the transformation of soil P and thus an integral part of the soil P cycle (Chen et al., 2006). Fungi are also P-solubilizers and increase the yield of crops (Adesemoye and Kloepper, 2009).

**Phytopathogen control**: The use of natural PGPR strains in plant frontline defense may offer a practical way to deliver immunisation. PGPR have been reported to increase plant resistance to fungal, bacterial and viral diseases, insects and nematodes. Mode of actions of PGPR for biological control includes:

(1) antibiotic synthesis (Hebbar et al., 1992);

(2) secretion of iron binding siderophores to obtain soluble iron from the soil and provide it to a plant, making it less available to certain members of the native pathogenic microflora (Subba Rao, 1993) and thereby deprive fungal pathogens in the vicinity of soluble iron (Loper and Buyer, 1991);

(3) production of low molecular weight secondary metabolites, such as hydrogen cyanide, with antifungal activity (Dowling and O'Gara);

(4) production of enzymes including chitinase, β-1,3-glucanase, protease, or lipase, which can lyse some fungal cells (Chet and Inbar, 1994);

(5) production of extracellular lytic enzymes (Fridlender et al., 1993);

(6) out-competing phytopathogens for nutrients and niches on the root surface (Loper et al., 1997);

(7) lowering the production of (pathogen) stress ethylene in plants with the enzyme ACC deaminase (Glick et al., 1998, Penrose et al., 2001);

(8) manipulation of the host plant's physical and biochemical properties (induced systemic resistance (ISR)) in which non-infected parts of previously pathogen-infected plants become more resistant to further infection (Pieterse et al., 2003).

PGPR as a component in integrated management systems in which reduced rates of agrochemicals and cultural control practices are used as biocontrol agents (Kloepper et al., 2004). Several species of *Pseudomonas*, namely *P. fluorescens*, *P. putida*, *P. cepacia* (*Burkholderia cepacia*) and *P. aeruginosa* have been reported as potential biocontrol agents of several phytopathogenic fungi (Thomashow et al., 1990). Antifungal activity of *Azotobacter* strains is also common (Brown, 1974). Inoculant development has been most successful to deliver biological control agents of plant disease i.e. organisms capable of killing other organisms pathogenic or disease causing to crops. At present, there are fewer than 20 different biocontrol PGPR strains that are commercially available (Penrose and Glick, 2003).

**Root colonization**: The colonization of plant roots by the introduced bacteria is an important step in establishing an effective plant-bacteria interaction (Schippers et al., 1987). The presence of flagella (de Weger et al., 1987) and *O*-antigens of lipopolysaccharide (de Weger et al., 1989), and the ability to synthesize amino acids (Simons et al., 1997) are important bacterial traits for effective root colonization. *Pseudomonas* spp. and *Azospirillum* strains are also known as good colonizers of many crops. A two-steps attachment mechanism is proposed for plant root colonization by *Azospirillum*. In the first step, *Azospirillum* rapidly and weakly binds

to the root surface; this is mediated by the polar flagellum (Croes et al., 1993). The second step occurs in a high C/N ratio medium and is mediated by bacterial polysaccharide, which helps the bacteria to become firmly attached to the plant root (Michiels et al., 1991).

## 2.10 PGPR and inoculation with PGPR as an alternative

PGPR can affect plant growth and yield in a number of ways, and thus, they are also referred as yield increasing bacteria (YIB). Enhancement of vegetative and reproductive growth by PGPR is documented in a range of crops like cereals, pulses, ornamentals, vegetables, plantation crops and some trees. However, very small portions (about 2-5%) of the total rhizobacterial community are PGPR (Antoun and Kloepper, 2001). Treatments with PGPR increase germination percentage, seedling vigor, emergence, plant stand, root and shoot growth, total biomass of the plants, seed weight, early flowering, grains, fodder and fruit yields etc., (Ramamoorthy et al., 2001). Therefore, the application of PGPR in plant cultivation is one of the most promising methods for increasing agricultural productivity and the efficiency of soil pollutant biodegradation (Lugtenberg et al., 2002).

Several PGPR inoculants that currently commercialized seem to promote growth through at least one mechanism; suppression of plant disease (termed Bioprotectants), improved nutrient acquisition (Biofertilizers), or phytohormone production (Biostimulants) (Zhang et al., 1996). These products are mainly applied as seed treatment, soil amendment or soil drench at the time of seeding or immediately after transplanting, to promote plant growth and effectively suppress several diseases in a number of crops (Kloepper et al., 2004).

Various species of bacteria like *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Bacillus*, and *Serratia* those have been applied to various crops to enhance growth, seed emergence and crop yield, and reported to enhance the plant growth, and some have been commercialized (Herman et al., 2008). It is also crucial for the microbial inoculants used as biofertilizers, biocontrol agents, phytostimulators, and bioremediators (Lugtenberg et al., 2001).

## 2.11 Co-inoculation of *Rhizobium*/*Bradyrhizobium* and PGPR

In recent years, several PGPR-based products became commercially available in many countries, and more are currently under development (Choudhary and Johri 2009). Because of the effective properties of PGPR, those bacteria have potential to be use in combination with rhizobial isolates and obtained the positive responses in several research on different leguminous crops (Table 1).

**Table 1.** Co-inoculation effects of *Rhizobium*/*Bradyrhizobium* and PGPRs on leguminous plants

| Sr No. | Crop | Co-inoculation | Positive effect | References |
|---|---|---|---|---|
| 1 | Soybean | *Bradyrhizobium japonicum* and *Azospirillum* or PGPR | increases in nodulation, nitrogenase activity, and plant growth | Li and Alexander, 1988 |
| 2 | different forage and grain legumes | *Rhizobium* with *Azotobacter* or *Azospirillium* strains | favorable influence of the free-living diazotrophic bacteria on nodule weight and number, $N_2$-fixation, plant dry-matter accumulation and N content | Yahalom et al. 1987 |
| 3 | Chickpea | *Azospirillum brasilense* and *Rhizobium* strains | increase in grain yield, nodule dry matter, and nitrogenase activity | Rai, 1983 |
| 4 | Chickpea | *Rhizobium* and *Bacillus* strains or *Rhizobium* and *Pseudomonas* strains | stimulate the plant growth, nodulation and nitrogen fixation | Parmar and Dadarwal,1999 |
| 4 | Alfalfa | *Pseudomonas* sp. with rhizobia | enhance nodulation, $N_2$-fixation, plant dry matter and grain yield | Derylo and Skorupska,1993 Dashti et al., 1997 |
| 5 | Clover | | | |
| 6 | Pea | | | |
| 7 | Soybean | | | |
| 8 | Common bean | *Rhizobium tropici* and *Paenibacillus polymyxa* | greater growth and nitrogen content | Figueiredo et al., 2008 |
| 9 | Soybean | Inoculated with crude or formulated metabolites, or with cells *Bacillus subtilis* | increase the contribution of the biological nitrogen fixation processes | Araújo and Hungria, 1999 |
| 10 | Faba bean | *Rhizobium leguminosarum* bv. *viceae* with *Azotobacter* and *Azospirillum* strains | changes on the concentration, content and/or distribution of several mineral nutrients in roots and/or shoots of plants | Rodelas et al., 1999 |

## 2.12 Role of PGPR in co-inoculation with rhizobia on legume nodulation and plant growth promotion

Generally, PGPR improve nodulation and that enhanced nodulation allows higher nitrogenase activity resulting in superior dry matter yield. PGPR may increase the efficiency of *Rhizobium* inoculation in legumes through the production of antibiotics, siderophore, and certain enzymes. They also enhance the infection sites for *Rhizobium* by colonizing the root surface (Contesto et al., 2008) which may have contributed to increase the formation of nodule primordia and early nodule development. Plant root flavonoids are the inducers of nodulation gene (nod genes) expression in *Rhizobium* (de Rijke et al., 2006). Therefore, co-inoculation with PGPR promotes root hair growth and enhances root flavonoids secretion (Dardanelli et al., 2008) which is needed for early events of nodule formation. In legume root nodules, IAA produced by most of PGPR activates the enzyme $H^+$-ATPase, which is fundamental for energy production in the nodules (Rosendahl and Jochimsen, 1995).

Compared to single *Rhizobium* inoculation, co-inoculation of *Rhizobium* spp. and *Azospirillum* spp. can enhance the number of root hairs, the amount of flavonoids exuded by the roots and the number of nodules formed (Remans et al., 2008). Moreover, the presence of azospirilla in the rhizosphere was reported to activate the hydrolysis of conjugated phytohormones and flavonoids in the root tissue, thus leading to the release of compounds in their active forms (Dobbelaere and Okon, 2007).

In the case of increased nodulation, the significant increase in root hairs number and length in the presence of the *Rhizobium-Azospirillum* mixture suggested that *Azospirillum* can create additional infection sites, which can be occupied later by

rhizobia (Tchebotar et al., 1998). Bellone et al. (1997) also reported that the young and appropriate new roots are one of the key factors for sufficient infectivity by *Bradyrhizobium* in most of the legumes, because it becomes attached to new roots and root hairs, producing root hair curling followed by infection thread development for nodulation. This hypothesis is strengthened by a further study using a *gus*-reporter gene (Tchebotar et al., 1998), in which an equal mixture of *Azospirillum lipoferum*-*R. leguminosarum* bv. *trifolii* increased nodulation in clovers, and *Azospirillum* was observed colonizing tap root, root hairs and sites near or on the nodules.

In the case of *Azotobacter*, azobacterization with auxin biosynthesis might have provided improved colonization niches through root proliferation to introduced *Rhizobium* (*Mesorhizobium*) in the rhizosphere of chickpea and which reflecting-in better nodulation and yield as compared to their individual inoculations (Qureshi et al., 2009). Therefore, co-inoculation of legumes with symbiotic and free living microbes like *Azotobacter*, *Azospirillum* and *Acetobacter* has received great attention because free-living diazotrophs increase the lateral roots and root hair density resulting in more infection sites for rhizobia and thus enhancing the $N_2$-fixing ability of legumes (Parmar and Dadarwal, 1999). The root length and mass enhancement owing to the changes in the root system architecture resulted in increased root density, root hairs and surface area due to interaction of microbes with plant roots. This increase in root surface area resulted in better acquisition of nutrients (Qureshi et al., 2011).

Chebotar et al. (2001) suggested that *Pseudomonas fluorescens* strain 2137 could enhance nodulation by the release of growth promoting substances that stimulate *B. japonicum*. In *P. fluorescens*, growth promotion mechanism 2,4-diacetyl-

phloroglucinol (DAPG) can act as a plant hormone-like substance, including morphological changes in the plant that can lead to enhanced infection and nodulation by *Rhizobium* in pea (de Leij et al., 2002).

Some rhizobacterial strains promote legume nodulation and nitrogen fixation by producing flavonoid-like compounds and/or stimulating the host legume to produce more flavonoid signal molecules (Parmar and Daderwal, 1999). Lian et al. (2001) observed that a strain of *Bacillus circulans* produces a chemical compound analog to the nod factor of *B. japonicum*. This compound causes root hair deformation activity on soybean. Other reasons which increase in plant growth in combined inoculations of *Rhizobium* and *Azospirillum* may be ascribed for enhanced N and P nutrient uptake or it might be due to synthesis and oxidation of plant growth promoting substances like IAA and GA that are known to enhance the shoot elongation, root elongation and plant growth (Spaepen et al., 2007).

## 2.13 Soil microbial community structures

Soils cover almost all of the terrestrial area on earth and have an indispensable ecological function in the global cycles of carbon, nitrogen and sulfur. Due to their physico-chemical complexity with many microniches, they teem with biodiversity, both phylogenetically and functionally. A single gram of soil has been estimated to contain thousands to millions of different bacterial, archaeal and eukaryotic species (Torsvik et al., 2002) interwoven in extremely complex food webs. Communities of soil microbes carry out a multitude of small-scale processes that underlie many environmentally important functions (Fierer et al., 2007).

A higher density and a higher number of microbial species are always measured in the rhizosphere compared with the phyllosphere or in the endorhiza compared with the endosphere. Composition, abundance, and dynamics of the microbial community in the rhizosphere play an important role and may have a positive or negative influence on plant growth (Copenhagen, 1997). Both the bacterial and fungal communities in soil play important roles in soil functioning, for instance, in key steps of mineralization processes. Both groups of organisms are thus important for the growth and development of plants (crops and trees), and also for the maintenance of soil structure (Uroz et al., 2007).

## 2.14 Active shift of rhizosphere soil microbial community structures by PGPR

PGPR must be rhizospheric competent, able to survive and colonize in the rhizospheric soil (Cattelan et al., 1999). Among the microorganisms, bacterial communities respond quickly to environmental changes because of their high growth rate and short life span (Øvrea°s, 2000). Changes in soil microbial biomass are associated with shifts in the microbial community structure, in particular the ratio of bacteria to fungi (Bardgett et al., 1999). Composition, abundance, and dynamics of the microbial community in the rhizosphere play an important role and may have a positive or negative influence on plant growth (Lynch, 1990). Microbial soil characteristics may indicate changes in resource availability, soil structure, or pollution and represent one important key to understanding impacts of environmental and anthropogenic factors (DeLong and Pace, 2001).

## 2.15 Factors affecting the rhizosphere soil microbial community structures

Soil microorganisms play a fundamental role in driving carbon turnover and nutrient cycling in all terrestrial ecosystems. Rhizosphere microbial communities perform fundamental processes that contribute to nutrient cycling, healthy root growth, and promotion of plant growth (Buchenauer, 1998). Changes in land use and cover, management and plant productivity may influence the biomass, structure, and functional processes of soil microorganisms through modification of the quantity and types of organic matter inputs (Steenwerth et al., 2003).

Modifications in the soil-plant-microorganisms partnership bring about intricate reaction mechanisms. However, effects on rhizosphere microorganisms when a PGPR is introduced at high levels in the rhizosphere may be depended on interactions within and between indigenous populations. Unfortunately, the interaction between associative PGPR and plants can be unstable. In relation to the soil-plant-environment background, certain groups may be enhanced, while others may be inhibited, or the introduced PGPR may not affect population structure (Dobbelaere et al., 2003).

**Plant Factors:** Plant factors that have an influence upon microbial communities include plant age (Herschkovitz et al., 2005a, b), plant species or even plant genotype (Dalmastri et al., 1999) and root exudates (rhizodeposition) (de Weert et al., 2002). Plant roots release a wide variety of compounds into the rhizosphere that create unique microenvironments for soil microorganisms. The root surrounding rhizosphere contains compounds such as free amino acids, proteins, carbohydrates, alcohols,

vitamins, and hormones which are important sources of nutrients for the microorganisms present in the rhizosphere and attract a great diversity and population density of microorganisms (Han et al., 2005). Plant species are also importance because of differences in root exudation and rhizodeposition in different root zones (Brimecombe et al., 2001). Since bacteria respond differently to the compounds released by roots, different compositions of root exudates are believed to explain the plant-specific bacterial communities in the rhizosphere (Smalla et al., 2001).

**Soil type:** Soil type is another important factor in the determination of rhizosphere bacterial communities (Kowalchuk et al., 2000), as different soils display different particle size distribution, pH, aeration, and physico-chemical characteristics that can affect bacterial communities either directly, by providing a specific habitat for selecting specific bacteria, or indirectly, by affecting plant root exudation (Garbeva et al., 2004). Disturbances through agricultural treatments such as soil tillage, fertilization, and plant protection may favor certain species, resulting in reduced complexities of these communities (Torsvik et al., 2002).

Agricultural treatments have been reported to influence soil microbial community structures (Widmer et al., 2006) and to decrease soil bacterial diversity (Torsvik et al., 2002). Soil carbon inputs in a variety of forms can significantly impact soil microbial biomass, composition, and activities (Brant et al., 2006) and that shifts in soil microbial community structure may occur with changes in substrate types (Fontaine et al., 2004).

## 2.16 Approach for soil microbial community structure analysis

Only a small percentage of the indigenous soil bacteria are culturable from environmental sample. Evaluation of changes in the structure of bacterial communities using only culturing methods is inadequate because those can analyze only a minor fraction of the microbial community. Therefore, the use of the microbial diversity of soil to ensure environmental sustainability is a major challenge in agriculture. Recently, culture-independent methods have become commonly applied for studying the composition of bacteria in samples (Tringe and Hugenholtz, 2008) such as (1) Methods using nucleic acids (gene); DNA reassociation analysis, DNA (G+C%) density fraction analysis, cross DNA hybridization analysis, PCR-amplified DNA clone library method, and various genetic fingerprint analyses (denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), amplified ribosomal DNA restriction analysis (ARDRA), single strand conformation polymorphism (SSPC), ribosomal intergenic spacer analysis (RISA), and random amplified polymorphic DNA (RAPD)) (Ranjard et al., 2000a), (2) methods using cellular components (biomarkers): phospholipid fatty acid (PLFA) analysis (Arao et al., 1998), quinine profile analysis (Fujie et al., 1998), and (3) methods using carbon source- assimilating property: diversity analysis based on the carbon source utilization patterns of isolates (Yokoyama, 1996), and community-level physiological profile (CLPP) analysis (Konopka et al., 1998). These techniques allow the analysis of only a minor fraction of the microbial community.

The determination of soil microbial biomass often is combined with a characterization of the physiological status of the microorganism community. Characterization of other biomass parameters such as the relation to respiration,

energetic state, and soil nutrients cannot be used to describe changes in the microbial communities, in the diversity of the community or in the activities of single species and physiological groups. In these cases, specific biochemical constituents, 'signature chemicals' which are restricted to certain species or groups can be used. In the serological approach, polyclonal as well as monoclonal antibodies are used to investigate population dynamics of microbes in soil. As compared to polyclonal antisera, monoclonal antibodies offer the lowest level of cross-reaction to non-target organisms, which is very important in complex systems (Bohlool and Schmidt, 1980).

DNA fingerprinting analyzes part of the genetic information, mostly the ribosomal operon, contained in nucleic acids directly extracted from environmental samples. Simple and reliable methods to be rapidly investigated even when there are a large number of samples are rRNA intergenic spacer analysis (RISA), and automated RISA (ARISA) method. Due to the high resolution of the gels and the high sensitivity of fluorescence detection, the number of peaks detected is much higher on ARISA profiles than on RISA profiles. Similarly, differences in the intensity of the bands can be estimated precisely, which allows a finer comparison of the profiles. However, this level of sensitivity might have some drawbacks because it may introduce a variability within profiles that has no biological origin (Ranjard et al., 2001).

The diversity of target genes, such as the 16S rRNA or 18S rRNA genes, can be assessed by means of molecular fingerprinting techniques such as DGGE (Heuer and Smalla, 1997) in which DNA fragments obtained after PCR amplification of target genes from complex microbial communities are separated according to their sequence (G+C content). It was originally developed to detect specific mutations within genomic genes due to one base mismatch. The separation of the different

DGGE bands depends on the melting behavior of the PCR product and not on the size of the fragment. These methods are useful for simultaneous analysis of large numbers of samples and the comparison of microbial communities based on temporal and geographical differences an essential requirement for ecological studies (Myers et al., 1985).

Furthermore, the method enables sequence data to be obtained on the DNA of dominant species from individual bands. The advantage of this technique is that DGGE bands of interest can be excised from the gel and further analyzed by cloning and sequencing (Nakatsu, 2007). Although this tool has many advantages, as mentioned above, a few biases derived from PCR and heterogeneity of copy number of 16S rDNA among bacterial species have been reported (Ranjard et al., 2000b).

Changes in microbial community structures may not necessarily lead to altered diversities, because changes of some taxonomic groups may be compensated by changes of others. It has been suggested that, for instance, species richness may exhibit less variability in response to environmental factors than species composition (Ernest and Brown, 2001).

## 2.17 References

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# CHAPTER III

# CO-INOCULATION EFFECTS OF *Bradyrhizobium japonicum* WITH PLANT GROWTH PROMOTING RHIZOBACTERIA UNDER SOYBEAN-NODULATING BRADYRHIZOBIA-NON-ESTABLISHED SOIL CONDITIONS

## 3.1 Abstract

Co-inoculation of rhizobia with PGPR plays an important role in cultivation of leguminous plants for both promotion of nodulation and plant growth. In this study, rhizobacteria were screened for their capacity to promote the nodule formation when co-inoculated with *Bradyrhizobium japonicum* on soybean under aseptic condition. The obtained rhizobacteria were further screened in soybean-nodulating bradyrhizobia-free soils to evaluate their co-inoculation effects on enhancement of soybean nodulation, plant growth and on rhizosphere soil microbial community structures. By co-inoculation either of *B. japonicum* strain CB 1809 or USDA 110 under pot conditions, *Azospirillum* sp. gave more benefits in nodulation and plant growth than *Bacillus solisalsi* did. Moreover, *Azospirillum* sp. co-inoculated with either *B. japonicum* CB 1809 or USDA 110 under field conditions gave 32.23% and 16.85% of nodulation, 26.51% and 18.83% of nodule dry weight, and 23.65% and 34.92% seed yield increasing over single inoculation of CB 1809 and USDA 110,

respectively. Denaturing Gradient Gel Electrophoresis (DGGE) and Principle Component Analysis (PCA) in both pot and field experiments were shifted by plant growth stages but not by bacterial inoculation. In contrast, neither inoculation of tested bacteria nor plant growth stages shifted the rhizosphere soil fungal community structures.

## 3.2 Introduction

*Bradyrhizobium japonicum* forms a symbiotic relationship with soybean (*Glycine max*) and gives an increase in nodulation which leads to increases in plant fresh weight, seed protein, and seed yield. However, not all the rhizobial inoculation gives positive response to nodulation because a variety of biotic or abiotic factors affects nodulation of plants. There were many approaches which tried to overcome this problem. Among them, co-inoculation of rhizobia with proper 'Plant Growth Promoting Rhizobacteria' (PGPR) is one of the popular methods. For instance, inoculation with mixed culture of *B. japonicum* containing either *Azotobacter vinelandii* or *Azospirillum brasilense* gave increased yields in soybean (Crossman and Hill, 1987; Herschkovitz et al., 2005). Improvement in crop production of groundnut and mungbean due to *Rhizobium* and *Azotobacter* inoculation has been reported by Sethi and Adhikary (2009). *Pseudomonas fluorescens* showed the best compatible with *B. japonicum* among tested beneficial microorganisms (Belkar and Gate, 2012). Anandaraj and Leema Rose Delapierre (2010) reported that bacterization of green gram with the composite inoculants of *Rhizobium* sp., *Pseudomonas fluorescens* and *Bacillus megaterium* were highly beneficial for enhancing the plant growth and yield of green gram besides effecting a reduction in the cost of inorganic fertilizers.

