การปรับปรุงประสิทธิภาพของหัวเชื้อแบรดดีไรโซเบียมโดยใช้ไฮโดรเจนออกซิไดซิงแบคทีเรียที่มีคุณสมบัติในการส่งเสริมการเจริญเติบโตของพืช

ว่าที่ร้อยตรีณรงค์ฤทธิ์ สกุลโพน

มหาวิทยาลัยเทคโนโลยีสุรนารี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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IMPROVEMENT OF BRADYRHIZOBIAL INOCULANT EFFICIENCY BY USING H_2-OXIDIZING PGPR

Narongrit Sakunpon

A Thesis Submitted in Partial Fulfillment of the Requirements for the

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IMPROVEMENT OF BRADYRHIZOBIAL INOCULANT EFFICIENCY BY USING H_2-OXIDIZING PGPR

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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ณรงค์ฤทธิ์ สกุลโพน : การปรับปรุงประสิทธิภาพของหัวเชื้อแบรดดีไรโซเบียมโดยใช้ไฮโดรเจนออกซิไดซิงแบคทีเรียที่มีคุณสมบัติในการส่งเสริมการเจริญเติบโตของพืช (IMPROVEMENT OF BRADYRHIZOBIAL INOCULANT EFFICIENCY BY USING H_2-OXIDIZING PGPR) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.พรรณลดา ติตตะบุตร, 77 หน้า.

หัวเชื้อแบรดดีไรโซเบียมมีบทบาทสำคัญในการปลูกพืชตระกูลถั่ว ทั้งนี้หัวเชื้อแบรดดีไรโซเบียมควรมีปริมาณเซลล์มีชีวิตที่สูง เพื่อให้สามารถแข่งขันเข้าสร้างปมกับเชื้อแบรดดีไรโซเบียมท้องถิ่นได้ งานวิจัยนี้พบว่า เชื้อแบรดดีไรโซเบียมท้องถิ่นที่พบในดินในประเทศไทยมักจะมีกิจกรรมของเอนไซม์ไนโตรจีเนสในระดับต่ำ แต่มีความสามารถในการแข่งขันเข้าสร้างปมกับถั่วเหลืองที่สูงกว่าหัวเชื้อแบรดดีไรโซเบียมที่ใช้เป็นการค้า โดยเชื้อแบรดดีไรโซเบียมท้องถิ่นที่คัดแยกได้จากดินส่วนใหญ่ (ร้อยละ 69) ไม่มีเอนไซม์ไฮโดรจีเนส (Hup^-) ที่สามารถตรึงไฮโดรเจน (H_2) กลับมาใช้ใหม่ได้ ดังนั้นไฮโดรเจนซึ่งเป็นของเหลือจากกระบวนการตรึงไนโตรเจนทางชีวภาพ จะถูกปลดปล่อยออกมาจากปมถั่วที่ถูกสร้างโดยเชื้อแบรดดีไรโซเบียมท้องถิ่น (Hup^-) ในปริมาณมาก พบว่าก๊าซไฮโดรเจนที่ปลดปล่อยออกมาไม่มีผลกระทบโดยตรงต่อการส่งเสริม การเจริญของพืช รวมทั้งไม่มีผลกระทบโดยตรงต่อกิจกรรมเอนไซม์ไนโตรจีเนสของเชื้อแบรดดีไรโซเบียม ที่สามารถนำก๊าซไฮโดรเจนกลับไปใช้ในเซลล์ใหม่ได้ (Hup^+) แต่พบว่าก๊าซไฮโดรเจนที่ปลดปล่อยออกมาอาจมีประโยชน์ต่อกลุ่มแบคทีเรียที่อาศัยบริเวณรอบรากพืช ที่มีคุณสมบัติในการส่งเสริมการเจริญของพืช (Plant Growth Promoting Rhizobacteria: PGPR) โดยแบคทีเรียเหล่านี้สามารถนำก๊าซไฮโดรเจนไปใช้เป็นแหล่งพลังงานได้ ดังนั้นจึงเป็นอีกทางหนึ่งในการใช้ (Hup^+ PGPR) ในการส่งเสริมการเจริญเติบโตของถั่วเหลือง วัตถุประสงค์โดยรวมของงานวิจัยนี้คือการคัดเลือก PGPR ที่มีคุณสมบัติเป็น Hup^+ ที่มีประสิทธิภาพต่อการส่งเสริมการเจริญของพืช เช่น การสร้าง indole-3-acetic acid (IAA) และมีกิจกรรมของเอนไซม์ 1-aminocyclopropane-1-carboxylate (ACC) deaminase เมื่อนำเชื้อ PGPR นี้ ใช้ร่วมกับหัวเชื้อ *Bradyrhizobium diazoeficiens* USDA110 โดยเชื้อ Hup^+ PGPR ที่คัดเลือกได้ถูกนำมาตรวจสอบประสิทธิภาพการส่งเสริมการเจริญของถั่วเหลืองในสภาวะทั้งที่ไม่ใช้ หรือใช้ร่วมกับแบรดดีไรโซเบียม Hup^+ และ Hup^- ผลการทดลองพบว่าการใช้เชื้อ Hup^+ PGPR สามารถส่งเสริมการเจริญของถั่วเหลืองได้ทั้งภายใต้สภาวะที่มีการให้ก๊าซไฮโดรเจน หรือให้อากาศ โดยเชื้อ Hup^+ PGPR สายพันธุ์ 2H17 (*Bacillus* sp.) และ H39 (*B. megaterium*) สามารถส่งเสริมการเจริญเติบโตของถั่วเหลือง ที่ปลูกภายใต้สภาวะที่มีก๊าซไฮโดรเจนได้ดีกว่าการปลูกภายใต้สภาวะใช้อากาศ