Moreover, co-inoculation of Phosphate Solubilizing Bacteria (PSB) *Pseudomonas* sp. and *B. japonicum* (TAL 379) significantly increased nodulation, plant total N and P uptake, seed yield and yield components of soybean over negative control and chemical fertilizers (Argaw, 2012).

Although the inoculation of plants with PGPR may occur naturally, it is mainly an artificial agricultural procedure. To commercialize PGPR, 'effective strategies' for initial selection and screening of rhizobacterial isolates are required (Nelson, 2004) because exploitation of PGPR as biocontrol or biofertilizer inoculants has been shown to be hampered by inconsistent results at the field scale (Mark et al., 2006). Moreover, soil is considered to be the richest environment, with a high diversity of microorganisms (Fierer and Jackson, 2006), and PGPR that have been added to soil or seeds to improve plant growth and/or health will also modify the composition of the resident bacterial community of the rhizosphere.

The interaction of $N_2$-fixing bacteria with other bacteria can inhibit or promote their diazotrophic activity (Isopi et al., 1995). In this study, selection of native PGPR strains which suppose to be good strains in Thailand soil was conducted with the main purpose on co-inoculating the soybean with bradyrhizobia. In addition, the changes of microbial community structures of soybean rhizosphere by this co-inoculation under soybean-nodulating bradyrhizobia-free soil conditions were also investigated.

## 3.3 Materials and Methods

### 3.3.1 Bacterial strains, media, and growth conditions

Two *B. japonicum* strains (CB 1809 and USDA 110) and a total of 200 rhizobacterial isolates were used in this study. CB 1809 was supplied by Department of Agricultural Research (DAR), Myanmar. *B. japonicum* strain USDA 110 and rhizobacterial isolates were sourced from School of Biotechnology Laboratory, Suranaree University of Technology (SUT), Nakhon Ratchasima, Thailand (Piromyou et al., 2011). Bradyrhizobia and rhizobacterial isolates were maintained on Yeast Extract Mannitol agar (YEM) medium (Appendix 1) (Vincent, 1970) and LG (N-free) medium (Appendix 2) (Hirschi et al., 1991), respectively by periodically transferring and storing those isolates in the refrigerator for further studies.

### 3.3.2 Antagonistic test between *B. japonicum* and rhizobacteria

*B. japonicum* and rhizobacteria were cultured in YEM and LG broth, and shaken at 180 rpm at room temperature (28±2°C) for 7 days and 2 days, respectively. To determine the antagonistic effects of rhizobacteria on bradyrhizobia, each of bradyrhizobial broth cultures (containing 1 x $10^8$ colony forming unit (cfu) $ml^{-1}$) was separately swept on duplicate YEM agar plates by using cotton stick, and incubated for two days. Twelve rhizobacterial isolates were spotted onto a lawn of bacterial cells per plate and incubated to observe their antagonisms. Only the *Bradyrhizobium* non-inhibitors which did not give the clear zone were selected for co-inoculation with *B. japonicum* on soybean.

Soybean seeds (*Glycine max*, Chiang Mai 60) obtained from Department of Agriculture (DOA), Thailand were pre-sterilized, pre-germinated, and grown into

the growth media (vermiculite) under aseptic conditions in sterilized Leonard's Jar (Leonard, 1943) at the rate of 3 seeds $jar^{-1}$. Each seed was inoculated with 1 ml bacterial culture ($10^8$ cfu $ml^{-1}$) of *B. japonicum* alone (CB 1809 or USDA 110) or co-inoculated by mixing of selected rhizobacterial and bradyrhizobial cultures in a ratio of 1:1 (v/v). Non-inoculated treatment was also included as a control. The experiment was laid out in a Completely Randomized Design (CRD) with three replications. Plants were cultivated on a growth shelf at 27/20°C under 16/8 h light/dark photoperiod. The N-free nutrient solution (Appendix 3) (Broughton and Dilworth, 1971) in the lower part was supplemented whenever necessary. At 21 days after inoculation (DAI), two rhizobacterial isolates with better nodulation were selected for co-inoculation under pot conditions.

### 3.3.3 Characterization of selected bacteria

#### 3.3.3.1 Acetylene reduction assay (ARA)

The selected bradyrhizobia and rhizobacteria were cultured in 5 ml of LG (N-free) broth in 21 ml test tube and incubated for 7 and 2 days, respectively at 28±2°C. Ten percentage (v/v) of gas phase in the headspace was replaced with acetylene and further incubated at 28±2°C for 24 h, and the free-living $N_2$-fixing activity was examined by acetylene reduction assay (ARA) following Hardy et al. (1968). Ethylene production was measured by gas chromatograph (GC) with a flame ionization detector equipped with PE-Alumina column (50 m x 0.32 mm x 0.25 μm) (Perkin Elmer, USA). Standard curve of ethylene was constructed by varied concentration of pure ethylene following Nuntagij et al. (1997).

#### 3.3.3.2 Indole-3-acetic acid (IAA) production

IAA production of selected bacterial strains was colorimetrically determined as described by Fukuhara et al. (1994). Pure IAA at different concentrations of 0, 10, 20, 50, 100, 150, and 200 μM were used as a standard.

After completion of ARA and IAA assays, total protein concentrations of the concerned cell suspensions were determined using Lowry's method (Lowry et al., 1951).

#### 3.3.3.3 Identification of selected bacteria

The chromosomal DNA of the selected rhizobacterium (Isolate 3) was extracted following Prakamhang et al. (2009) and 16S rRNA gene was amplified by using the primer pair fD1 and rP2 (Weisburg et al., 1991). The resulted PCR product was purified by using the QIA quick PCR purification kit (Qiagen, Hilden, Germany) and ligated into the pGEM-T Easy Vector System (Promega, USA) for further transformation into *Escherchia coli* DH5α competent cells by following the manufacturer's instructions. DNA sequencing was performed by MACROGEN Company (Korea) and the most closely related sequences were obtained from the NCBI database.

### 3.3.4 Single and co-inoculation effects of selected rhizobacteria and *B. japonicum* strains

The experimental soils used in both pot experiment and the field experimental sites were selected from non-soybean growing area of Muang District, Nakhon Ratchasima, Thailand (14° 52' 10" N and 102° 00' 42.24" E) which had no history of any leguminous crops cultivation.

#### 3.3.4.1 Quantification of indigenous soybean-nodulating bradyrhizobia

Soil samples were collected from 15 randomized sites of the experimental field. The amount of indigenous soybean-nodulating bradyrhizobia present in experimental soil samples was determined by a modification of the plant infection test using the most probable-number (MPN) technique (Vincent, 1970). Plants were grown on a growth shelf at 27/20°C under 16/8 h light/dark photoperiod. MPN estimations based on nodulation were determined at three weeks after inoculation.

### 3.3.5 Pot experiment

The soils were amended with eucalyptus compost, $P_2O_5$, $K_2O$, $CaSO_4$. $2H_2O$, and $CaCO_3$ at the rate of 37.50, 0.75, 0.75, 15.00, and 2.50 g Kg $soil^{-1}$, respectively. The physicochemical analysis of amended soil showed loamy sand in texture, having a pH 5.25, 0.39% organic matter, 4.03 and 34.5 ppm of available P and exchangeable K, respectively. Nine kilograms of amended soils were filled into pots (20 cm diameter x 20 cm height), and ten pre-sterilized and pre-germinated soybean seeds (Chiang Mai 60) were sown in each pot.

*B. japonicum* strains (CB 1809 and USDA 110), *Azospirillum* sp., (AB 114190), and *Bacillus solisalsi* Isolate 3 were cultured as described before, and single or mixed bacterial broth culture was inoculated onto seed ($10^8$ cfu $ml^{-1}seed^{-1}$). The treatments included 1- 4) single inoculation of each of *Azospirillum* sp., *B. solisalsi* Isolate 3, USDA 110, and CB 1809, 5-7) co-inoculation in 1:1 (v/v) of CB 1809 with each of USDA 110, *Azospirillum* sp. and *B. solisalsi* Isolate 3, 7-9) co-inoculation in 1:1 (v/v) of USDA 110 with each of *Azospirillum* sp. and *B. solisalsi* Isolate 3,

10-12) co-inoculation in 1:1:1 (v/v/v/) of CB 1809, USDA 110, and either *Azospirillum* sp. or *B. solisalsi* Isolate 3, 13) combined inoculation in 1:1:1:1 (v/v) of all tested bacterial cultures, and 14) bulk soil (no planted and non-inoculated control).

The pots were laid out in a CRD design with three replications. Plants were thinned down to uniformity (six plant $pot^{-1}$) and watered by tap water whenever necessary. Regular agricultural practices were done except pesticide spraying. Plants were sampled and nodule number, nodule dry weight, and biomass dry weight (dried at 70ºC) were recorded at 30 and 45 DAI. Statistical significance was determined by analysis of variance (Steel et al., 1980) and means were compared by the Duncan's Multiple Range Test (DMRT) ($p \leq 0.05$) (Duncan, 1955). Based on this experiment, the most effective rhizobacteria was selected to evaluate its potential under field conditions.

### 3.3.6 Field experiment

Before sowing, the field soil was fertilized with 50 kg $ha^{-1}$ of each $P_2O_5$ and $K_2O$ fertilizers. The soil was sandy soil in texture, having pH 6.41, 0.39% organic matter, and available P and exchangeable K was 4.78 and 70.64 ppm, respectively. Each subplot size was 2 and 3 $m^2$ in size with four rows. The experiment was arranged in a Randomized Complete Block Design (RCBD) with three replications. The treatments consisted of non-inoculated control, single inoculation with USDA 110, CB 1809, and *Azospirillum* sp. alone, and co-inoculated in 1:1 (v/v) ratio of *Azospirillum* sp. and each of CB 1809 and USDA 110. Soybean seeds (Chiang Mai 60) were inoculated with bacterial broth cultures (approximately $10^6$ bacterial cells $seed^{-1}$) just prior to sowing.

During the experiment, regular agricultural practices were done as needed. At 30, 45, and 70 DAI, five soybean plants per each plot were randomly sampled for assessment of nodulation and plant growth parameters. At 70 DAI, the dried plant materials were analyzed for dry matter and total plant nitrogen percent. Soybean yield and yield components were determined from a random sample of 10 plants from two inner rows per plot at maturity (90 DAI). Statistical significance was determined as described in pot experiment.

### 3.3.7 Denaturing gradient gel electrophoresis (DGGE) and principle component analysis (PCA) from pot and field experiments

Total genomic DNAs of selected bacteria which were used for inoculation in pot and field experiments were extracted following Prakamhang et al. (2009) and kept at -20$^{o}$C before using as the marker. Both eubacterial and fungal community structures were evaluated from pot experiment and only eubacterial community structure was analyzed in field experiment at 0, 7, 14, 30, and 45 DAI. Soil microbial DNAs were directly extracted from 0.5 g rhizosphere soils by using the Ultra Clean Soil DNA kit (MoBio Laboratories, Solana Beach, Califonia, USA) following the manufacturer's instructions. Eubacterial 16S rRNA (V6-V8 variable regions, ~ 400 bp) and fungal 18S rRNA (~1,650 bp) gene fragments were amplified by using universal primers F984 and R1378 (Heuer et al., 1997) and fungus-specific primers NS1 and FR1 (Oros-Sichler et al., 2006), respectively. A GC-clamp (Costa et al., 2006) was added to the 5'end of the forward primers F984 and NS1 to prevent the complete melting of PCR products during separation in the denaturing gradient gel.

PCR products were subjected separately for DGGE analysis by using a Dcode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA). About 45 µl of PCR products were loaded onto 1 mm thick (20 x 20 cm) gel with 6% (w/v) polyacrylamide gel (37.5:1 of Acrylamide: Bis-acrylamide, Bio-Rad Laboratories, Inc.) prepared with a linear denaturing gradient ranging from 40-70% denaturant (100% denaturant consisted of 40% (v/v) formamide and 7M urea) and 10% (w/v) polyacrylamide gel with 18-43% denaturant for 16S rRNA and 18S rRNA, respectively. PCR products from inoculated bacteria were loaded at the both left and right sides of the sample lanes as markers.

DGGE was performed in 1x TAE buffer at 60°C with constant voltage of 75V for 10 min and thereafter 110V for 18 h for eubacteria PCR and at 180 V for 16 h for fungal PCR. The gels were stained with SYBR Green (3µl in 15µl 1x TAE buffer) for 30 min and rinsed for 3 min in running water before photographing. DNAs from excised bands of interest in DGGE gels were eluted by incubation in 30 µl dd$H_2O$ at 4°C overnight. Supernatant (~0.5 µl) was used as a template for PCR amplification as described above by using with the same primer pair without a GC-clamp. The PCR products were purified by using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) followed by sequencing and analyzing of DNA as described above.

Cluster analysis and principle components analysis (PCA) were performed according to the presence and absence of bands occurred in DGGE gels based on Unweighted Pair Group Method with Arithmetic Means (UPGMA) algorithms by the NTSYSpc (2.2, Exeter Software, USA) (Rohlf, 2000). Based on the DGGE results, the Shannon index (H') (Shannon and Weaver, 1963) was calculated according to the following equation:

$H' = -\sum P_i \log P_i$

where $P_i$ is the proportion represented by each DGGE band relative to the total number of bands. The indices obtained were statistically analyzed as described for other univariate data.

## 3.4 Results

### 3.4.1 Antagonistic test and screening of rhizobacterial isolates for co-inoculation with *B. japonicum* strains

Totally 152 out of 195 tested rhizobacterial isolates were detected as '*Bradyrhizobium* non-inhibitors' and those were screened for co-inoculation with *B. japonicum* on soybean under controlled conditions. Among them, Isolates 1, 3, 13 and 15 showed an increase in nodule numbers when each was co-inoculated with either CB 1809 or USDA 110; however, those numbers were not significantly higher than that of individual bradyrhizobial inoculation (data not shown). Therefore, five additional rhizobacterial strains which did not inhibit against tested bradyrhizobia in *in vitro* cultures; namely, *Bacillus* sp. SUT 1, *Pseudomonas* sp. SUT 16 and SUT 19, which are prominent in most of the experimental research at Laboratory of School of Biotechnology, SUT (Piromyou et al., 2011), *Azotobacter* sp., and *Azospirillum* sp. which are being commercialized as PGPR inocula for various crops cultivation by Suranaree University of Technology (Teaumroong et al., 2009), were selected to be added in screening test. Among the tested isolates, the *Bacillus* sp. SUT 1 gave the maximum nodule number when co-inoculation with CB 1809, whereas, it decreased to minimum nodule formation when co-inoculated with USDA 110. Out of the nine rhizobacterial isolates, co-inoculation of *Azospirillum* sp. or Isolate 3 with either CB 1809 or USDA 110 gave significantly higher nodule numbers than bradyrhizobial

single inoculation, and thus those two isolates were selected for further experiments (Table 2).

**Table 2.** Single or co-inoculation effects of *B. japonicum* strain (CB 1809 or USDA 110) and promising rhizobacterial isolates on nodulation of soybean (Chiang Mai 60) under controlled environmental conditions at 21 DAI

| **Treatment (Bacterial isolate no.)** | **Nodule No. plant$^{-1}$ CB 1809** | **Nodule No. plant$^{-1}$ USDA 110** |
|---|---|---|
| Isolate 1 | $14.3^{cd} \pm 2.9$ | $14.8^{ab} \pm 2.3$ |
| Isolate 3 | $24.0^{ab} \pm 2.7$ | $20.1^{a} \pm 2.7$ |
| Isolate 13 | $19.6^{bc} \pm 2.7$ | $11.8^{bc} \pm 1.1$ |
| Isolate 15 | $14.2^{cd} \pm 2.6$ | $14.8^{ab} \pm 4.7$ |
| SUT 1 (*Bacillus* sp.) | $26.8^{a} \pm 3.3$ | $6.2^{c} \pm 2.7$ |
| SUT 16 (*Pseudomonas* sp.) | $16.9^{cd} \pm 4.3$ | $9.3^{bc} \pm 0.9$ |
| SUT 19 (*Pseudomonas* sp.) | $14.4^{cd} \pm 1.7$ | $18.9^{a} \pm 2.8$ |
| *Azotobacter* sp. | $17.4^{cd} \pm 2.7$ | $18.8^{a} \pm 3.7$ |
| *Azospirillum* sp. | $19.2^{bc} \pm 2.7$ | $19.2^{a} \pm 3.3$ |
| None (*B. japonicum* inoculation alone) | $12.4^{d} \pm 1.7$ | $11.1^{bc} \pm 5.3$ |
| *F*- test | ** | ** |

Values followed by the same letter within the same columns are not significantly different by Duncan's multiple range test ($P \leq 0.05$).

### 3.4.2 Characterization of selected bacteria

Based on 16S rRNA sequence analysis, Isolate 3 was related to *Bacillus solisalsi* with 98 % homology (JX 290169). This *B. solisalsi* Isolate 3 gave the significantly highest IAA production, and *Azospirillum* sp. produced higher but not significantly different amount of IAA compared to *B. japonicum* CB 1809 and USDA 110 (Table 3). ARA results revealed that CB 1809 gave the maximum $N_2$-fixation in

free-living bacterial stage followed by USDA 110. $N_2$-fixation given by CB 1809 was significantly different from those by *Azospirillum* sp. and *B. solisalsi* Isolate 3. The *B. solisalsi* Isolate 3 has the lowest $N_2$-fixation ability when compared with others.

**Table 3**. Characterization of selected bacteria for nitrogenase activity and IAA production

| Treatment | Nitrogenase activity of free-living bacteria (nmole of ethylene mg protein $^{-1}$ hr$^{-1}$) | IAA (µM mg protein$^{-1}$) |
|---|---|---|
| *Azospirillum* sp. | 3.08$^{c}$ ± 0.5 | 0.25$^{b}$ ± 0.2 |
| *Bacillus solisalsi* Isolate 3 | 1.19$^{d}$ ± 0.1 | 0.78$^{a}$ ± 0.1 |
| *B. japonicum* CB 1809 | 8.21$^{a}$ ± 0.0 | 0.13$^{b}$ ± 0.0 |
| *B. japonicum* USDA 110 | 4.12$^{b}$ ± 0.0 | 0.10$^{b}$ ± 0.0 |
| *F*- test | ** | ** |

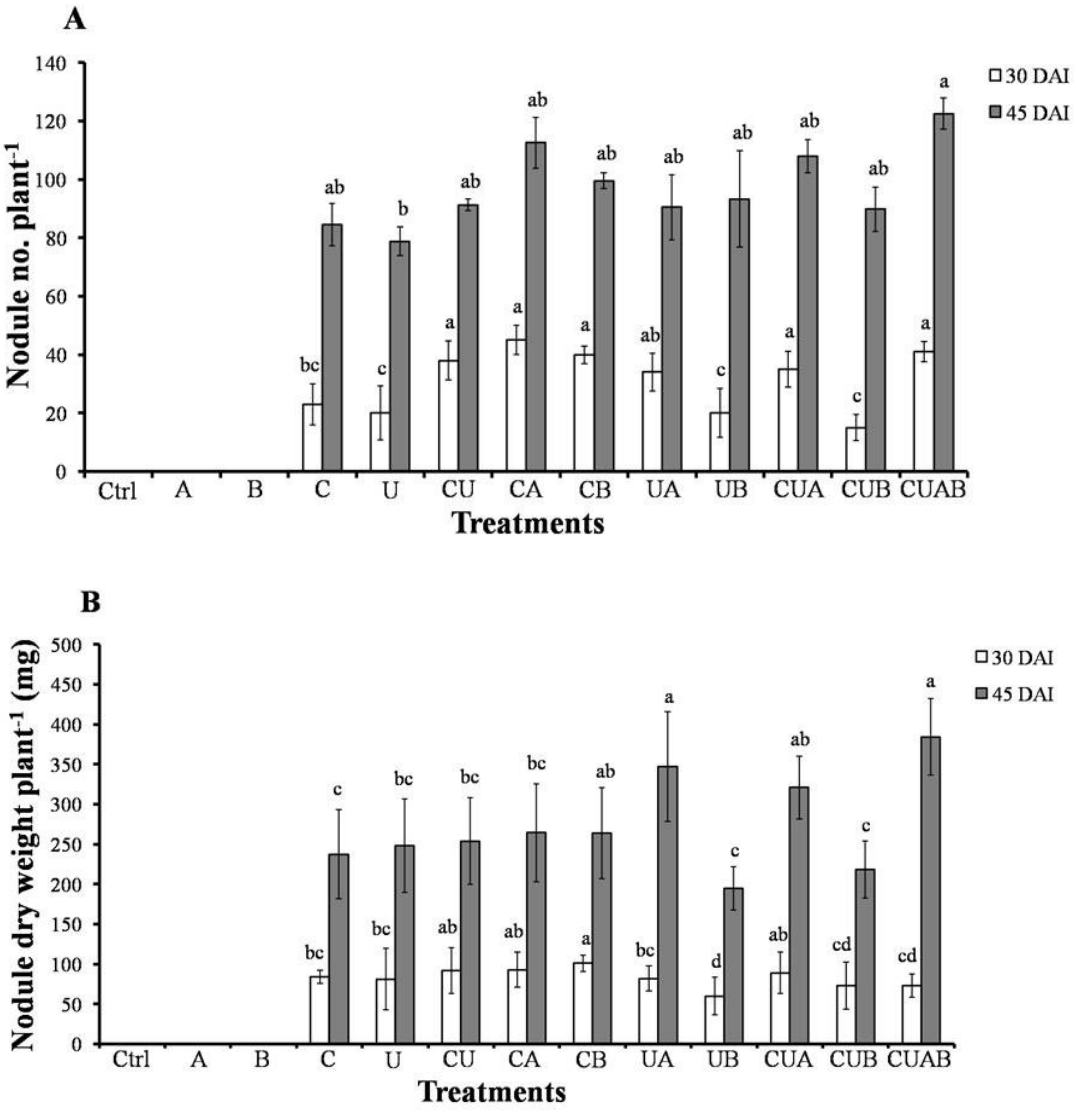
Different letters in the same column indicate significantly differences among treatments ($P \leq 0.05$).

### 3.4.3 Pot experiment

MPN plant infection counting from collected soil samples gave no nodule formation. In pot experiment, nodule formation was not observed in non-inoculated control and rhizobacterial inoculation alone as expected. The lowest shoot dry weight was noted in non-inoculated control.

Either single bradyrhizobial inoculation or co-inoculation with tested rhizobacteria gave the significantly highest biomass dry weight compared to PGPR

inoculation alone or non-inoculated control (Figure 1C). The nodule formation was significantly increased when *B. solisalsi* Isolate 3 was co-inoculated with CB 1809; however, a similar trend was not observed in co-inoculation with USDA 110 (Figure 1A). Maximum nodulation, nodule dry weight, and biomass dry weight of soybean were accomplished by altogether combined inoculation of tested bradyrhizobia and rhizobacterial isolates (Figure 1A, B, and C). Positive responses on nodule number and shoot dry weight of soybean were observed by co-inoculation of either *B. japonicum* CB 1809 or USDA 110 with *Azospirillum* sp. at 45 DAI.



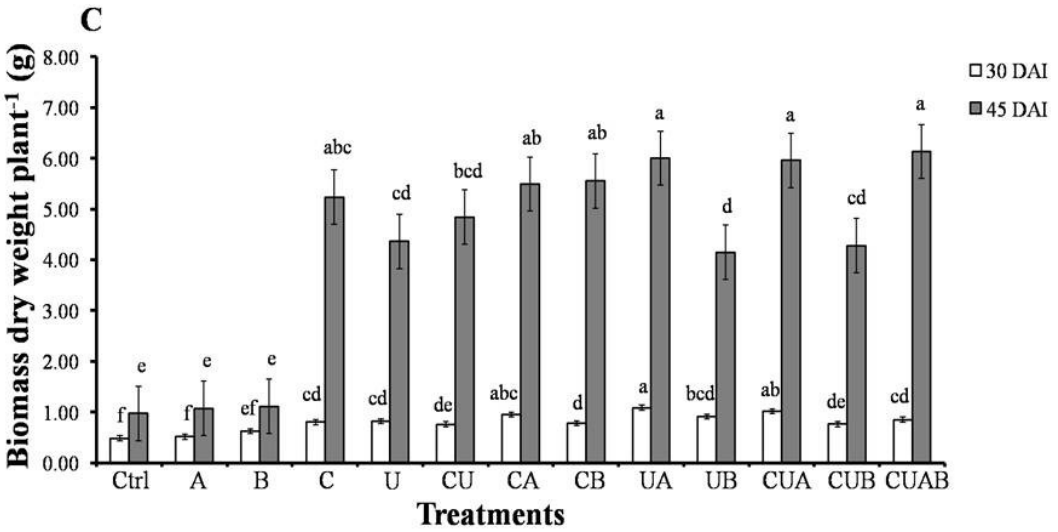



**Figure 1.** Co-inoculation effects of *B. japonicum* (CB 1809 and USDA 110) and selected rhizobacteria (*Azospirillum* sp. and *Bacillus solisalsi* Isolate 3) on soybean nodulation and plant growth under soybean-nodulating bradyrhizobia-free pot condition at 30 and 45 DAI. (Ctrl) Control; (A) *Azospirillum* sp.; (B) *Bacillus solisalsi* Isolate 3; (U) *Bradyrhizobium japonicum* USDA 110; (C) *B. japonicum* CB 1809; and coupled-letters referred to co-inoculated with indicated labels. **A.** Nodule number plant$^{-1}$, **B**. Nodule dry weight plant$^{-1}$ (mg), and **C.** Biomass dry weight plant$^{-1}$ (g).

### 3.4.4 DGGE and PCA analysis from pot experiment

DGGE profiles of eubacterial community structures were divided into two main clusters. The first cluster mainly included the samples from 0, 7, and 14 DAI samples with 78% similarity and the latter included those mainly from 30 and 45 DAI samples with 81% similarity (Figure 2). Eubacterial community structure in bulk soil samples did not form a separate branch from the clustering tree of bacterial inoculation treatments. A clear separation of the DGGE profiles was observed at different sampling times as well as different plant growth stages in 0, 7, 14, and 45

DAI except in 30 DAI (Figure 2). PCA result did not provide any clear separation among treatments (Figure 3).

Banding patterns of the bulk soil and rhizosphere soil samples from 7, 14, 30 and 45 DAI also revealed that there were considerable differences among the sampling times varying from 7-23, 10-22, 13-25 and 16-27 bands, respectively. However, the Shannon diversity indices (H' values) calculated from DGGE profiles of each treatment were not different significantly from each other in each sampling time (data not shown). Highly recovery of the DGGE bands of the inoculated bacteria was observed at the same position of the reference markers in all plant growth stages (line 1, 2, and 3 in Figure 2). There was only one common band that appeared in all samples and that is 100% similar to *Burkholderia* sp. (JX 290164) (PB1). Other two bands which were homologous to *Clostridium* sp. (JX 290165) (98% homology) (PB 2) and *Parasegitibacter luojiensis* (JX 290166) (95% homology) (PB 3) were observed in most of the samples.

The cluster analysis on DGGE banding profiles of 18S rRNA genes showed different but not clear effects of bacterial inoculation and sampling times on rhizosphere soil fungal community structures except that they shared some 2-3 common bands (Figure 4). Some bands were widely distributed and found in more than half of the samples. The number of bands corresponded to the number of predominant members in the microbial communities. However, most of the excised bands failed to be amplified and could not be sequenced. Two dominant bands which could be sequenced successfully were uncultured ascomycetes (JX 290170) (95% homology) (PF1) and *Fusarium oxysporum* (JX 290168) (99% homology) (PF2) (Figure 4). PCA result did not showed any clear separation among treatments (Figure 5).

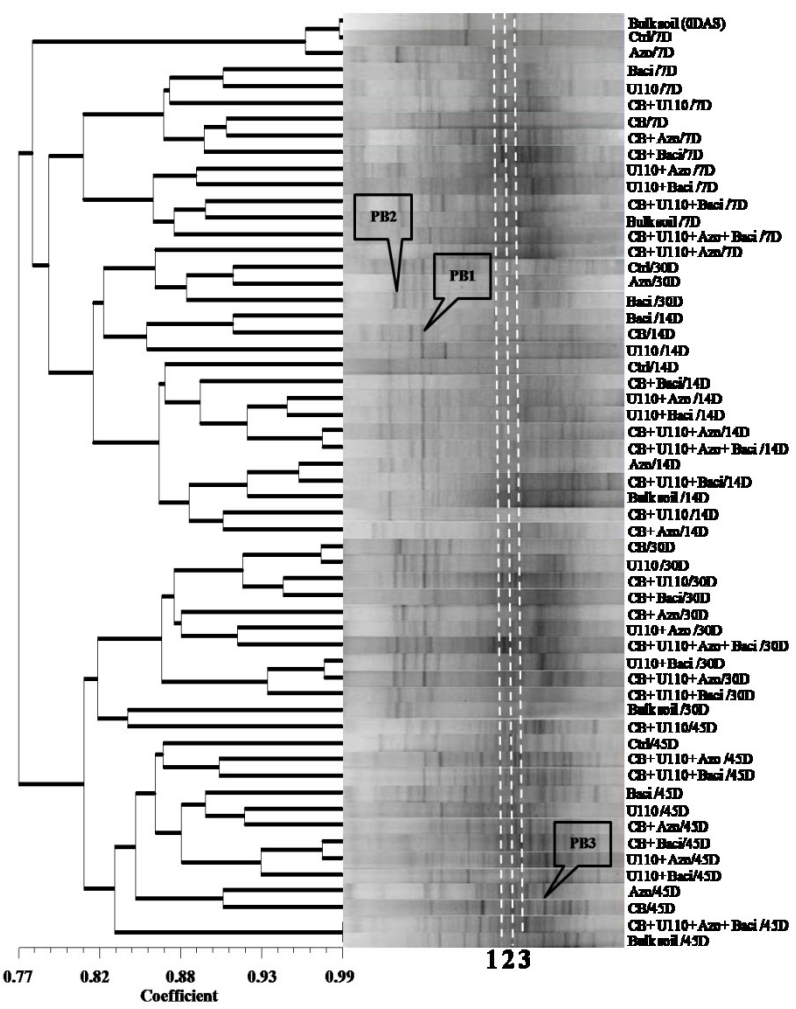



**Figure 2**. Cluster analysis of eubacterial community structures of partial 16S rRNA PCR-DGGE fingerprints of different soybean rhizosphere samples after inoculation with different bacterial inocula i.e., (Bulk) Bulk soil; (Ctrl) Control; (Azo) *Azospirillum* sp.; (Baci) *Bacillus solisalsi* Isolate 3; (CB) *B. japonicum* CB 1809; (U110) *B. japonicum* USDA 110 and (+) co-inoculation refer to indicated labels at different sampling times (0, 7, 14, 30, and 45 DAI) under soybean-nodulating bradyrhizobia-free pot conditions. Labels on fingerprints were subjected to sequence. Line 1, 2 and 3 refer to inoculated bacteria, *Azospirillum* sp., *B. solisalsi* Isolate 3 and *B. japonicum* (CB 1809 or USDA 110) respectively.

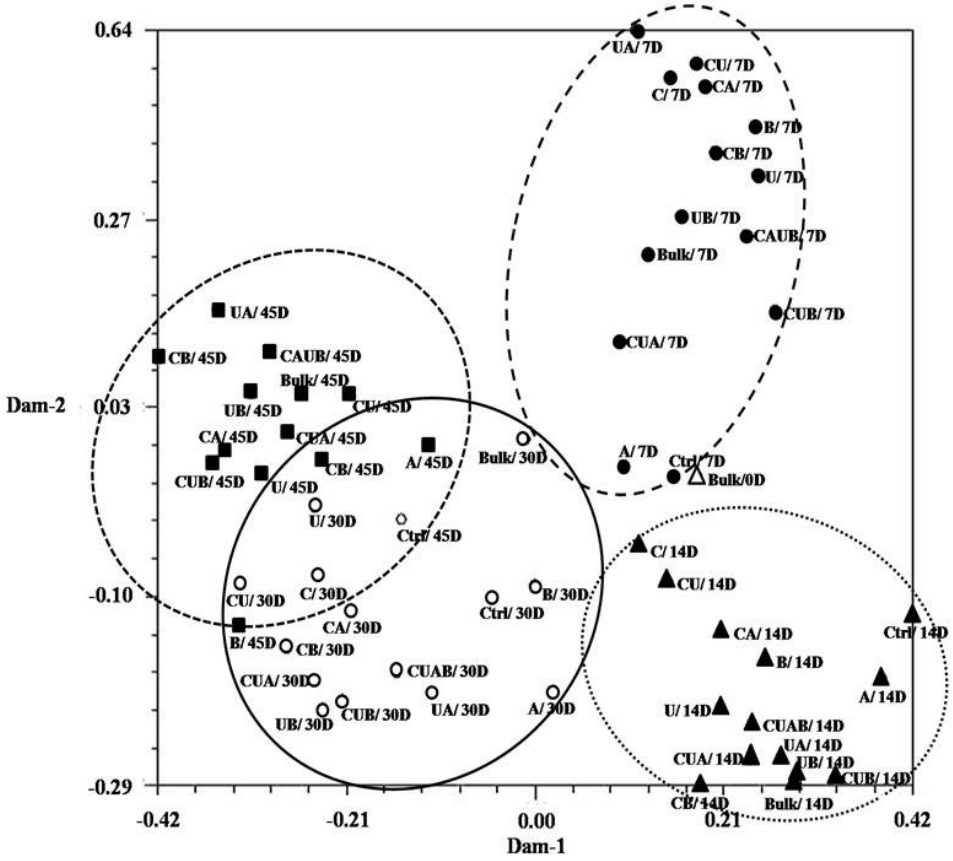



**Figure 3.** Community analysis derived from PCA of partial 16S rRNA banding profiles of soybean rhizosphere soil under soybean-nodulating bradyrhizobia-free pot conditions. Letters adjacent to marks indicate the treatments: (Bulk) Bulk soil; (Ctrl) Control; (A) *Azospirillum* sp.; (B) *Bacillus solisalsi* Isolate 3 (C) *B. japonicum* CB 1809; (U) *B. japonicum* USDA 110; and coupled-letters refer to co-inoculation due to indicated labels) at different sampling times: (▫) 0DAI; (■) 7DAI; (■) 14 DAI; (o) 30 DAI and (■) 45 DAI. Different samples formed a cluster which is circled by (- - - - , ……,– – – – , and ——), which shows in a trend of 7, 14, 30 and 45 DAI, respectively.

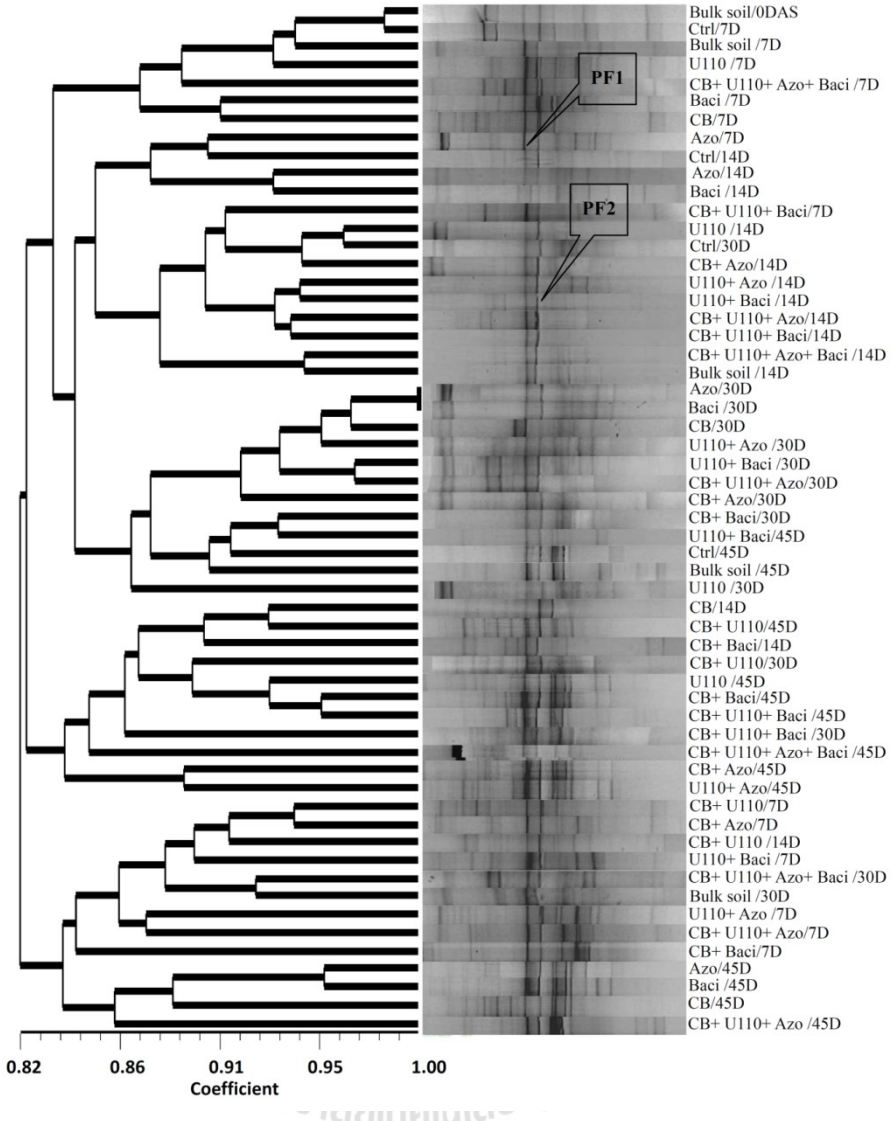



**Figure 4.** Cluster analysis of fungal community structures of partial 18S rRNA PCR-DGGE fingerprints of different soybean rhizosphere samples after inoculation with different bacterial inocula i.e., (Ctrl) Control; (Azo) *Azospirillum* sp.; (Baci) *Bacillus solisalsi* Isolate 3; (CB) *B. japonicum* CB 1809; (U110) *B. japonicum* USDA 110; and (+) co-inoculation refer to indicated labels at different sampling times (0, 7, 14, 30, and 45 DAI) under soybean-nodulating bradyrhizobia-free pot conditions. Labels on fingerprints were subjected to sequence.

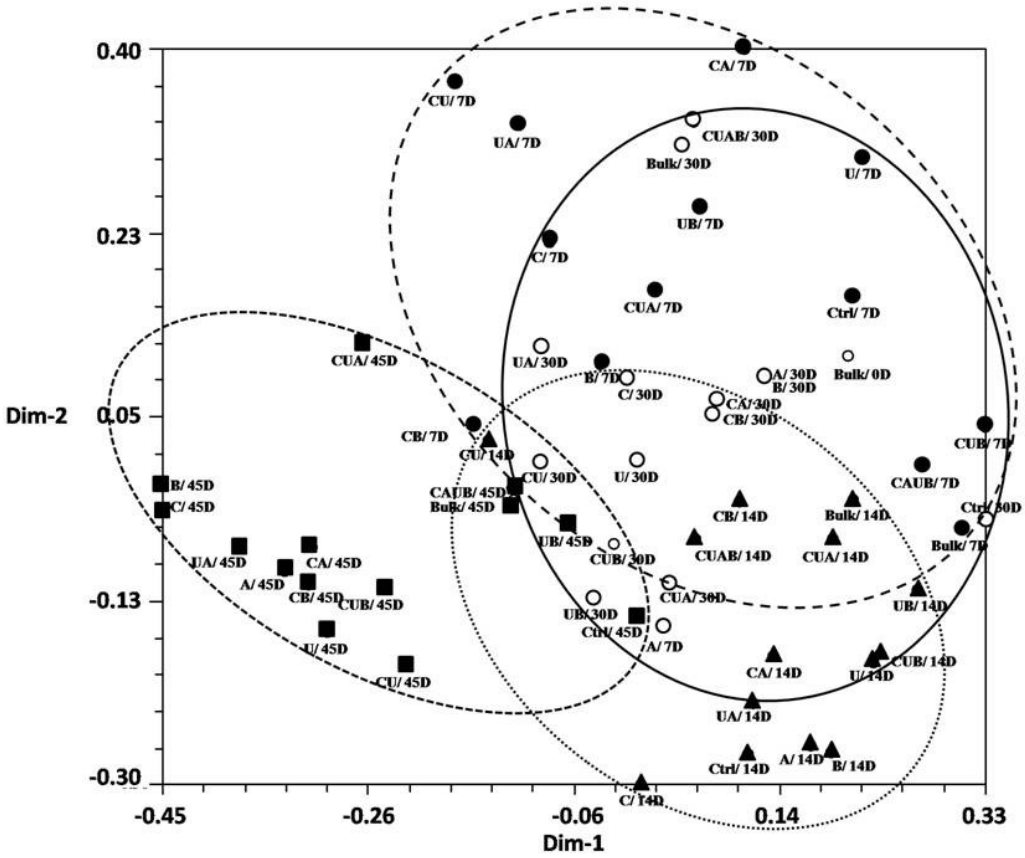



**Figure 5.** Community analysis derived from PCA of partial 18S rRNA banding profiles of soybean rhizosphere soil under soybean-nodulating bradyrhizobia-free pot conditions. Letters adjacent to marks indicate the treatments: (Bulk) Bulk soil; (Ctrl) Control; (A) *Azospirillum* sp.; (B) *Bacillus solisalsi* Isolate 3 (C) *B. japonicum* CB 1809; (U) *B. japonicum* USDA 110; and coupled-letters refer to co-inoculation due to indicated labels) at different sampling times: (▯) 0DAI; (■) 7DAI; (■) 14 DAI; (o) 30 DAI and (■) 45 DAI. Different samples formed a cluster which is circled by (- - - - , ……,– – – – – , and ——), which shows in a trend of 7, 14, 30 and 45 DAI, respectively.

### 3.4.5 Field experiment

Based on pot experiment results, *Azospirillum* sp. was selected for further study under field condition as it has nodulation and plant growth promoting ability on soybean when co-inoculated with bradyrhizobia. The soybean plants which were obtained by without bradyrhizobial inoculation in field experiment and those from MPN plant-infection count were free of nodules. Single inoculation of *Azospirillum* sp. has no prominent effects on soybean plant growth compared to non-inoculated control (Figure 6C). However, when it was co-inoculated with either CB 1809 or USDA 110, nodulation and plant growth were significantly increased when compared with non-inoculated control or *Azospirillum* sp. inoculation alone at 30, 45, and 70 DAI (Figure 6A, B, and C).

Better in root development were observed in co-inoculation with *Azospirillum* sp. compared to single inoculation of *B. japonicum* (Figure 7). Based on all sampling times, co-inoculation of either CB 1809 or USDA 110 with *Azospirillum* sp. increased 32.23% and 16.85% of nodulation and 26.51% and 18.83% of nodule dry weights over single inoculation of CB 1809 and USDA 110, respectively. Co-inoculation of USDA 110 with *Azospirillum* sp. increased ~36.99% of soybean nodulation over USDA 110 single inoculation at 45 DAI, leading to significantly higher and evident response to biomass dry weight.

Percentages of total plant nitrogen of soybean given by CB 1809 or USDA 110 inoculation and their co-inoculation with *Azospirillum* sp. were higher than those given by non-inoculated control and inoculation of *Azospirillum* sp. alone at 70 DAI (Table 4). Although the nodules obtained by inoculation with CB 1809 or co-inoculation with *Azospirillum* sp. gave the effective nodules and significantly

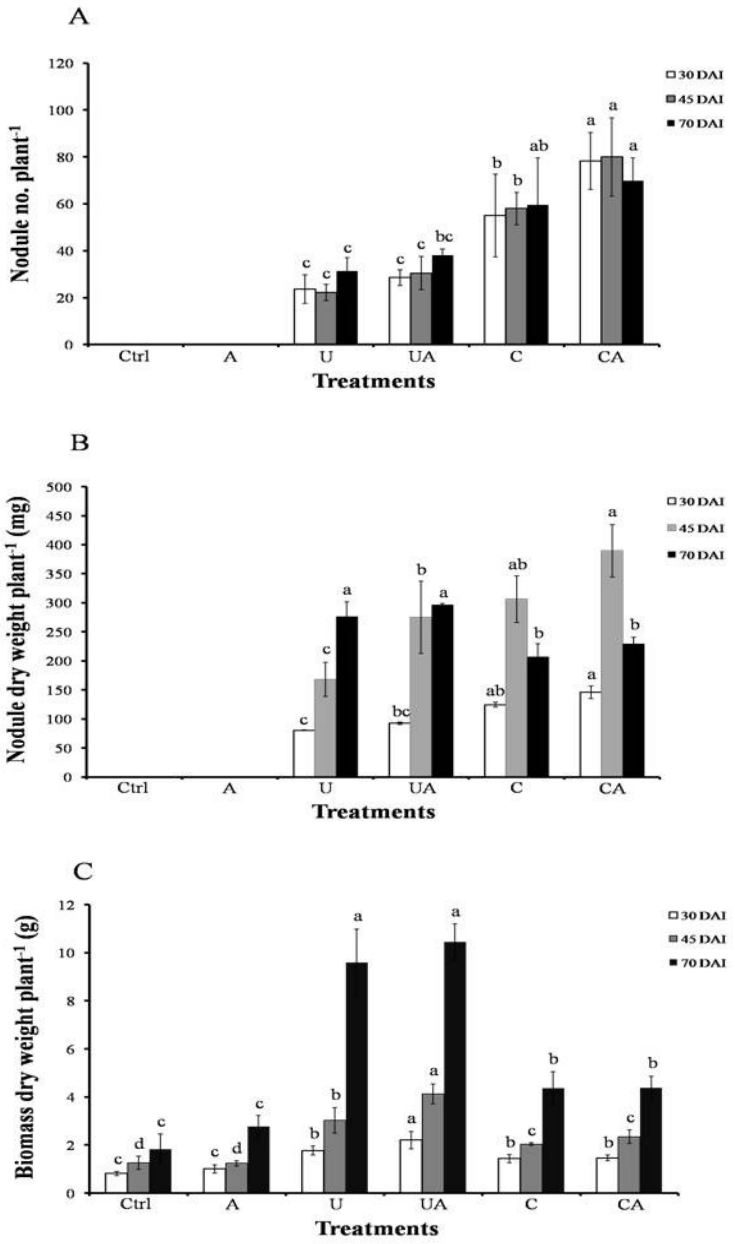



**Figure 6.** Co-inoculation effects of *B. japonicum* (CB 1809 and USDA 110) and *Azospirillum* sp. on soybean nodulation and plant growth (soybean-nodulating bradyrhizobia-free field conditions) at 30, 45, and 70 DAI. (Ctrl) Control; (A) *Azospirillum* sp.; (U) *B. japonicum* USDA 110; (C) *B. japonicum* CB 1809; and coupled-letters referr to co-inoculation related with indicated labels. **A**. Nodule number plant$^{-1}$, **B**. Nodule dry weight plant$^{-1}$ (mg), and **C.** Biomass dry weight plant$^{-1}$ (g).


**Figure 7.** Root development of soybean obtained by inoculation with **(A)** *B. japonicum* CB 1809; **(B)** *B. japonicum* CB 1809 and *Azospirillum* sp.; **(C)** *B. japonicum* USDA 110; **(D)** *B. japonicum* USDA 110 and *Azospirillum* sp at 45 DAI under field conditions.

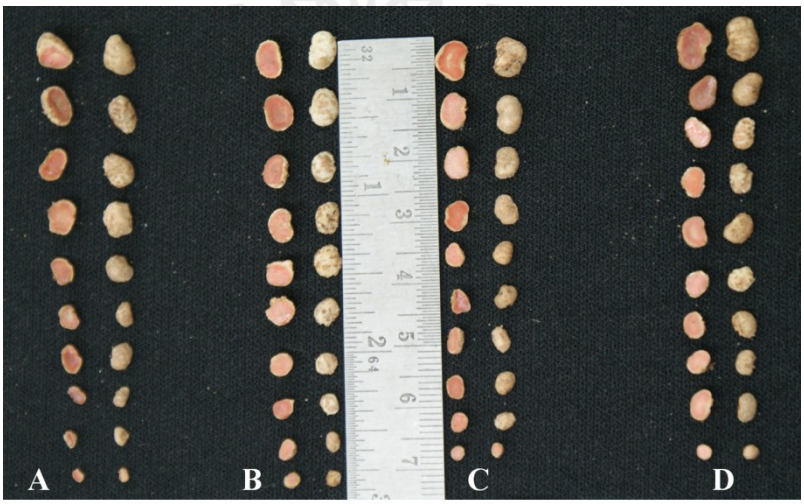



**Figure 8.** Cross-section of soybean nodules obtained by inoculation with **(A)** *B. japonicum* USDA 110 alone; **(B)** *B. japonicum* USDA 110 and *Azospirillum* sp.; **(C)** *B. japonicum* CB 1809 alone; **(D)** *B. japonicum* CB 1809 and *Azospirillum* sp at 70 DAI under field conditions.


**Figure 9.** Soybean plant growth under field conditions by inoculation with: **(A)** None (non-inoculated control); **(B)** *Azospirillum* sp.; **(C)** *B. japonicum* CB 1809; **(D)** *B. japonicum* CB 1809 and *Azospirillum* sp.; **(E)** *B. japonicum* USDA 110; **(F)** *B. japonicum* USDA 110 and *Azospirillum* sp. at 45 DAI.

highest number with appearance of pink-red color inside the nodules (Figure 8), the plant growth were less than those in USDA 110 and its co-inoculation (Figure 9) .

Enhancement in root dry weight of soybean obtained by co-inoculation of *B. japonicum* USDA 110 and *Azospirillum* sp. were significantly different from those of single inoculation at 30 and 45 DAI (Table 4); however, similar trend was not observed in *B. japonicum* CB 1809 and its co-inoculation. Moreover, noticeably increasing in plant height was not observed in *B. japonicum* CB 1809 and its co-inoculation. Co-inoculation of USDA 110 with *Azospirillum* sp. gave the significantly highest in plant height at 45 and 70 DAI (Table 4), and also gave the significantly highest number of seeds per plant and higher number of pods, 100 seeds weight, and seed weight per plant at harvest; however, those were not significantly different from those of USDA 110 inoculation alone (Table 5). The lowest yield (304

kg $ha^{-1}$) was obtained in non-inoculated control. Co-inoculation of *Azospirillum* sp. and either of CB 1809 or USDA 110 gave 23.65% and 34.92% higher seed yields over CB 1809 or USDA 110 single inoculation, respectively. Healthier and bigger seed size obtained by co-inoculation of USDA 110 and *Azospirillum* sp. gave the significantly highest yield with 1727.00 kg $ha^{-1}$ and it was almost 5-6 times more yields with respect to the control plants.

### 3.4.6 DGGE and PCA analysis from field experiment

DGGE profiles of soil eubacteria community structures in the field experiment revealed two main clusters with 76% similarity; one included the samples from four sampling times (0, 7, 30 and 45 DAI), and later from the sampling times at 14 DAI (Figure 10). Except at 14 DAI, the DGGE patterns generated in the rhizosphere soil samples of *Azospirillum* sp. inoculated and its co-inoculation with bradyrhizobia were clearly separated into small cluster with 88-91% similarity at different sampling times. The detected band numbers were increased from 7-14 DAI and, generally, most of the treatments gave higher number of band detection at 14 DAI and decreased at later stages. PCA analysis provided the grouping of the DGGE band profiles into four main groups and the changes were influenced by plant age (Figure 11).

Sequencing of partial 16S rRNA genes from the commonly detected bands revealed that *Streptococcus agalactiae* (JQ. 990157. 1) (99% homology) (FB1) and *Bacillus* sp. (JX 290163) (99% homology) (FB2) (Figure 10) were detected in all samples at all sampling times except the band intensities appeared different. However, *Propionibacterium freudenreichii* (JX 290167) (95% homology) (FB3) was detected in late sampling (30 and 45 DAI) and that band seems to be propagated later season of soybean growing.

**Table 4.** Co-inoculation effects of *B. japonicum* (CB 1809 and USDA 110) and *Azospirillum* sp. on soybean root dry weight and plant height under rhizobia-free field conditions (September-December, 2011)

| Treatments | Root dry weight per plant (g plant$^{-1}$) | | | Plant height (cm plant$^{-1}$) | | |
|---|---|---|---|---|---|---|
| | 30 DAI | 45 DAI | 70 DAI | 30 DAI | 45 DAI | 70 DAI |
| Non-inoculated control | 0.17$^{c}$± 0.02 | 0.29$^{b}$ ± 0.04 | 0.99$^{c}$ ± 0.20 | 17.07$^{c}$ ± 1.07 | 18.83$^{c}$ ± 0.53 | 18.03$^{c}$ ± 0.27 |
| *Azospirillum* sp. alone | 0.23$^{b}$± 0.02 | 0.27$^{b}$ ± 0.01 | 1.54$^{bc}$± 0.20 | 19.31$^{c}$ ± 1.50 | 19.17$^{c}$ ± 0.58 | 19.03$^{c}$ ± 0.85 |
| USDA 110 alone | 0.25$^{b}$± 0.02 | 0.30$^{b}$ ± 0.01 | 2.03$^{ab}$± 0.21 | 27.57$^{a}$ ± 0.71 | 30.78$^{a}$ ± 2.62 | 36.87$^{a}$ ± 0.42 |
| USDA 110 and *Azospirillum* sp. | 0.34$^{a}$± 0.02 | 0.44$^{a}$ ± 0.01 | 2.53$^{a}$ ± 0.31 | 28.43$^{a}$ ± 0.52 | 31.45$^{a}$ ± 2.04 | 37.30$^{a}$ ± 1.41 |
| CB 1809 alone | 0.25$^{b}$± 0.02 | 0.26$^{b}$ ± 0.02 | 1.20$^{c}$ ± 0.07 | 25.76$^{ab}$ ± 1.09 | 23.89$^{b}$ ± 1.13 | 23.53$^{b}$ ± 1.68 |
| CB 1809 and *Azospirillum* sp. | 0.24$^{b}$± 0.01 | 0.30$^{b}$ ± 0.02 | 1.20$^{c}$ ± 0.03 | 23.20$^{b}$ ± 0.80 | 24.50$^{b}$ ± 0.95 | 23.83$^{b}$ ± 0.53 |

Values followed by the same letter within the same columns are not significantly different by Duncan's multiple range test ($P \leq 0.05$).

**Table 5.** Single and co-inoculation effects of *B. japonicum* CB 1809, USDA 110, and *Azospirillum* sp. on $N_2$-fixation, plant growth, yield and yield components of soybean under soybean-nodulating bradyrhizobia-free field conditions.

| Treatments | No. of pods plant$^{-1}$ | No. of seeds plant$^{-1}$ | 100 seeds weight (g) | Seed weight (g plant$^{-1}$) | Yield (kg ha$^{-1}$) | ARA (nmole plant$^{-1}$) | Total N (% ) |
|---|---|---|---|---|---|---|---|
| Non-inoculated control | 4.7$^{e}$ ± 0.4 | 7.8$^{d}$ ± 0.3 | 12.7$^{d}$ ± 1.0 | 0.62$^{c}$ ± 0.5 | 304$^{e}$ ± 12.6 | 0.00$^{d}$ ± 0.00 | 0.45$^{c}$ ± 0.1 |
| *Azospirillum* sp. alone | 5.7$^{de}$ ± 0.5 | 9.2$^{d}$ ± 1.4 | 13.7$^{cd}$ ± 0.7 | 0.71$^{c}$ ± 0.6 | 353$^{e}$ ± 47.0 | 0.00$^{d}$ ± 0.00 | 0.83$^{b}$ ± 0.3 |
| USDA 110 alone | 10.3$^{ab}$ ± 0.5 | 19.9$^{b}$ ± 2.1 | 15.4$^{ab}$ ± 0.7 | 2.56$^{ab}$ ± 0.5 | 1280$^{b}$ ± 62.5 | 0.95$^{b}$ ± 0.08 | 1.03$^{ab}$ ± 0.2 |
| USDA 110 + *Azospirillum* sp. | 12.3$^{a}$ ± 2.8 | 27.2$^{a}$ ± 2.9 | 16.5$^{a}$ ± 0.7 | 3.45$^{a}$ ± 1.3 | 1727$^{a}$ ± 186.5 | 1.30$^{a}$ ± 0.01 | 1.29$^{a}$ ± 0.2 |
| CB 1809 alone | 7.2$^{cd}$ ± 0.7 | 13.1$^{c}$ ± 1.2 | 14.1$^{bcd}$ ± 0.9 | 1.56$^{bc}$ ± 0.1 | 778$^{d}$ ± 54.6 | 0.58$^{c}$ ± 0.07 | 1.25$^{a}$ ± 0.1 |
| CB 1809 + *Azospirillum* sp. | 8.8$^{bc}$ ± 0.9 | 15.0$^{c}$ ± 0.9 | 15.4$^{ab}$ ± 0.7 | 1.93$^{bc}$ ± 0.2 | 962$^{c}$ ± 17.4 | 0.51$^{c}$ ± 0.00 | 1.07$^{ab}$ ± 0.2 |
| *F*- test | ** | ** | ** | ** | ** | | ** |

Values followed by the same letter within the same columns are not significantly different by Duncan's multiple range test ($P \leq 0.05$).

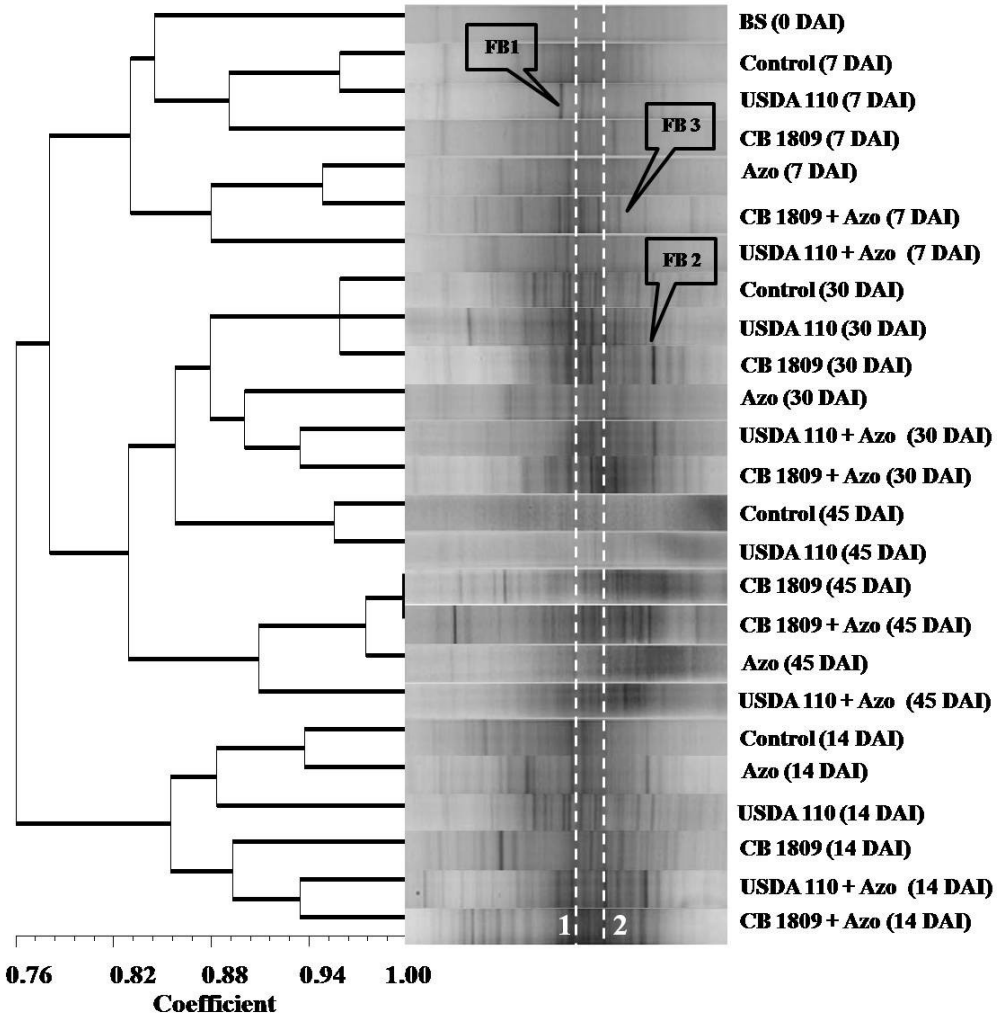



**Figure 10**. Cluster analysis of eubacterial community structures of partial 16S rRNA PCR-DGGE fingerprints of different soybean rhizosphere samples after inoculation with different bacterial inocula: (BS) Bulk soil; (Ctrl) Control; (Azo) *Azospirillum* sp.; (CB 1809) *B. japonicum* CB 1809; (USDA110) *B. japonicum* USDA 110; and (+) refer to co-inoculation of indicated labels at different sampling times (0, 7, 14, 30, and 45 DAI) under soybean-nodulating bradyrhizobia-free field conditions. Line 1 and 2 refer to inoculated bacteria *Azospirillum* sp. and *B. japonicum*, respectively.

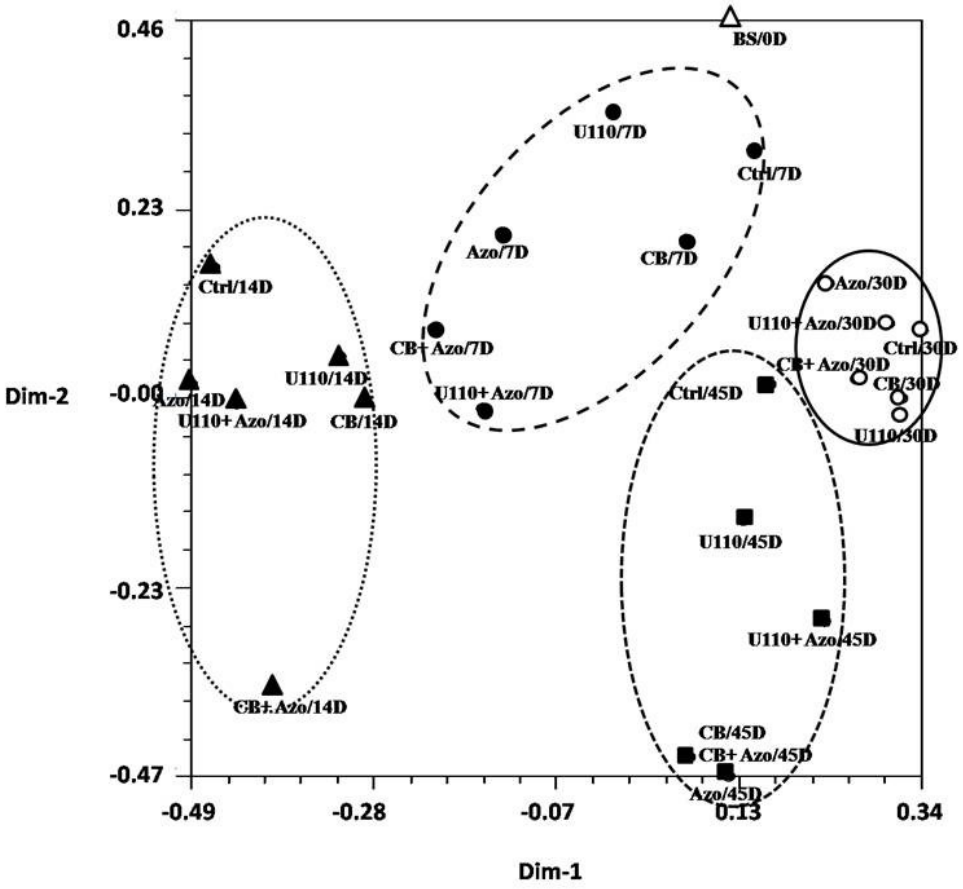



**Figure 11.** Community analysis derived from PCA of partial 16S rRNA banding profiles of soybean rhizosphere soil samples under soybean-nodulating bradyrhizobia-free field conditions. Letters adjacent to marks indicate the treatments: (BS) Bulk soil; (Ctrl) Control; (Azo) *Azospirillum* sp.; (CB) *B. japonicum* CB 1809; (U110) *B. japonicum* USDA 110; and (+) co-inoculation refer to co-inoculation of indicated labels at different sampling times: (△) 0DAI; (■) 7DAI; (■) 14 DAI; (0) 30 DAI and (■) 45 DAI. Different samples formed a cluster which is circled (- - - - , ……, —— , and – – – – –), which shows in a trend of 7, 14, 30 and 45 DAI, respectively.

## 3.5 Discussion

### 3.5.1 Screening of rhizobacteria for co-inoculation with *B. japonicum* on soybean

The compatibility of the microorganisms needs to be evaluated before they are used as co-inoculants because of the possibility of antagonistic interactions among them, (Abd-Alla et al., 2001). In this study, totally 43 isolates out of 200 tested rhizobacterial strains showed inhibition on tested bradyrhizobia and 157 isolates which did not inhibit the two tested *B. japonicum* growth in *in vitro* cultures were selected as *Bradyrhizobium* non-inhibitors for further co-inoculation studies under controlled (aseptic) conditions.

The *Azotobacter* sp. and *Azospirillum* sp. used in this screening study are being commercialized as PGPR inocula for various crops cultivation by SUT (Piromyou et al., 2011; Teaumroong et al., 2009) and their positive responses on soybean nodulation were observed in this study. Rhizospheric microorganisms 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 (Rautela et al., 2001; Gupta et al., 2003). Different responses on co-inoculation as such as interactions among different *B. japonicum* and PGPR strains were observed in this study. In the case of *Bacillus* sp. SUT 1, it gave different responses on nodulation (nodule number) of soybean when it was co-inoculated with *B. japonicum* CB 1809 and USDA 110. It can be possible that SUT 1 did not support nodulation sites on tested soybean roots for USDA 110 as in CB 1809, or it competed for nutrient absorption instead of sharing nutrients with USDA 110, or plant autoregulation system control the amount of

nodule in different combinations of two bacteria. In this study, two isolates out of 157 *Bradyrhizobium* non-inhibitors; namely, *Azospirillum* sp. and Isolate 3 (sequenced as *Bacillus solisalsi*), were selected as soybean nodulation enhancers. It has been reported that co-inoculation of *Azospirillum lipoferum* with rhizobia stimulates the formation of epidermal cells that become infected root hair cells, or create additional infection sites that are later occupied by rhizobia (Tchebotar et al., 1998). Araújo and Hungria (1999) demonstrated the viability of co-inoculating soybean seeds with crude or formulated metabolites, or with cells of *Bacillus subtilis*, to increase the contribution of the biological $N_2$-fixation process.

When compared the characteristics of selected bacteria, *Azospirillum* sp. and *B. solisalsi* Isolate 3 produced high amount of IAA. However, the lowest level of ARA was detected in *B. solisalsi* Isolate 3. Adesemoye and Kloepper (2009) confirmed that PGPR such as *Bacillus amyloliquefaciens* and *B. pumilis* can fix nitrogen and can increase plant N uptake from fertilizer via other mechanisms but not with their own $N_2$-fixing capability. In this study, free-living *Azospirillum* sp. gave higher acetylene reduction activity than *B. solisalsi* Isolate 3, and similar result was reported by Piromyou et al. (2011) that the *Azospirillum* sp. showed the highest $N_2$-fixation ability in free-living compared to *Azotobacter* sp. and other PGPR isolates including *Bacillus* sp. Moreover, Spaepen et al. (2009) also reported that effects of *Azospirillum* inoculation are mainly attributed to improved root development and enhanced water and mineral uptake and those effects were responsible by plant growth promoting substances, mainly IAA secreted by *Azospirillum*. As nodulation promoting rhizo-bacteria increase nodulation leading to increased plant growth (Zhang et al., 1997), effects on nodulation and plant growth of soybean by dual inoculation of selected PGPR and *B. japonicum* were evaluated under pot conditions.

### 3.5.2 Screening of rhizobacteria for co-inoculation with *B. japonicum* on soybean under pot conditions

No nodule formation in both MPN plant infection counting from tested soil samples, and non-inoculated control and rhizobacterial inoculation alone in pot experiment indicated that there has no specific indigenous soybean-nodulating bradyrhizobia present in tested soils because of the nodulation of soybean requires specific *Bradyrhizobium* species (Abaidoo et al., 2007). Bashan et al. (2004) reported that inoculation of plants with *Azospirillum* sp. alters the root morphology, increases numerous plant shoot growth parameters, and eventually increases the yield of many crops. However, no prominent enhancement in plant growth of soybean by inoculating the soybean with *Azosprillum* sp. alone was observed in this experiment. This may be due to very low organic matter content (~0.39%) in tested soil and could not accumulate the fixed-nitrogen in plants. However, co-inoculation of *Azospirillum* sp. and either CB 1809 or USDA 110 enhanced root growth (data not shown), gave higher nodule numbers and plant growth than single inoculation of *B. japonicum*. It may be due to *Azospirillum* ensuring the availability of appropriate type of roots for effective infection when co-inoculated with *Bradyrhizobium* in legumes. Similar findings were reported on co-inoculation of soybean with *B. japonicum* and *Azospirillum* sp. which increases nodulation, nitrogenase activity, and plant growth (Zhang et al., 1996; Dashti et al., 1998).

There were many possibilities that inoculated PGPR could enhance nodulation which led to enhancement in plant growth. For instance, Poi et al. (1989) reported that the presence of *Azospirillum* sp. in the rhizosphere makes the root hairs more susceptible to rhizobial infection that is reflected in better plant growth. In this

study, not only different in root and shoot development significantly but also in nodulation of soybean were observed in co-inoculation with *B. japonicum* and *Azospirillum* sp. Remans et al. (2008) pointed out the effects of co-inoculation of *Rhizobium* spp. and *Azospirillum* spp. on common bean which can increase the number of root hairs, the amount of flavonoids exuded by the roots and the number of nodules formed compared to single *Rhizobium* inoculation. In co-inoculation, *Azospirillum* promoted epidermal cell differentiation in root hairs that increased the number of potential sites for Bradyrhizobial infection (Yahalom et al., 1990) and as a result more nodules were developed (Andreeva et al., 1993). It may be due to *Azospirillum* ensuring the availability of appropriate type of roots for effective infection when co-inoculated with *Bradyrhizobium* in legumes.

While *Azospirillum* sp. did not vary its effectiveness when co-inoculated with any of both bradyrhizobia, *B. solisalsi* vary its effects on co-inoculation with different bradyrhizobia. The negative effect on nodule formation offered by co-inoculation of *B. solisalsi* with both bradyrhizobia was found to be recovered by *Azospirillum* sp. that showed clearly in the all together co-inoculation of all tested bacteria. Therefore, it could be concluded that co-inoculation with *Azospirillum* sp. enhanced nodulation and nodule dry weight better than *B. solisalsi* did. In spite of no emphasized on the detail mechanisms of nodule enhancement in this study, there were many reports stated that when co-inoculated with rhizobia, *Azospirillum lipoferum* stimulates the formation of epidermal cells that become infected root hair cells, or creates additional infection sites that are later occupied by rhizobia (Tchebotar et al., 1998). Therefore, *Azospirillum* sp. was selected as a more effective PGPR for co-inoculation with both tested *B. japonicum* CB 1809 and USDA 110.

**DGGE and cluster analysis of rhizosphere soil microbial community structures from pot experiment**

In field study, the whole rhizosphere soil samples were intended to be studied and cluster analysis did not allow a clear distinction of eubacterial community structures in bulk soil samples from the clustering tree of bacterial inoculation treatments. Costa et al. (2006) reported that no differences encountered between the microenvironments were due to the absence of clear characteristic patterns. Four main groups obtained by PCA analysis confirmed that the differences were mainly due to plant growth stages rather than bacterial inoculation. Similar result was reported by Herschkovitz et al. (2005) that *Azospirillum brasilense* inoculation had no effect on the size or on the structure of the bacterial communities. They also supposed that variation of microbial communities with the progression of growth stages may be related to two separate mechanisms, i.e., environmental changes such as soil temperature and soil moisture with the growth stages (Nazih et al., 2001) and the changes in the quality and quantity of root exudates of rhizodepositions with the growth stages (Garbeva et al., 2004). More abundant and numerous bands detected in later plant growth stages than early stages suggested that bacterial communities are more complex in later plant growth stages. Xu et al. (2009) also suggested that the growth stage is the second major factor in shaping bacterial communities in the soybean rhizosphere because compositions of the root exudates were shown to vary depending on the plant species and the stage of the plant development (Heulin et al., 1987).

High recovery of the inoculated bacterial bands at the same position of the concerning markers confirmed that the introduced bacteria were able to establish

along with the plant growth stages. *Burkholderia* sp. (JX 290165) was found to be an indigenous in tested soil as it was detected in all samples. This β-proteobacterial (β-Rhizobia) *Burkholderia* form effective nodules on species of *Mimosa* (Parker et al., 2007), *Acacia*, and *Prosopis* (Talbi et al., 2010), *Dalbergia* (rose wood legume trees) (Lu et al., 2012), and some other leguminous plants such as common bean (Talbi et al., 2010). Moreover, *Clostridium* sp. (JX 290165) and *Parasegitibacter luojiensis* (JX 290166) were found to be as dominant bacteria as those that appeared in most of the samples. The composition of the exudates has been shown to exert selective effects towards certain bacterial groups, such as the *Proteobacteria* (Smit et al., 2001).

The fungi represent a dominant component of the soil microflora (Thorn, 1997). However, there are relatively few studies on the effects of bacterial inoculation on the soil fungal community compared with the number of studies reporting the effects on specific target plant pathogens (Takehara et al., 2003; Browning et al., 2006) and on the bacterial community (Dungan et al., 2003). The 18S rRNA gene of fungi contains a lower amount of variation than others such as 16S rRNA gene across bacteria (Anderson and Cairney, 2004). In this study, the detected density of fungal community was higher than that of eubacterial community. Soils used in this pot experiment were collected from the field with the history of cassava (*Manihot esculenta*) cultivation (Dahniya, 1994). *Fusarium oxysporum* (JX 290168) that was dominantly detected in this pot experiment, and *Fusarium* species are a significant component of the set of fungi associated with cassava root rot (Bandyopadhyay et al., 2005). However, no wilt symptom was observed during the plant development. *Burkholderia* sp. was detected by DGGE analysis in this pot experiment as described

above. *Burkholderia cepacia* is recognized as a biological control agent for the control of plant pathogens (Nion and Toyota, 2008; Sijam and Dikin, 2005). In contrast to eubacterial communities, bacterial inoculation and sampling times did not clearly affect soil fungal communities.

### 3.5.3 Screening of rhizobacteria for co-inoculation with *B. japonicum* on soybean under field conditions

The selected *B. japonicum* strains and *Azospirillum* sp. were continued to test their co-inoculation effects under field condition because 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 (Navarro et al., 1999). There is an agreement that improved plant growth is attributed to *Azospirillum* through subsequent increase of lateral root number and root hair formation, alter the root morphology, water and mineral uptake and $N_2$-fxation, and eventually increases the yield of many crops (Bashan, 1999) while no prominent enhancing in plant growth of soybean by inoculating with *Azosprillum* sp. alone was observed in this field experiment. It may be because of the tested soil has very low in organic matter content (~0.39%) and cannot accumulate the fixed nitrogen. However, detection of enhancement in root growth, nodule number and plant growth by co-inoculation of soybean with *Azosprillum* sp. and either of *B. japonicum* CB 1809 or USDA 110 support the fact pointed out by Poi et al. (1989) that the presence of *Azospirillum* sp. in the rhizosphere makes the root hairs more susceptible for rhizobial infection that is reflected in better plant growth.

In field experiments, non-inoculated control plants provide the

information about the effects of single *B. japonicum* inoculation and its co-inoculaion on $N_2$-fixation capacity of soybean because no chemical source of N was applied during the experiment. Increasing about 20.16% of total plant N in co-inoculating soybean with *B. japonicum* USDA 110 and *Azospirillum* sp. than USDA 110 single inoculation lead to give higher plant growth and seed yield in this study. Similar result was reported by Groppa et al. (1998) that nitrogen content of dual (*B. japonicum* and *Azospirillum brasilense*) inoculated soybean plants in pot condition was significantly increased 23% over *B. japonicum* single inoculated plants; however, no significant difference in total dry matter production could be detected in their study. They suggested that co-inoculation leads to an increased number of the most active nodules, therefore, to a greater $N_2$-fixation and assimilation. Similar finding was reported by Galal (1997) that the superior dual inoculation effects of *B. japonicum* and *Azospirillum brasilense* over single inoculation with *B. japonicum* with regards to nitrogen fixation and dry biomass of soybean, and Bashan et al. (1990) supposed that which may be attributed to a stimulating effect of hormones excreted by *Azospirillum* on both nodulation and nutrient uptake.

Higher nodulation with pink-red colored appearance in the cross-section of soybean nodules obtained by CB 1809 inoculation alone and its co-inoculation with *Azospirillum* sp. supposed to be processed $N_2$-fixation properly; however, plant growth was not as high as USDA 110 and/or its co-inoculated soybean plants. Related to plant growth, the highest yield was observed by co-inoculation with USDA 110 and *Azospirillum* sp. among the treatments because *Azospirillum* sp. are capable of increasing the yield of important crops growing in various soils and climatic regions and significant increases in yield in the order of 5-30% in 60-75% of the published reports (Fuentes-Ramirez and Coballero-Mellado, 2006).

However, soybean yields in this research were much more less than those from commercial production because this study was mainly emphasized on effects of bradyrhizobial and its co-inoculation with PGPR, not much input of inorganic nitrogen fertilizers were applied before sowing. Soybean plant can assimilate the N from three sources; 1) N derived from symbiotic $N_2$ fixation by root nodules, 2) absorbed N from soil mineralized N, and 3) N derived from fertilizer when applied. For the maximum seed yield of soybean, it is necessary to use both $N_2$-fixation and absorbed N from roots (Harper, 1987). Soybean plants assimilate a large amount of nitrogen during both vegetative and reproductive stages, and the total amount of N assimilated in plant is highly correlated with the soybean yield. Generally, soybean seed yield depends mostly on pod number per area and average seed weight is affected by growing conditions in late growth stages.

At the time of pod fill, nodules on legume lose their ability to fix $N_2$ because the plant feeds the developing seeds rather than nodules. To obtain high seed yield of soybean, good nodulation and high and long lasting nitrogen fixation activity are very important. When only $N_2$-fixation is available to the plant, vigorous vegetative growth does not occur, which results in reduced seed yield. When compared the $N_2$-fixation ability and seed yields obtained by USDA 110, CB 1809, and their related co-inoculations, there were prominent variations. *B. japonicum* USDA 110 and its co-inoculation gave more effective and functional nodules and which leading to increased in seed yield in contrast to CB 1809. Somasegaram and Bohlool (1990) reported the similar results that *B. japonicum* USDA 110 maintained its high effectiveness and superiority in colonization in a comparison with strains USDA 138 and CB 1809 under conditions of soil mineral N availability and immobilization.

CB 1809 inoculated soybean plants become nitrogen deficient in the middle of the growing season because nitrogen demands are greatest and inefficient $N_2$-fixation in that stage seems that CB 1809 could not fix enough $N_2$ to support the plant at the pod filling stage. There are many possibilities such as drought stress, decrease in oxygen supply, a high or low pH, nutrient imbalance etc., which may depress nodule formation and nitrogen fixation activity. Molybdenum (Mo) is the least abundant of the trace elements in soil, however, very little is present in forms that are available to plants. In plants, Mo is an essential mineral nutrients involved in the reduction of nitrate. In rhizobia, it is a part of the enzyme nitrogenase that is essential for $N_2$-fixation (Fox and Whitney, 1978). However, there will be limited amount of this nutrients in this experimental field sites may be one of the possibilities which did not gave effective $N_2$-fixation by CB 1809.

Inoculation of soybean with PGPR in the presence of *B. japonicum* increased soybean grain yield, grain protein yield, and total plant protein production in short season areas (Dashti et al., 1997). Inoculation of soybean crops with effective *B. japonicum* strains singly (Galal-Gorchev, 1993) or in combination with *Azospirillum brasilense* (El-Mokadem et al., 1986; Bashan et al., 1990) was found to be important for improving and maximizing the plant growth and $N_2$-fixation potential of the crop either in soil which lacks indigenous *B. japonicum* (Singleton and Tavares, 1986) or in those soils high in indigenous *Bradyrhizobium* spp., but less effective than the introduced bacteria (Kucey et al., 1988). Therefore, more additional studies and efforts should be focused on co-inoculation effects of *B. japonicum* and *Azospirillum* sp. on the nodulation and plant growth of soybean in soil conditions with high indigenous bradyrhizobial population and different environmental conditions.

**DGGE and cluster analysis of rhizosphere soil microbial community structures from field experiments**

In the field conditions, the structural and functional diversity of rhizosphere populations is supposed to be affected by differences in root exudation and rhizodeposition in different root zones and in relation to soil types, plant species, growth stages, cultural practices such as tillage and crop rotation, and other environmental factors (Horwath et al., 1998). DGGE patterns of *Azospirillum* sp. inoculated treatments were clearly separated from non-inoculated and bradyrhizobial inoculation alone. Because of the inoculation with azospirilla also leads to an increase in plant root exudation (Landa et al., 2003), both changes in root structure and exudation are potential factors influencing the type of microorganisms colonizing the radicular environment. *Streptococcus agalactiae* and *Bacillus* sp. were detected in all samples in all sampling times. Such two strains supposed to be dominant strains in tested soil and *Bacillus* sp. is soil dwelling bacteria mostly can be found as rhizobacteria. *Propionibacterium freudenreichii* was detected in late sampling time, and it seemed to be propagated in later soybean growing season. In the soils, humic substances have important roles in soil fertility and they are considered to have primal relevance for the stabilization of soil aggregation, and also sources of carbon or micronutrients for growth of microorganisms. *Propionibacterium freudenreichii* bacteria are fermenting bacteria (Reid et al., 2004) and are capable of channeling electron from anaerobic conditions via humic acid towards iron-reduction (Benz et al., 1998). Gradual and continuous changes from first to last sampling times in PCA analysis were supposed to be dominated by changes of eubacterial community by plant ages and not by bacterial inoculation.

## 3.6 Conclusion

Co-inoculation of *B. japonicum* and *Azospirillum* sp. gave positive responses in nodulation and plant growth, and did not shift the soil microbial community structures noticeably under soybean-nodulating bradyrhizobia-free soils. Therefore, *Azospirillum* sp. was selected as the most effective PGPR that has a potential to be used in co-inoculants with *B. japonicum* strains. However, on-farm competition trials in soybean-nodulating bradyrhizobia-established soil in soybean growing areas are also necessary to determine their potential for competitiveness against native strains.

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# CHAPTER IV

# CO-INOCULATION EFFECTS OF *Bradyrhizobium japonicum* AND *Azospirillum* sp. ON COMPETITIVE NODULATION AND RHIZOSPHERE EUBACTERIAL COMMUNITY STRUCTURES OF SOYBEAN UNDER RHIZOBIA-ESTABLISHED SOIL CONDITIONS

## 4.1 Abstract

Bradyrhizobial inoculants used for soybean seed inoculation to maximize the benefit of $N_2$-fixation should include bradyrhizobial strain with high $N_2$-fixation rates and ability to compete with the indigenous rhizobial populations. In this study, co-inoculation of Plant Growth Promoting Rhizobacteria (PGPR) *Azospirillum* sp. with either of *Bradyrhizobium japonicum* CB 1809 or USDA 110 increased shoot and root dry weight of soybean over non-inoculated control under pot condition with no indigenous soybean- nodulating bradyrhizobia. Moreover, competition for nodulation and the effects on rhizosphere soil eubacterial community structures by using single or co-inoculation of *B. japonicum* and *Azospirillum* sp. under rhizobia-established Myanmar and Thailand soils were investigated. By inoculation of *gus*-marked USDA 110 singly or its co-inoculation gave 93.21-94.75% and 74.21-100% in nodule

occupancy, and 23.50-41.95% and 50.37-73.24% promoting in biomass dry weight over non-inoculated control in Myanmar and Thailand soil samples, respectively. Each of all tested inoculum levels, i.e., $10^6$, $10^7$ and $10^8$ cfu $ml^{-1}$ of *Azospirillum* sp., enhanced nodulation in combination with USDA 110 with a corresponding increase in 73.8%, 62.25% and 95.34%; and 51.52%, 62.38% and 79.46% over non-inoculated control, respectively in Myanmar and Thailand soil, respectively. In addition, soybean rhizosphere soil eubacterial community structures were not shifted by bacterial inoculation. Therefore, *Azospirillum* sp. could be used in co-inoculant production with *B. japonicum* for soybean.

## 4.2 Introduction

Maximum benefit of $N_2$-fixation by soybean often requires the inclusion of selected strains of *Bradyrhizobium* in seed inoculants. The main criterion used in selection of *Bradyrhizobium* strains for legume inoculation is the ability to form an effective symbiosis with the hosts for which the inoculants is recommended. However, inoculation may not always lead to improved nodulation or enhanced $N_2$-fixation because of the presence of indigenous rhizobia which are more competitive than the inoculants strain (Roughley et al., 1976). Both competitiveness and symbiotic effectiveness were independent traits (Castro et al., 2000); therefore, the *Rhizobium* strain selected for inoculants should not only has high $N_2$-fixation rates, but also be able to compete with the indigenous rhizobia populations (Vlassak and Venderleyden, 1997).

Nowadays, Plant Growth Promoting Rhizobacteria (PGPR) play an important role as they have several mechanisms to promote the plant growth (Glick, 1995).

*Azospirillum* is one of the PGPR and considered as a *Rhizobium* helper by stimulating nodulation, nodule function, and possibly plant metabolism (Andreeva et al., 1993). Effects of *Azospirillum* inoculation are mainly attributed to improved root development and enhanced water and mineral uptake. Secretion of plant growth promoting substances, mainly indole-3-acetic acid (IAA), is strongly associated with the positive response by the plant (Spaepen et al., 2009). Phytohormones produced by *Azospirillum* promoted epidermal-cell differentiation in root hairs that increased the number of potential sites for rhizobial infection (Yahalom et al., 1991) leading to forming more nodules (Andreeva et al., 1993). *A. brasilense* Az39 and *B. japonicum* E109 inoculated singly or in combination have the capacity to promote seed germination and early seedling growth in soybean and corn (Cassan et al., 2009). Moreover, dual inoculation of soybean with *B. japonicum* and *A. brasilense* gave a significantly higher proportion of nodules attached to the main root, and increased number of the most active nodules, and increased 23% of nitrogen content of soybean plants over *B. japonicum* single inoculated plant (Groppa et al., 1998).

Currently, *B. japonicum* strains CB 1809 and USDA 110 are being used in "Rhizobial Inoculant Production" for soybean in Myanmar and Thailand, respectively. However, in both countries, there were no reports on promotion effects on soybean through co-inoculation with *B. japonicum* and any PGPR, and no literature on studying of rhizosphere soil microbial community structures in any leguminous plants with respect to rhizobial inoculations. In this study, *Azospirillum* sp., one of the effective PGPR which was being commercially used in PGPR inoculants production by Suranaree University of Technology (SUT), Thailand (Teaumroong et al., 2009), was selected for co-inoculation with *B. japonicum*.

Moreover, it is needed to study the changes of microbial community caused by inoculation of rhizobial inoculants as their potential ecological risks on microbial diversity should not be neglected. Therefore, this study was aimed to evaluate the co-inoculation effect of *B. japonicum* and *Azospirillum* sp. on soybean nodulation and plant growth under no indigenous soybean nodulating bradyrhizobia soil conditions and to detect the competitive nodulation occupancy of co-inoculated *B. japonicum* strain USDA 110 and *Azospirillum* sp. on soybean as well as to observe the changes of rhizosphere soil bacterial community structures.

## 4.3 Materials and Methods

### 4.3.1 Bacterial strains, media, and growth conditions

Two *B. japonicum* strains of CB 1809 and USDA 110 those were currently using in rhizobial inoculants production for soybean at Department of Agricultural Research (DAR), Myanmar and Thailand were cultured in Yeast Extract Manitol (YEM) media (Appendix 1) (Vincent, 1970) and *Azospirillum* sp. that was supported from School of Biotechnology Laboratory, Suranaree University of Technology (SUT), Thailand was cultured in Nutrient broth (Appendix 4). Those cultures were maintained by periodic transferred and stored in the refrigerator for further studies.

### 4.3.2 Soil samples collection and analysis

The soil samples for preliminary pot experiment with minimum or absence of indigenous soybean nodulating bradyrhizobia were collected from the field of Muang District, Nakhon Ratchasima, Thailand (14° 52' 10" N and 102° 00' 42.24"

E) which has no history of leguminous cultivation. The soil was loamy sand in texture, having a pH 5.25 with 0.42% organic matter content and 4.03 and 34.5 ppm of available P and exchangeable K, respectively.

For nodulation competition study, two soil samples from soybean nodulating bradyrhizobia-established soils were collected from Kyauk Me Agricultural Research Farm (22° 32' 20.93" N and 97° 01' 42.10" E), Department of Agricultural Research (DAR), Kyauk Me Township, Myanmar and Farmer's soybean field, Chiang Mai (18° 48' 01.28" N and 98° 39' 59.00" E), Thailand while soybean was grown as a standing crop to maximize the rhizobial and soil bacterial population. Soil samples were kept in clean polyethylene bags and stored at 4°C until used. Soil physicochemical characterization showed that Myanmar soil has pH 4.72 with 2.88% organic matter; and 21.43 and 164.38 ppm of available P and exchangeable K, respectively. In Thailand soil, soil pH was 4.96 with 2.46% organic matter content, and available P and exchangeable K contents were 27.27 and 73.47 ppm, respectively.

### 4.3.3 Quantification of the number of indigenous soybean nodulating rhizobia

The number of indigenous soybean nodulating rhizobia in experimental soil samples was determined by a modification of the plant infection test using a most probable-number (MPN) technique (Vincent, 1970). One milliliter aliquot of each dilution was inoculated onto pre-sterilized soybean seeds in sterilized growth pouch and grown axenically in light room condition. Two seeds per pouch were grown and four seeds (quadruplicate) were inoculated for each dilution. Non-inoculated control was also included. Plants were grown in growth chamber at 27/20°C light room under

16/8 h light/dark photoperiod, and MPN estimations based on nodulation were determined at 3 weeks after inoculation.

### 4.3.4 Co-inoculation effects of *B. japonicum* and *Azospirillum* sp. on soybean under indigenous soybean nodulating rhizobia non-established soil

A preliminary pot experiment was conducted during June-July, 2011 to evaluate the co-inoculation effects of *B. japonicum* CB 1809 and USDA 110, and *Azospirillum* sp. on soybean. Nine kg of soils were put into the pot (20 cm diameter x 20 cm height). Ten pre-sterilized and pre-germinated soybean seeds (*Glycine max*, Chiang Mai 60) were sown in each pot and one milliliter of the bacterial broth culture ($10^8$ colony forming unit (cfu) $ml^{-1}$) was inoculated onto each seed according to treatments. For single inoculation, the seeds were inoculated separately with $10^8$ cfu $ml^{-1}$ of *Azospirillum* sp., CB 1809, and USDA 110. For co-inoculation, seeds were inoculated by 1:1 ratio of either of CB 1809 or USDA 110 with *Azospirillum* sp. Non-inoculated control was also included. The pots were laid out in a Completely Randomized Design (CRD) with three replications. The plants were watered by tap water whenever necessary and regular agricultural practices were done except pesticide spraying. Plants were sampled at 45 DAI and the nodule number, nodule dry weight, and shoot and root dry weights were recorded. Statistical significance was determined by analysis of variance (ANOVA) and means were compared by the Duncan's Multiple Range Test (DMRT) ($p \leq 0.05$) (Duncan, 1955).

#### 4.3.4.1 Rep-PCR amplication

The bacterial DNA were extracted from *B. japonicum* CB 1809 and USDA 110, and *Azospirillum* sp. Rep-PCR DNA fingerprint was used to investigate the genetic differences between *B. japonicum* strains USDA 110 and CB 1809. Rep-PCR fingerprints were obtained by using BOX-AIR primer (5′-CTA CGG CAA GGC GAC GCT GAC G- 3′) (Sadowsky et al., 1996). The PCR reaction contained 50 ng of DNA template, 50 pmol of primer, 2.5 mM of dNTP, 1x PCR buffer, and 2.5 U Taq DNA polymerase (Promega, USA) in total volume of 50 µl. Each PCR was performed with GeneAmpPCR system 9600 (Perkin Elmer, USA). The PCR reaction condition was used as follows: 95°C for 2 min 1 cycle, 94°C for 30 s, 53°C for 1 min, 56°C for 8 min 35 cycles and final 65°C for 16 min 1 cycle. Products from PCR were separated on 2% agarose gel, stained with ethidium bromide and viewed under UV light in gel documentation.

#### 4.3.4.2 Construction of *gus*-marked *B. japonicum* strains

Two bacterial strains, *Escherichia coli* S17-1 donor strain (harboring plasmid pCAM120, *Tn*5 fusion with *gus*-gene) which is resistant to 20 µg $ml^{-1}$ of both Streptomycin and Spectinomycin, and recipient *B. japonicum* strain USDA 110 which is resistant to Gentamycin (20 µg $ml^{-1}$), were grown to stationary phase in Luria-Bertani broth (LB) (Sambrook et al., 1989) and YEM broth for overnight and 7 days, at 37°C and 28±2°C, respectively. The method for biparental mating was followed by method of Krause et al. (2002). Blue forming colonies on (HEPES-MES) HM solid media (Cole and Elkan, 1973) containing Streptomycin (200 µg $ml^{-1}$), Gentamycin (30 µg $ml^{-1}$) and X-gluc (5-Bromo-4-chloro-3-indolyl-

beta-D-glucoside) (20 μg $ml^{-1}$) were selected as transconjugants and sub-cultured on YMA medium to check purity and *gus*-stability. Stable blue colonies were then picked up and inoculated into YEM broth with appropriate antibiotics and stored with 50% sterilized glycerol at -70°C until needed. The nodule formation of *gus*-marked *B. japonicum* strains were checked on both siratro (*Macroptilium atropurpureum*) and soybean hosts by using growth pouch method (Vincent, 1970).

### 4.3.5 Competitive nodulation ability of *B. japonicum* strain by co-inoculation with PGPR in rhizobia-established soils

Pot experiment was conducted to determine the competitive ability of single and/or co-inoculation effects of *B. japonicum* strain USDA 110 with *Azospirillum* sp. on soybean nodulation and rhizosphere eubacterial community structure. The *gus*-marked *B. japonicum* USDA 110, wild type USDA 110, and *Azospirillum* sp. were cultured in YEM broth containing appropriate antibiotics, normal YEM broth and LG (N-free) broth (Hirschi et al., 1991), respectively and shaken on the rotary shaker (180 rpm) at 28±2°C for 7-10 days for bradyrhizobia, and 2 days for *Azospirillum* sp. About 250 g of soil was put into the pre-sterilized modified Leonard's jar and four pre-sterilized and pre-germinated soybean seeds were grown in each jar. The cultures were centrifuged (4,000 x g for 5 mins) and washed with 0.85% (w/v) sterilized saline to remove the antibiotic and excess media from the culture media, and the cell pellet was resuspended in 0.85% (w/v) saline. One milliliter of the bacterial broth culture ($10^8$ cfu $ml^{-1}$) was inoculated onto each seed according to treatments. For the single inoculation, the seedlings were inoculated separately with $10^8$ cfu $ml^{-1}$ of *Azospirillum* sp., USDA 110 wild type (wt) and *gus*-

marked USDA 110 (tr). For the co-inoculation, $10^8$ cfu $ml^{-1}$ of USDA 110 (tr) were mixed in a ratio of 1:1 with three different inoculum levels ($10^6$, $10^7$, and $10^8$ cfu $ml^{-1}$) of *Azospirillum* sp. Bulk soil (no planted and non-inoculated control) and non-inoculated controls were also included.

The experiment was conducted as a CRD design with three replications. Plants were grown on a growth shelf at 27/20°C in light room condition under 16/8 h light/dark photoperiod. Additional experiment was set up as the same treatments; however, the vermiculite was used as growth media instead of soils under sterilized conditions. At 30 DAI, soybean plants were carefully uprooted from the jars from both sterilized and non-sterilized experiments, roots were gently washed with water not to remove the root hairs and nodules, and the nodulation competitiveness of inoculated bradyrhizobial strain was detected by *gus*-staining method. Nodule numbers per plant were counted and nodule dry weight per plant (mg) and biomass dry weight per plant (mg) were determined after oven dried at 70°C for 48 h. Total root length (m) for each fresh root samples was measured by scanning for three times with "Comair Root Measurement Scanner" (Commonwealth Aircraft Crop Ltd., Melbourne, Australia).

#### 4.3.5.1 Detection of *gus*-activity inside soybean root nodules

For the detection of inoculated *gus*-marked bradyrhizobia, root nodules from each treatment from non-sterilized soil experiments were cut in a half. The nodules were immersed in a microtiter plate containing the *gus*-assay solution (40 µl X-Gluc 20 mg $ml^{-1}$ in N, N-Dimethylformamide, SDS 20 mg, methanol 2 ml, 1M sodium phosphate buffer 0.2 ml and distilled water 7.76 ml), in vacuum for 2 h before

incubated for overnight at 28°C. Nodule formation by inoculated transconjugant *B. japonicum* USDA 110 was compared with those by normal *B. japonicum* USDA 110 (wt) and competitiveness was compared by non-inoculated control. Nodulation occupancy was calculated by percent nodulation formed by *gus*-marked USDA 110. Results were statistically analyzed by analysis of variance (ANOVA) and least significant different (LSD) test was applied at 0.05 level of significant. Root nodules from sterilized conditions were also stained by *gus*-buffer to calibrate the *gus*-activity expression in the soybean nodules.

### 4.3.6 Total community DNA extraction and Denaturing Gradient Gel Electrophoresis (DGGE) analysis

For soil microbial (eubacterial) community structure analysis by DGGE method, the sampling was done without disturbing the root system and the rhizosphere soil samples were taken weekly interval for five times including at the day of sowing until one month after inoculation, i.e., at 0, 1$^{st}$, 2$^{nd}$, 3$^{rd}$, and 4$^{th}$ weeks after inoculation. Total genomic DNA of *B. japonicum* strain USDA 110 and *Azospirillum* sp. were extracted (Prakamhang et al., 2009) and kept at -20$^{o}$C before using as markers for next experiments. Soil rhizosphere microbial DNA from plant samples was directly extracted from 0.5 g rhizosphere soil using with the Ultra Clean soil DNA kit (MO BIO Laboratories, Solana Beach, Califonia, USA), following the manufacturer's instructions. Group-specific PCR-amplification of eubacterial 16S rRNA gene fragments (V6-V8 variable regions of the 16S rRNA gene) which yielded the products of approximately 400 bp (Heuer et al., 1997) was done followed using universal primers F984 GC and R1378. A GC-clamp (Costa et al., 2005) was added to

the 5'end of the forward primer. The reaction mixture and PCR conditions were conducted along with the protocol of Piromyou et al. (2011). Aliquots (3 µl) of the amplification products were analyzed first by electrophoresis in 1% agarose gels and quantified using a 1 kb ladder marker and PCR products were store at -20°C before DGGE analysis.

The PCR products of inoculated bacteria and those of soil bacterial community were subjected separately to DGGE analysis. DGGE was performed using a Dcode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA). About 45 µl of PCR products were loaded onto 6% (w/v) polyacrylamide (Acrylamide: Bisacrylamide ratio, 37.5:1 Bio-Rad Laboratories, Inc.), and 1 mm thick (20 x 20 cm) gel in TAE buffer. The polyacrylamide gel was prepared with a linear denaturing gradient ranging from 40% to 70% (Urea and Formamide). A 100% denaturant consisted of 40% (v/v) formamide and 7M urea. PCR products of the rhizosphere soil eubacterial community were loaded in the middle lanes and those of inoculated bacteria were loaded at the both left and right sides of the sample lanes as "Marker bacteria". DGGE was conducted at a constant voltage of 75 V for 10 min and thereafter 110 V for 18 h maintained at 60°C. Subsequently, the gel was stained with SYBR Green (3µl in 15µl 1xTAE buffer) for 30 min and rinsed for 3 min in running water before photographing.

#### 4.3.6.1 Sequencing of DGGE bands

The microbial community composition in DGGE gel was analyzed by cloning and partial sequencing of the 16S rRNA genes. Bands of interest in DGGE gels were carefully excised from the UV illuminated acrylamide gels by

sterilized pipette tip (10 µl) and DNA was eluted from the excised gel by incubation in 30 µl dd$H_2O$ at 4°C overnight. Eluted DNA (~0.5 µl supernatant) was used as a template DNA for PCR amplification as described above by using with the same primer pair without GC-clamp, F984 and 1378 R for bacterial 16S rRNA genes amplifications. The purified PCR products were ligated into the pGEM®-T Easy Vector System (Promega, USA) and then further transformed into *E. coli* DH5α competent cells, following the manufacturer's protocol. PCR amplification and DNA sequencing was performed by MACROGEN Company (Korea). Sequences were generated and the most closely related sequences were obtained from the NCBI database.

### 4.3.7 Statistical analyses

The experimental data of nodulation and plant growth parameters were statistically analyzed as described by Stell et al., 1980, and means were compared by DMRT (Duncan, 1955). The cluster analysis and dendrogram generation of DGGE fingerprint profiles, and Principle Component Analysis (PCA) were carried out by the NTSYSpc (2.2, Exeter Software, USA) (Rohlf, 2000). The Shannon index (H') (Shannon and Weaver, 1963) was calculated according to the following equation:

$$H' = -\sum P_i \log P_i$$

where $P_i$ is the proportion represented by each DGGE band relative to the total number of bands. The indices obtained were statistically analyzed as described for other univariate data.

## 4.4 Results

### 4.4.1 Co-inoculation effects of *B. japonicum* and *Azospirillum* sp. on soybean under indigenous soybean nodulating rhizobia non-established soil

The soils used in this study were collected from the field of Muang District, Nakhon Ratchasima, Thailand with no history of leguminous cultivation and thus no nodule formation was observed in both MPN plant infection counting (data not shown) and preliminary pot experiment as expected. Increases in numbers of nodule and nodule dry weight were observed by both co-inoculations even those were not significantly different from bradyrhizobial single inoculation (Table 6). Positive responses on shoot and root dry weights of soybean were obtained by co-inoculation of *Azospirillum* sp. with either of USDA 110 or CB 1809 (Figure 12). Combined inoculation of USDA 110 and *Azospirillum* sp. gave the maximum shoot and root dry weight and that was significantly higher than USDA 110 inoculation alone. Shoot and root growth was increased from 4.77 to 6.51 and from 2.32 to 3.27 times upon non-inoculated control, respectively. Although co-inoculation of CB 1809 with *Azospirillum* sp. promoted the nodulation and plant growth, it gave less benefit compared to those of USDA 110 and *Azospirillum* sp. co-inoculation.

**Table 6.** Co-inoculation effects of *B. japonicum* (CB 1809 and USDA 110) and selected PGPR on soybean nodulation and plant growth under pot conditions at 45 DAI (June-July, 2011)

| Treatment | Nodule No. per plant | Nodule dry weight per plant (mg) | Biomass dry weight per plant (mg) | Root dry weight per plant (mg) |
|---|---|---|---|---|
| Non-inoculated control | $0.00^{b}$±0.00 | $0.00^{e}$±0.00 | $812.20^{c}$±35.84 | $217.80^{c}$±8.43 |
| *Azospirillum* sp. | $0.00^{b}$±0.00 | $0.00^{e}$±0.00 | $1054.35^{c}$±111.27 | $202.48^{c}$±6.43 |
| CB 1809 | $84.50^{a}$±4.21 | $237.37^{b}$±27.98 | $4598.30^{ab}$±387.70 | $611.60^{ab}$±53.98 |
| USDA 110 | $78.75^{a}$±19.20 | $248.03^{ab}$±29.38 | $3873.68^{b}$±327.09 | $506.30^{b}$±24.03 |
| CB 1809 + *Azospirillum* sp. | $112.50^{a}$±10.90 | $264.25^{ab}$±30.54 | $4892.85^{a}$±305.24 | $640.63^{a}$±59.35 |
| USDA 110+ *Azospirillum* sp. | $90.50^{a}$±7.82 | $346.90^{a}$±34.35 | $5289.80^{a}$±666.61 | $712.33^{a}$±46.97 |

Values followed by the same letter within the same columns are not significantly different by Duncan's multiple range test ($P \leq 0.05$).

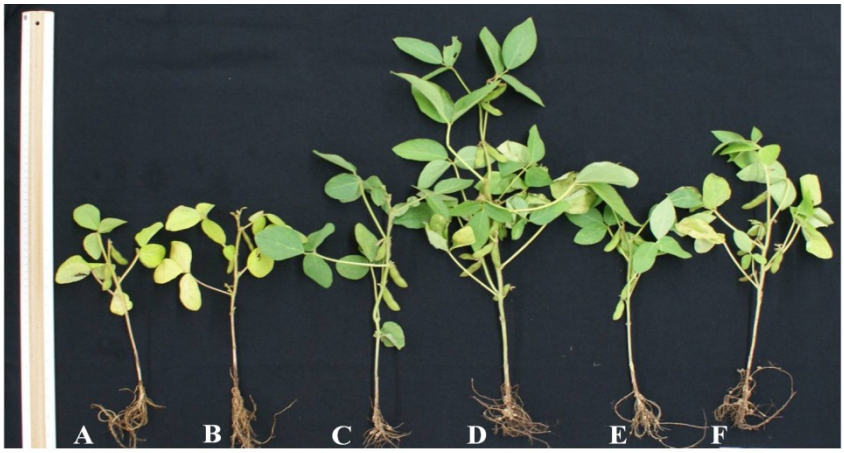


**Figure 12.** Co-inoculation effects of *B. japonicum* and *Azospirillum* sp. on soybean plant growth under indigenous soybean nodulating rhizobia non-established soil**: A.** Non-inoculated control, **B.** *Azospirillum* sp. inoculation alone**, C.** *B. japonicum* USDA 110 inoculation alone, **D.** co-inoculation of *B. japonicum* USDA 110 and *Azospirillum* sp., **E.** *B. japonicum* CB 1809 inoculation alone, **F.** co-inoculation of *B. japonicum* CB 1809 and *Azospirillum* sp.

#### 4.4.1.1 Rep-PCR amplification and genetic marking of *B. japonicum* strain

Results from preliminary screening tests showed the different responses of *B. japonicum* strains CB 1809 and USDA 110 on co-inoculation with different rhizobacteria. Therefore, Rep-PCR fingerprinting was used to investigate the genetic differences between *B. japonicum* strains CB 1809 and USDA 110. The resulted BOX-PCR fingerprints of two bradyrhizobia showed differences in banding patterns (Figure 13). The previous results from pot experiment revealed that *Azospirillum* sp. co-inoculated with *B. japonicum* strain USDA 110 gave higher shoot and root dry weight than with CB 1809. Therefore, only USDA 110 was selected for further studies.

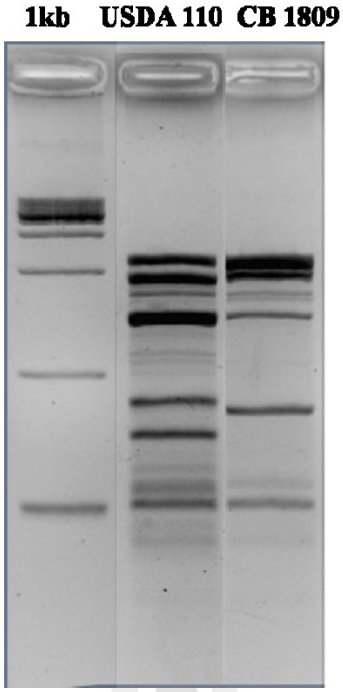


**Figure 13.** Comparison of Rep-PCR products of *B. japonicum* USDA 110 and CB 1809 with 1 kb ladder marker.

### 4.4.2 Competition for nodule occupancy analysis in rhizobia-established Myanmar and Thailand soils

Plant infection test used to assess the presence of indigenous soybean-nodulating bradyrhizobial populations in tested soil samples showed that both Myanmar and Thailand soils have indigenous soybean rhizobial population ($3.1 \times 10^6$ and $1.7 \times 10^5$ cells per gram of dry soil, respectively). Under sterilized conditions, USDA 110 (tr) gave 100% nodule occupancy on soybean. Moreover, soybean inoculated with USDA 110 (tr) produced similar nodule number and biomass compared to those of the unmarked USDA 110 (wt) strain in both sterilized growth media and un-sterilized soil conditions (Table 7, 8 and 9).

Under sterilized growth media conditions, significant differences in nodulation were observed among the treatments. Co-inoculation of USDA 110 (tr) with *Azospirillum* sp. ($10^7$ cfu $ml^{-1}$) gave the significantly highest nodule number (Table 7). Maximum and significantly highest biomass dry weight was given by co-inoculation of USDA 110 (tr) and *Azospirillum* sp. ($10^8$ cfu $ml^{-1}$).

All of the inoculation treatments increased in nodule number and nodule dry weight in compared to non-inoculated control in both Myanmar and Thailand soils (Table 8 and 9). Although when soybean seeds were inoculated singly with PGPR strain *Azospirillum* sp., the plants showed different responses on growth, root development (Figure 14) and increased the number of nodules compared to non-inoculated control in both tested soils. The root length measured by using "Comair Root Measurement Scanner" clearly showed the positive response to inoculation of *Azospirillum* sp. In rhizobia-established Myanmar soil, co-inoculation of *B. japonicum* strain USDA 110 (tr) with *Azospirillum* sp. in $10^8$ cfu $ml^{-1}$ gave maximum nodule formation and it was significantly different compared to non-inoculated control. Combined inoculation of USDA 110 (tr) with *Azospirillum* sp. ($10^8$) gave the maximum enhancement of soybean nodulation and plant growth followed by $10^6$ and/or $10^7$ cfu $ml^{-1}$ of *Azospirillum* sp. In rhizobia-established Thailand soil, co-inoculation of USDA 110 (tr) with different tested inoculum levels of *Azospirillum* sp. ($10^6$-$10^8$ cfu $ml^{-1}$) gave significantly higher nodule formation and biomass dry weight compared to those of non-inoculated control.

Each of all tested inoculum levels; i.e., $10^6$, $10^7$, and $10^8$ cfu $ml^{-1}$ of *Azospirillum* sp. enhanced nodulation in combination with *B. japonicum* USDA 110 with a corresponding increase in 73.80, 62.25 and 95.34%; and 51.52, 62.38 and

79.46% over non-inoculated control in Myanmar and Thailand soil, respectively. Overall, the results obtained in the present study clearly indicated that all of the tested inoculum levels of PGPR *Azospirillum* sp. influenced the biomass development and nodulation when co-inoculated with *B. japonicum* USDA 110 ($10^8$ cfu $ml^{-1}$). In term of nodulation occupancy in rhizobia-established Myanmar soil, 93.21-94.75% of the nodules were occupied by *gus*-marked *B. japonicum* USDA 110 when inoculated singly or combination with and percent occupancies were not significantly different among them. However, in rhizobia-established Thailand soil, significant differences in competitive abilities of *gus*-marked *B. japonicum* were observed in a range of 74.21-100% nodule occupation when co-inoculated with different inoculum levels of *Azospirillum* sp. In addition, co-inoculations gave 23.50-41.95% and 50.37-73.24% biomass dry weight over non-inoculated control in rhizobia-established Myanmar and Thailand soil, respectively.

**Table 7. Single and co-inoculation of *B. japonicum* and *Azospirillum* sp. on soybean nodulation in sterilized growth media**

| Treatments | Nodule No. per plant | Nodule dry weight per plant (mg) | Biomass dry weight per plant (mg) |
|---|---|---|---|
| Control | $0.00^{d} \pm 0.00$ | $0.00^{c} \pm 0.00$ | $241.30^{d} \pm 53.70$ |
| *Azospirillum* sp. ($10^8$) | $0.00^{d} \pm 0.00$ | $0.00^{c} \pm 0.00$ | $249.52^{d} \pm 19.68$ |
| USDA 110 wt ($10^8$) | $12.67^{c} \pm 2.09$ | $30.75^{ab} \pm 1.28$ | $517.20^{bc} \pm 34.64$ |
| USDA 110 tr ($10^8$) | $18.33^{c} \pm 1.45$ | $27.65^{b} \pm 0.80$ | $449.15^{c} \pm 18.14$ |
| USDA 110 tr ($10^8$) + *Azospirillum* sp. ($10^6$) | $32.00^{b} \pm 2.08$ | $27.03^{b} \pm 3.68$ | $494.13^{bc} \pm 91.38$ |
| USDA 110 tr ($10^8$) + *Azospirillum* sp. ($10^7$) | $44.83^{a} \pm 4.91$ | $39.40^{a} \pm 5.38$ | $662.38^{c} \pm 87.50$ |
| USDA 110 tr ($10^8$) + *Azospirillum* sp. ($10^8$) | $32.50^{b} \pm 1.32$ | $42.08^{a} \pm 1.52$ | $873.92^{a} \pm 49.84$ |

Values followed by the same letter within the same columns are not significantly different by Duncan's multiple range test ($P \leq 0.05$).

**Table 8.** Competitive ability, nodulation efficiency, and plant growth enhancement of *gus*-marked *B. japonicum* strain USDA 110 and *Azospirillum* sp. inoculation on soybean in rhizobia-established Myanmar soil (30 DAI)

| Treatments | Nodule number per plant | % nodule occupancy by *gus*-marked USDA 110 | Nodule dry weight (mg) | Plant height per plant (cm) | Biomass dry weight per plant (mg) | Root length per plant (m) |
|---|---|---|---|---|---|---|
| Control | $19.31^{b}$±3.60 | - | $28.90^{b}$±7.11 | $30.33^{d}$±0.75 | $568.20^{c}$±41.69 | $15.85^{b}$±1.74 |
| *Azospirillum* sp. ($10^8$) | $25.67^{ab}$±5.78 | - | $31.82^{b}$±7.73 | $29.75^{d}$±0.36 | $593.22^{bc}$±29.27 | $29.90^{ab}$±2.94 |
| USDA 110 wt ($10^8$) | $30.21^{ab}$±3.63 | - | $41.36^{ab}$±5.11 | $32.50^{cd}$±0.73 | $701.70^{ab}$±35.62 | $24.28^{ab}$±1.00 |
| USDA 110 tr ($10^8$) | $34.58^{ab}$±2.52 | $94.50^{a}$±3.67 | $33.71^{b}$±3.33 | $30.25^{d}$±0.70 | $705.12^{ab}$±39.26 | $29.58^{ab}$±7.70 |
| USDA 110 tr ($10^8$)+ *Azospirillum* sp. ($10^6$) | $29.11^{ab}$±3.36 | $96.31^{a}$±1.87 | $44.93^{ab}$±5.25 | $36.58^{ab}$±1.14 | $755.28^{a}$±61.45 | $38.67^{a}$±7.68 |
| USDA 110 tr ($10^8$)+ *Azospirillum* sp. ($10^7$) | $31.33^{ab}$±3.51 | $93.21^{a}$±3.92 | $39.80^{ab}$±4.68 | $34.25^{bc}$±0.76 | $748.16^{a}$ ±40.18 | $32.38^{a}$±4.71 |
| USDA 110 tr ($10^8$)+ *Azospirillum* sp. ($10^8$) | $37.72^{a}$ ± 6.67 | $94.75^{a}$±2.24 | $58.00^{a}$±6.98 | $38.83^{a}$±2.34 | $806.58^{a}$±30.32 | $32.43^{a}$±2.12 |

Values followed by the same letter within the same columns are not significantly different by Duncan's multiple range test ($P \leq 0.05$).

**Table 9.** Competitive ability, nodulation efficiency, and plant growth enhancement of *gus*- marked *B. japonicum* strain USDA 110 and *Azospirillum* sp. inoculation on soybean in rhizobia-established Thailand soil (30 DAI)

| Treatments | Nodule number per plant | % nodule occupancy by *gus*-marked USDA 110 | Nodule dry weight (mg) | Plant height per plant (cm) | Biomass dry weight per plant (mg) | Root length per plant (m) |
|---|---|---|---|---|---|---|
| Control | $20.79^{c}\pm4.10$ | - | $26.15^{b}\pm5.68$ | $29.30^{c}\pm1.86$ | $502.85^{b}\pm24.55$ | $14.25^{c}\pm1.18$ |
| *Azospirillum* sp. ($10^8$) | $25.28^{bc}\pm2.67$ | - | $32.05^{ab}\pm2.82$ | $35.25^{b}\pm2.09$ | $690.89^{a}\pm39.60$ | $17.18^{bc}\pm3.41$ |
| USDA 110 wt ($10^8$) | $31.87^{ab}\pm3.41$ | - | $33.02^{ab}\pm2.35$ | $37.75^{ab}\pm2.22$ | $761.82^{a}\pm43.08$ | $21.03^{a}\pm1.90$ |
| USDA 110 tr ($10^8$) | $28.71^{abc}\pm3.69$ | $86.77^{ab}\pm3.46$ | $37.68^{a}\pm2.22$ | $35.67^{b}\pm2.36$ | $756.14^{a}\pm86.09$ | $15.63^{bc}\pm1.09$ |
| USDA 110 tr ($10^8$)+ *Azospirillum* sp. ($10^6$) | $31.50^{ab}\pm3.48$ | $74.21^{b}\pm4.60$ | $34.68^{ab}\pm3.14$ | $43.08^{a}\pm1.91$ | $805.92^{a}\pm75.16$ | $16.45^{bc}\pm0.34$ |
| USDA 110 tr ($10^8$)+ *Azospirillum* sp. ($10^7$) | $33.75^{ab}\pm3.48$ | $100.00^{a}\pm0.00$ | $33.02^{ab}\pm4.24$ | $43.08^{a}\pm1.65$ | $836.03^{a}\pm67.43$ | $19.88^{abc}\pm2.15$ |
| USDA 110 tr ($10^8$)+ *Azospirillum* sp. ($10^8$) | $37.31^{a}\pm4.64$ | $95.38^{ab}\pm2.75$ | $42.83^{a}\pm2.56$ | $40.17^{ab}\pm1.66$ | $871.13^{a}\pm56.98$ | $24.56^{a}\pm0.61$ |

Values followed by the same letter within the same columns are not significantly different by Duncan's multiple range test ($P \leq 0.05$).

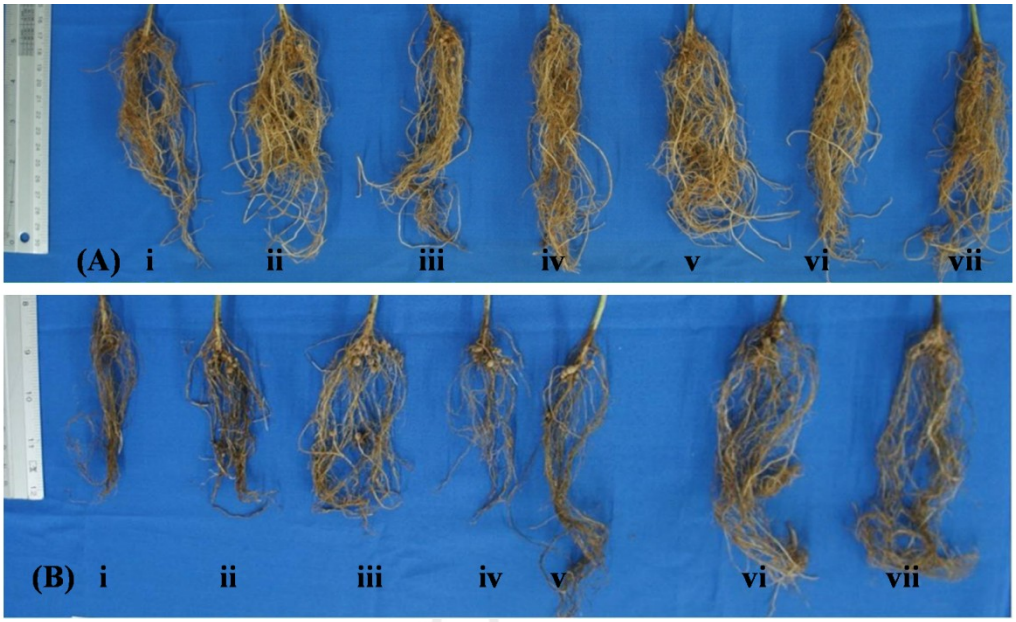


**Figure 14** Soybean root development in **(A)** Myanmar Soil and **(B)** Thailand Soil by inoculation with **(i)** None; **(ii)** *Azospirillum* sp.; **(iii)** *B. japonicum* USDA 110 (wt); **(iv)** *B. japonicum* USDA 110 (tr); **(v)** *B. japonicum* USDA 110 (tr) ($10^8$ cfu $ml^{-1}$) and *Azospirillum* sp. ($10^6$ cfu $ml^{-1}$); **(vi)** *B. japonicum* USDA 110 (tr) ($10^8$ cfu $ml^{-1}$) and *Azospirillum* sp. ($10^7$ cfu $ml^{-1}$); **(vii)** *B. japonicum* USDA 110 (tr) ($10^8$ cfu $ml^{-1}$) and *Azospirillum* sp. ($10^8$ cfu $ml^{-1}$).

### 4.4.3 DGGE analysis

There were 22-39 bands observed in 16S rRNA eubacterial community profiles of Myanmar soil generated by DGGE analysis and it was clearly classified into two main groups with 68% similarities (Figure 15) by cluster analysis. The first cluster included ~73% similarity of week zero bulk soil sample, 1st, and 3rd week samples, and the second cluster included the 2nd and 4th week samples with ~75%

similarity. Except from the 4th week samples, bulk soil samples from other sampling times were clearly separated from inoculated and non-inoculated samples. Prominent DGGE bands were excised for nucleotide sequence determination. Four bands presented in nearly all profiles in Myanmar soil samples were *Bacillus cecembensis* (JX 290163), *Azotobacter nigricans* (JX 290160), *Bradyrhizobium elkanii* (JX 290163) and *Burkhoderia* sp. (JX 290164) with 99, 98, 100 and 95% similarity, respectively.

In the case of Thailand soil, 12-20 bands were observed in DGGE fingerprints of different sampling times which were 76-100% similarities and grouped into two main clusters with 76% similarity, i.e., first cluster included week 0, 1st, 2nd, and 3rd week samples with 80% similarity and second included only 4th week samples (Figure 17). The presence of 100% similarity among the DGGE patterns of different treatments indicated that the eubacterial community structures were not significantly shifted by bacterial inoculation. In Thailand soil samples, two prominent bands were sequenced to be *Bradyrhizobium* sp. (NR 0417851) and *Nitrospira moscoviensis* (JX 290162) with 100 and 99 % similarity, respectively.

Principal component analysis (PCA) separated the DGGE profiles of both Myanmar and Thailand soil samples into four groups. It was gradually and continuously changed from first week to last week sampling in Myanmar soil samples (Figure 16). However, in the case of Thailand soil samples, there were two groups those were not clearly separated between 1st week and 3rd week samples (Figure 18).

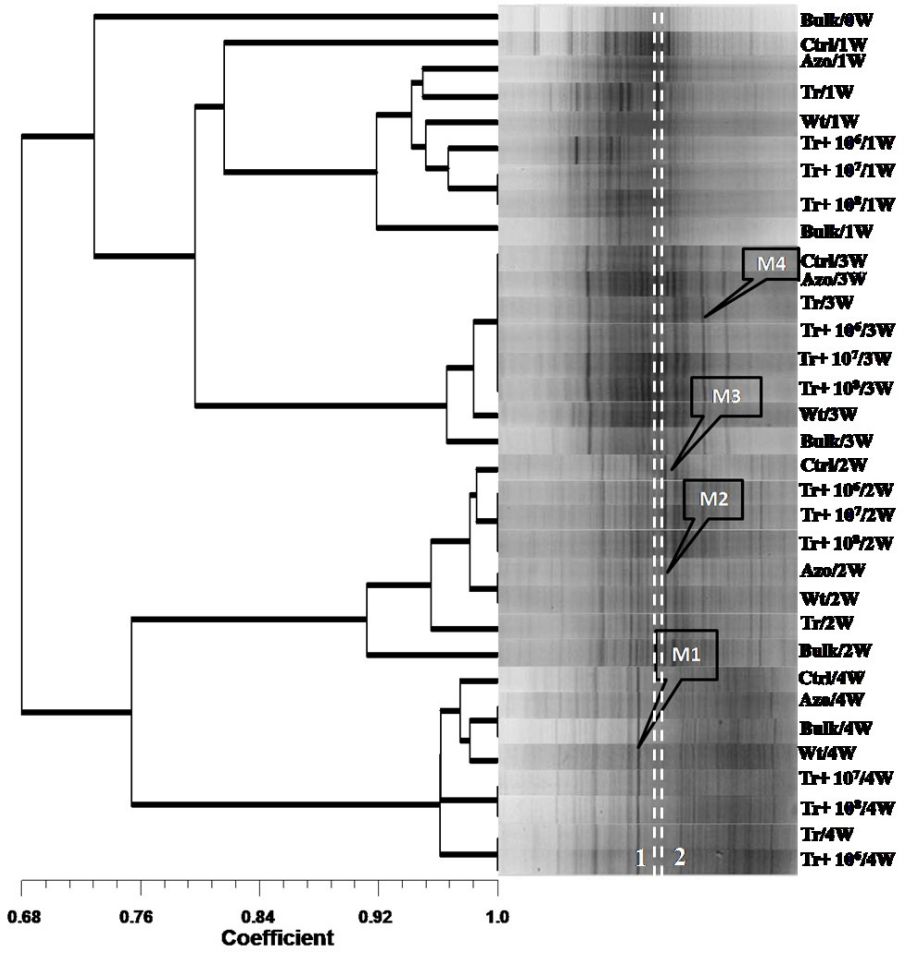


**Figure 15.** Cluster analysis of eubacterial community structures of partial 16S rRNA PCR- DGGE fingerprints of different soybean rhizosphere samples after inoculation with different bacterial inocula, i.e., (Bulk), Bulk Soil; (Ctrl), Control; (Azo), *Azospirillum* sp.; (Wt), USDA 110 wild type; (Tr), *gus*-marked USDA 110; Tr + ($10^6$-$10^8$), co-inoculation of *gus*-marked USDA 110 ($10^8$) with different inoculum levels of *Azospirillum* sp. ($10^6$-$10^8$) at different sampling times (0, 1st , 2nd , 3rd , and 4th weeks after inoculation) under in rhizobia-established Myanmar soil. Labels on fingerprints were subjected to sequence for analysis. Line 1 and 2 refer to inoculated bacteria *Azospirillum* sp. and *B. japonicum*, respectively.

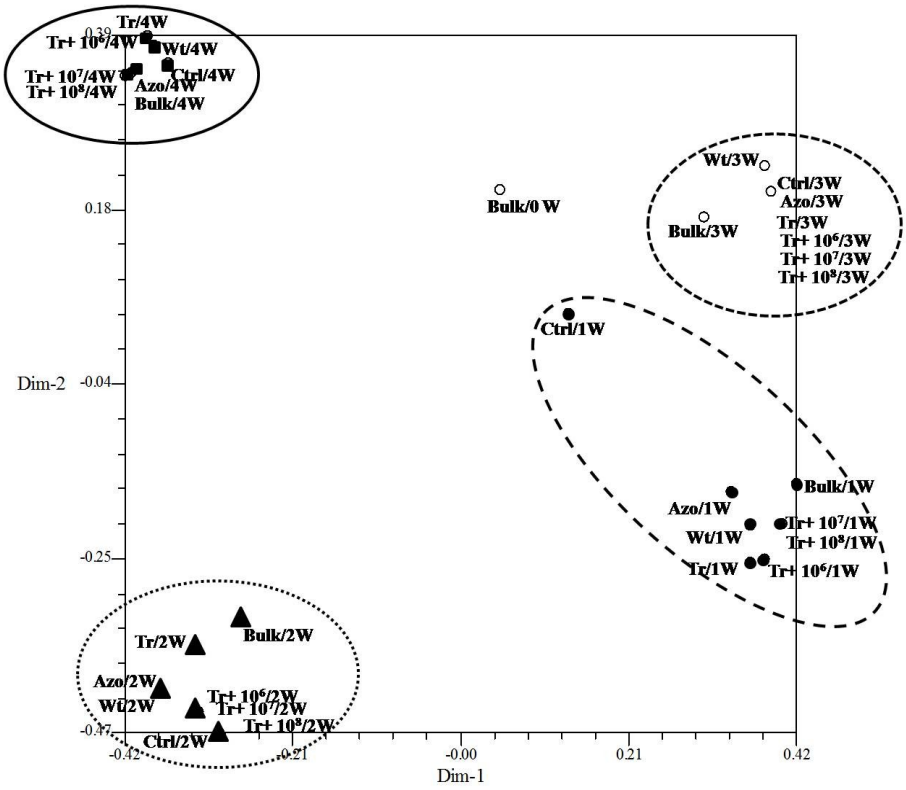


**Figure 16.** Community analysis derived from two-dimentional plot based on the first two principle coordinates from a principle coordinate analysis (PCA) of partial 16S rRNA banding profiles of soybean rhizosphere soil samples in rhizobia-established Myanmar soil. Letters adjacent to marks indicate the treatments: i.e., (Bulk), Bulk Soil; (Ctrl), Control; (Azo), *Azospirillum* sp.; (Wt), USDA 110 wild type; (Tr), *gus*-marked USDA 110; Tr + ($10^6$-$10^8$), co-inoculation of *gus*-marked USDA 110 ($10^8$) with different inoculum levels of *Azospirillum* sp. ($10^6$-$10^8$) at different sampling times represented as (0 Wk) week zero; (■) 1st week; (■) 2nd week; (o) 3rd week and (■) 4th week, respectively. Different samples formed a cluster which is circled by (- - - - , ……, ---- , and ——–) shows a trend of 1st , 2nd, 3rd and 4th week, respectively.

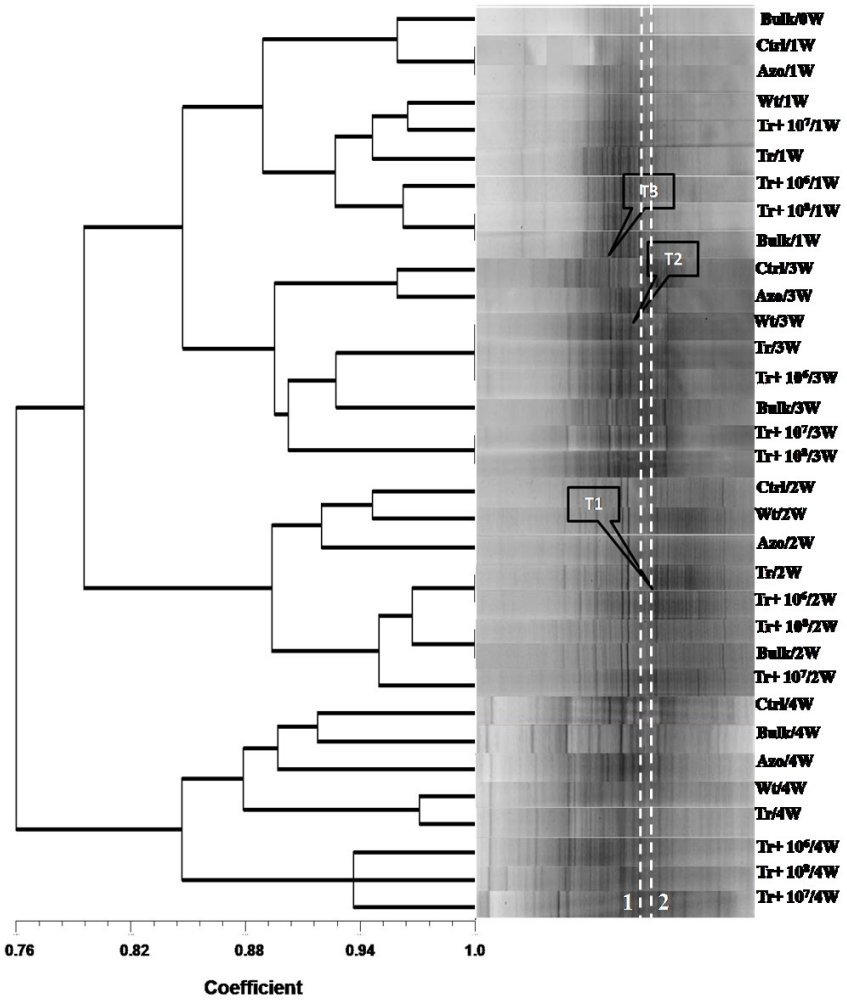


**Figure 17.** Cluster analysis of eubacterial community structures of partial 16S rRNA PCR- DGGE fingerprints of different soybean rhizosphere samples after inoculation with different bacterial inocula, i.e., (Bulk), Bulk Soil; (Ctrl), Control; (Azo), *Azospirillum* sp.; (Wt), USDA 110 wild type; (Tr), *gus*-marked USDA 110; Tr + ($10^6$-$10^8$), co-inoculation of *gus*-marked USDA 110 ($10^8$) with different inoculum levels of *Azospirillum* sp. ($10^6$-$10^8$) at different sampling times (0, 1st , 2nd , 3rd , and 4th weeks after inoculation) under in rhizobia-established Thailand soil. Labels on fingerprints were subjected to sequence for analysis. Line 1 and 2 refer to inoculated bacteria *Azospirillum* sp. and *B. japonicum*, respectively.

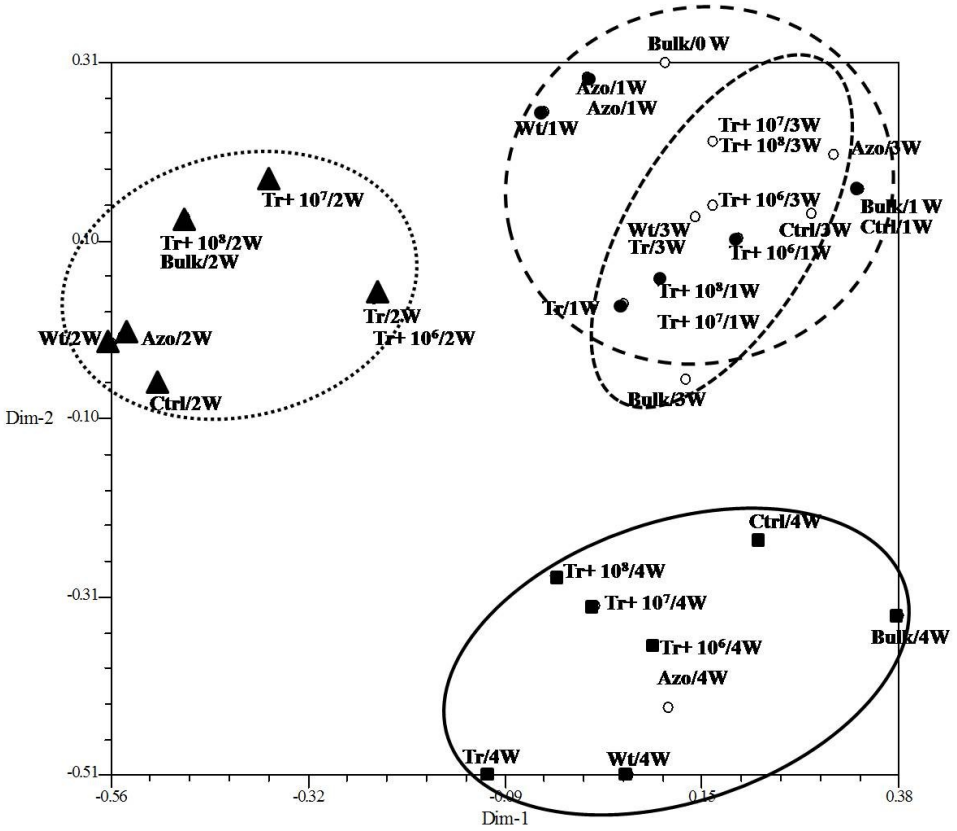


**Figure 18.** Community analysis derived from two-dimentional plot based on the first two principle coordinates from a principle coordinate analysis (PCA) of partial 16S rRNA banding profiles of soybean rhizosphere soil samples in rhizobia-established Thailand soil. Letters adjacent to marks indicate the treatments: i.e., (Bulk), Bulk Soil; (Ctrl), Control; (Azo), *Azospirillum* sp.; (Wt), USDA 110 wild type; (Tr), *gus*-marked USDA 110; Tr + ($10^6$-$10^8$), co-inoculation of *gus*-marked USDA 110 ($10^8$) with different inoculum levels of *Azospirillum* sp. ($10^6$-$10^8$) at different sampling times represented as (0 Wk) week zero; (■) 1st week; (■) 2nd week; (o) 3rd week and (■) 4th week, respectively. Different samples formed a cluster which is circled by (- - - - , ……, ---- , and ——-) shows a trend of 1st , 2nd, 3rd and 4th week, respectively.

## 4.5 Discussion

Prior to study on competitive ability of *B. japonicum* against indigenous bradyrhizobia, each *B. japonicum* strain CB 1809 or USDA 110 was co-inoculated with *Azospirillum* sp. on soybean under indigenous soybean nodulating rhizobia non-established soil conditions. Based on non-inoculated control in pot experiment and MPN plant infection count, the results provided the information about the absence of indigenous soybean nodulating-bradyrhizobia in the tested soils as no nodule formation was observed. In addition, it is indicated that although number of nodule formation was not different among single and co-inoculations, differences observed in nodule dry weight, and shoot and root dry weights revealed the efficient nodulation and $N_2$-fixation obtained by co-inoculation of soybean with *B. japonicum* and *Azospirillum* sp. Increasing of nodule number, nodule dry weight, and root dry weight given by single or co-inoculation either of *B. japonicum* strain CB 1809 or USDA 110 with *Azospirillum* sp. support plant growth of soybean. There have been several reports that described the beneficial effects of *Azospirillum* sp., a well documented member of PGPR, on the symbiosis between *Rhizobium* bacteria and legumes (e.g., Burdman et al., 1997; Tilak et al., 2006; Remans et al., 2007).

The *B. japonicum* strain CB 1809 and its co-inoculation with *Azospirillum* sp. could produce better nodulation but less plant growth compared to *B. japonicum* strain USDA 110 and its co-inoculation. Comparison between fingerprint patterns of CB 1809 and USDA 110 revealed that there will be some genetic differences between these two bradyrhizobia as the patterns of those were different from each other. Therefore, there will be some genes which differently response on co-inoculation of *Azospirillum* sp. As mentioned by Fages (1994) that more consistent results are

necessary for the commercial development of inoculants with *Azospirillum*, USDA 110 was selected to be a promising strain for further co-inoculation studies with *Azospirillum* sp. on competition for nodulation against indigenous bradyrhizobia.

Generally, indigenous soybean nodulating-bradyrhizobia establish in most of the soybean growing fields either with effective or ineffective $N_2$-fixation ability. Inoculation of soybean with rhizobial inoculants is a common practice in most of the soybean growing areas in Myanmar, but only a few percents of rhizobial inoculants for soybean are being produced by Department of Agricultural Research (DAR). However, since 10 years ago, there was not much information on competitive nodulation of inoculated *B. japonicum* strains against indigenous soybean rhizobia on field grown soybean in Myanmar. In Myanmar and Thailand, there has no literature on the bacterial and fungal community structures in soybean rhizosphere with respect to rhizobial inoculations. Plant infection tests revealed the presence of high populations of indigenous soybean-nodulating bradyrhizobia in soil samples of Myanmar and Thailand soybean growing fields. As mentioned by Shamseldin and Werner (2004), in major soybean crop regions, most of the ineffective indigenous rhizobial strains are prioritized over the inoculation strains because of their competitiveness for population and adaptation to the environment. The competition for nodulation is a complex phenomenon depending on soil parameters and genetic traits of both the *Rhizobium* symbiont and the host (Triplett and Sadowsky, 1992).

To evaluate the competition for nodulation of USDA 110, it was genetically marked by *gus*-marker gene. In this study, 10-100 times of *B. japonicum* was used in a full dose for single inoculation and in a half dose in combination with varied PGPR populations. The results indicated that the *gus*-marker is stably inherited and detected

in full percentage (100%) of soybean nodules under sterilized conditions. However, under non-sterilized conditions, nodulation occupancy is lower in Myanmar and Thailand soils compared to sterilized conditions which indicated the competition for nodulation by indigenous rhizobia. Based on data of Weaver and Frederick (1974), it can be predicted that an inoculation rate of at least 1000 times of the soil rhizobial population must be used in soils if the inoculum rhizobia to be formed 50% or more of the nodules. Dowdle and Bohlool, 1987 also illustrated high ratios of inoculum: indigenous numbers were required to displace indigenous rhizobia from nodules.

Strain USDA 110 was the predominant strain and the most competitive strain compared to USDA 138 and 136b in the nodules of all of the soybean varieties and at all of the sites (George et al., 1987). Similar result was observed with highly recoveries of USDA 110 (Kosslak and Bohlool, 1985). They found that USDA 110 to be highly competitive against USDA 123 in vermiculite and in Hawaiian soils devoid of *B. japonicum*. Payakapong et al. (2004) also reported that USDA 110 showed higher nodulation competitiveness than the other strains of bradyrhizobia, THA 5, THA 6, and SEMIA 5019 on three of the five cultivars.

In this study, the results demonstrate that there was a remarkable effect of PGPR *Azospirillum* sp. on enhancement of root development and nodulation by *B. japonicum* strain USDA 110. By single inoculation, although most of the parameters such as nodule number, nodule dry weight, plant height, and root length of soybean plants were not significantly different from those of non-inoculated control, highly significant differences were observed in biomass dry weight in both soils. It could suggest that increasing in nodule number was favored by increases in root growth that formed new root hairs. According to investigation on co-inoculation

effects, any tested level of *Azospirillum* sp. inoculum with *B. japonicum* can enhance on nodulation and plant growth over non-inoculated control in both soybean nodulating bradyrhizobia-established soils. Positive dual inoculation effects of *Rhizobium* and *Azospirillum* in various legume crops are recorded by several authors (Burdman et al., 1996; Iruthayathas et al., 1983). And this is attributed to early nodulation, increased number of total and upper nodules (probably due to an increase in the secretion of *nod* gene inducer signals by roots) and higher $N_2$-fixation rates (Burdman et al., 1996).

It is needed to study the changes of microbial community caused by inoculation of rhizobial inoculants as their potential ecological ricks on microbial diversity should not be neglected. Therefore, in addition to competition for nodulation, the changes of microbial communities of soybean rhizosphere soil were studied before and after inoculation of *B. japonicum* alone and its co-inoculation with *Azospirillum* sp. compared to both non-inoculation control and inoculation of PGPR in addition to bulk soil. High molecular weights of total community DNA extracts were recovered from soybean rhizosphere soil samples at four sampling times over the vegetation. From those total community DNAs, 16S rRNA fragments were amplified by PCR, and only eubacterial communities were analyzed by DGGE from pot experiment under control environment.

DGGE band patterns observed in bulk soil samples were even faint, noticeably affects were not occurred when applied the clustering methods, and it was evidence that inoculation has no affect on soil eubacterial community structures. The numbers of DGGE bands increased with the age of soybean roots for the 1st week and 2nd week rhizosphere samples, indicating the increase of the bacterial diversity along with the

root age at the early stage of soybean growth. Because of the root system releases a wide variety of organic materials and it differs during the development of the plant (time) (Swinnen et al., 1994) and for certain sites of the root system (space) (Lynch and Whipps, 1990), it can be expected that bacteria utilizing these materials as a substrate will vary in population composition and density during the development of plants (Bowen and Rovira, 1991).

Both soils used in this study were from the areas with a long history of soybean cultivation. Based on 16S rRNA genes, the closer related *Bradyrhizobium* spp. (*B. elkanii* in Myanmar soil and *Bradyrhizobium* sp. in Thailand soil) were detected and it was the predominant genus, representing in all samples in all sampling time and DGGE profiles. Highly distinct and high intensity bands were detected, and thus suggest that those bacteria colonized the soybean rhizosphere soils. This supports the reason of occurrence of nodulation on soybean in both non-inoculated control and *Azospirillum* sp. inoculation alone. An associative $N_2$-fixing bacterium *Azotobacter nigricans* was detected among the DGGE bands in Myanmar soil, and it can be supposed that is presented as a PGPR because the sampling field areas have been cultivated rice, maize, and sunflower as alternative crops. In 1982, FAO reported that *Azotobacter* spp. are found in the soil and rhizosphere of many plants, and their population ranges from negligible to $10^4$ $g^{-1}$ of soil depending upon the physico-chemical and microbiological (microbial interactions) properties. *Azotobacter* spp. are free-living, aerobic, plant growth promoting bacteria dominantly found in soils and shown to be antagonistic to pathogens. They are non-symbiotic heterotrophic bacteria capable of fixing an average 20 kg N $ha^{-1}$per $year^{-1}$. Furthermore, *Burkholderia* sp. was detected in Myanmar soil. *Burkholderia* can nodulate and form effective $N_2$-fixing symbioses with legumes most particularly those in the large mimosoid genus

*Mimosa* (Elliott et al., 2007). Shannon's index demonstrated that the species richnesses were not changed among the treatments (data not shown). Therefore, it can be supposed that changes in eubacterial communities in each sampling time were not affected by inoculation treatments.

In this study, soybean cultivar Chiang Mai 60 was used in both Myanmar and Thailand soil samples and DGGE profiles generated during the planting time were not clearly differences among the treatments. Thus, it can be assumed that the single bradyrhizobial inoculation or co-inoculation with any tested levels ($10^6$-$10^8$ cfu $ml^{-1}$) of *Azospirillum* sp. do not shift the soil eubacterial communities, however, the shifting in eubacterial community observed week after week in all treatments can be resulted from plant growth development. The dynamics of rhizosphere microbial communities is important for plant health and productivity, and can be influenced by soil type, plant species or genotype, and plant growth stage (Jin et al., 2009). The results of principal component analyses (PCA) from both soil types supported that the bacterial community structure changed with the growth stage and it was similar to the findings of Jin et al. (2009) and Piromyou et al. (2011).

Moreover, this experiment was conducted under control environment at light room condition which supported to create equal environmental effects; therefore, bacterial community changes might be according to inoculation and different sampling times. Generally, the variation of microbial communities with the progression of the growth stages may be related to two separate mechanisms. The first mechanism may involve environmental changes such as soil temperature and soil moisture with the growth stages (Nazih et al., 2001). However, this mechanism may only play a minor contribution, since the temperature and water regime were relatively uniform throughout the growth stages in the pot experiment. The second

mechanism may be ascribed to the changes in the quality and quantity of root exudates or rhizodepositions with the growth stages. Although they were not measured in the present study, there are several pieces of evidence that root exudates are strongly affected by the growth stages, which in turn can affect rhizosphere microbial communities over time (Duineveld et al., 2001; Garbeva et al., 2004). Thus, in this study, the succession of bacterial communities in the soybean rhizosphere may be due to the variations in root exudates or rhizodepositions at different plant growth stages.

## 4.6 Conclusions

Different inoculum levels of *Azospirillum* sp. and half ml of *B. japonicum* ($10^8$ cfu $ml^{-1}$) can enhance and compete for nodulation against indigenous rhizobia better than single inoculation of USDA 110 alone. Therefore, the selected USDA 110 and *Azospirillum* sp. in this research is prominent bacteria that can be applied for co-inoculant formulation for soybean. In addition, prior to large scale production of co-inoculants including *B. japonicum* and *Azospirillum* sp. for soybean, on-farm competition trials are also needed to determine the competitive ability against native strains in soybean growing areas and their effects on soil microbial community structures in Myanmar. Moreover, new high-yielding soybean cultivars are released year by year. Therefore, the competitiveness of introduced *B. japonicum* strain against indigenous strains for nodulation should be tested because of their host-specific legume-rhizobium symbiosis.

## 4.7 References

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# CHAPTER V

# GENERAL DISCUSSION, CONCLUSIONS AND PERSPECTIVES

To answer the first question in Chapter I, 200 rhizobacteria obtained from School of Biotechnology, SUT were screened for their antagonisms on bradyrhizobia *in vitro*. Those rhizobacteria were originally isolated from rhizosphere soil of rice, maize and vegetables. The results from Chapter III did evaluate that some of the tested rhizobacteria inhibit the tested rhizobial growth *in vitro*. It can suggest that not all rhizobacteria are suitable to be used in co-inoculants. However, if we want to use the rhizobacteria together with bradyrhizobia as co-inoculant on specific crop, other factors should also have to consider such as their abilities on nodulation enhancement, $N_2$-fixation, and plant growth promotion, etc.,. The selected *Azospirillum* sp. and *Bacillus solisalsi* Isolate 3 from tested rhizobacteria were effectively nodulated on soybean those were obtained from structural and sequential screening on the specific soybean host plant.

The results in Chapter III revealed that co-inoculation of *B. japonicum* and *Azospirillum* sp. gave positive responses in nodulation and plant growth under soybean-nodulating bradyrhizobia-free soils in both pot and field experiments. Co-inoculation of *B. japonicum* and *Azospirillum* sp. considerably and stably increased nitrogen fixation, yield and yield components of soybean under field condition. Because, there were many factors which affect the success of nodulation

under natural conditions, the results in this research fulfilled only some parts from those of several conditions.

Moreover, results from Chapter IV revealed that co-inoculation gave better competition for nodulation with tested different inoculums levels of *Azospirillum* sp. and half ml of *B. japonicum* ($10^8$ cfu $ml^{-1}$) against indigenous rhizobia than single inoculation of USDA 110 alone. The soil used in this study were already established with soybean-nodulating bradyrhizobia as shown in MPN plant-infection counting tests. The results from Chapter IV provided the information of the good in competitiveness of our *B. japonicum* USDA 110 and *Azospirillum* sp. in their combination.

The DGGE and PCA results from Chapter III and IV did illustrate that the selected rhizobacteria did not shift rhizosphere soil community structures as noticeably shifting of the rhizosphere soil microbial community structures by any co-inoculation was not detected. Plant age is the major factor that controls the community structures in all tested conditions.

In conclusion, prominent bacteria *Azospirillum* sp. was selected as the most effective PGPR that has a potential to be used in co-inoculants with *B. japonicum* strains that can be possible to apply for co-inoculant formulation for soybean. However, prior to large scale production of co-inoculants including *B. japonicum* and *Azospirillum* sp. for soybean, their survival and competition for nutrient in the inoculants and shelf-life of inoculants under different storage conditions should be evaluated. In addition, on-farm competition trial in soybean-nodulating bradyrhizobia-established soil in soybean growing areas is also necessary to determine their potential for competitiveness against native strains competitive ability

against native strains, effectiveness on new high-yielding soybean cultivars, and effects on soil microbial community structures in growing areas. Therefore, the competitiveness of introduced *B. japonicum* strain to indigenous strains for nodulation should be tested because of their host-specific legume-rhizobium symbiosis.

# APPENDICES

**Appendix 1. Yeast Manitol medium (YM) (Vincent, 1970)**

| | |
|---|---|
| D-manitol | 10.0 g |
| Yeast extract | 0.4 g |
| NaCl | 1.0 g |
| $MgSO_4 \cdot 7H_2O$ | 0.2 g |
| $H_2O$ | 1000 ml |
| pH | 6.8 |

**Appendix 2. LG medium (Hirschi et al., 1991)**

| | |
|---|---|
| Glucose | 10 g |
| $KH_2PO_4$ | 0.41 g |
| $K_2HPO_4$ | 0.52 g |
| $CaCl_2$ | 0.2 g |
| $Na_2SO_4$ | 0.05 g |
| $MgSO_4.7H_2O$ | 0.1 g |
| $FeSO_4.7H_2O$ | 0.005 g |
| $Na_2MoO_4.2H_2O$ | 0.0025 g |
| $H_2O$ | 1000 ml |

**Appendix 3. Composition of N-free Nutrient Solution (Broughton and Dilworth, 1971)**

| Stock Solution | Element | Form | g liter$^{-1}$ |
|---|---|---|---|
| 1 | Ca | $CaCl_2.2H_2O$ | 294.1 |
| 2 | P | $KH_2PO_4$ | 136.1 |
| 3 | Fe | Fe citrate | 6.7 |
| | Mg | $MgSO_4.7H_2O$ | 123.3 |
| | K | $K_2SO_4$ | 87.0 |
| | Mn | $MnSO_4.H_2O$ | 0.338 |
| 4 | B | $H_3BO_3$ | 0.247 |
| | Zn | $ZnSO_4.7H_2O$ | 0.288 |
| | Cu | $CuSO_4.7H_2O$ | 0.100 |
| | Co | $CoSO_4.7H_2O$ | 0.056 |
| | Mo | $NaMoO_2.2H_2O$ | 0.048 |

**Appendix 4. Nutrient Agar Medium (American Public Health Association, 1917)**

| | |
|---|---|
| Peptone | 5.0 g |
| Beef Extract | 3.0 g |
| Agar | 15 g |

**Appendix 5. HM medium (Cole and Elkan, 1973)**

| | |
|---|---|
| Sodium Glutamate | 1.0 g |
| $Na_2HPO_4$ | 0.125 g |
| $NaSO_4$ | 0.25 g |
| $NH_4Cl$ | 0.32 g |
| $MgSO_4 \cdot 7H_2O$ | 1.8 g |
| $FeCl_3$ | 0.004 g |
| $CaCl_2 \cdot 2H_2O$ | 0.013 g |
| HEPES | 1.3 g |
| MES | 1.1 g |
| Yeast extract | 1.0 g |
| L-arabinose | 1.0 g |
| $H_2O$ | 1000 ml |
| pH | 6.8 |

| | |
|---|---|
| $H_2O$ | 1000 ml |
| pH | 6.8 |

# BIOGRAPHY

Ms. Thi Thi Aung was born on April 9, 1976 in Ramree Province, Rakhine State, Myanmar. She received her Diploma in Agriculture (Dip. Agri) from State Agricultural Institute (SAI), Pwint Phyu, Myanmar in 1996 and Bachelor's Degree in Agriculture (B. Agr. Sc) form Yezin Agricultural University, Yezin, Myanmar in 2000. After graduation, she has been employed under the position of Research Technician at *Rhizobium* Inoculant Production Unit, Plant Pathology Section, Department of Agricultural Research (DAR), Yezin, Myanmar. She was trained for three months (July-September, 2004) for Starter Culture Development at Food Biotechnology Laboratory, BIOTEC, National Science and Technology Development Agency (NSTDA), Thailand for Human Resource Development program. She continued her graduate studies in the Yezin Agricultural University, Yezin (2004-2007) and her Doctoral Degree in School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Thailand (2008-2013). During her study, she presented her related work in Myanmar and her thesis work in Thailand in the title of "Improving Legume Inoculants in Myanmar" in 15$^{th}$ Australian Nitrogen Fixation Conference hold on 8$^{th}$ -13$^{rd}$ November, 2009.