อย่างมีนัยสำคัญทางสถิติ นอกจากนี้ระดับของการสร้าง IAA ในเชื้อสายพันธุ์ H39 ยังเพิ่มขึ้นเมื่อเจริญภายใต้สภาวะที่มีก๊าซไฮโดรเจน แสดงให้เห็นว่าเชื้อ PGPR สายพันธุ์ H39 สามารถนำก๊าซไฮโดรเจนไปใช้ได้จริง และมีศักยภาพในการส่งเสริมการเจริญของถั่วเหลืองในสภาวะที่มีไฮโดรเจน อย่างไรก็ตาม พบว่าการใช้เชื้อสายพันธุ์ H39 (Hup^+ PGPR) ร่วมกับ *B. diazoeficiens* USDA110 Hup^+ ไม่สามารถเพิ่มการเจริญเติบโตของถั่วเหลืองได้ แต่เมื่อใช้ร่วมกับ *Bradyrhizobium* sp. PC-5 Hup^- ซึ่งเป็นเชื้อแบรดดีไรโซเบียมท้องถิ่นที่ไม่สามารถตรึงไฮโดรเจนกลับเข้าสู่เซลล์ได้ สามารถเพิ่มการเจริญเติบโตของถั่วเหลืองได้ และเมื่อนำเชื้อสายพันธุ์ H39 ใช้ร่วมกับ *B. diazoeficiens* USDA110 ภายใต้สภาวะที่มีการแข่งขันเข้าสร้างปมกับเชื้อ *Bradyrhizobium* sp. PC-5 Hup^- พบว่าสามารถเพิ่มการเจริญเติบโตของถั่วเหลืองได้เช่นกัน ดังนั้นจึงเป็นไปได้ที่จะนำเชื้อสายพันธุ์ H39 ไปใช้ร่วมกับหัวเชื้อแบรดดีไรโซเบียมเพื่อเพิ่มประสิทธิภาพหัวเชื้อไรโซเบียมในการส่งเสริมการเจริญของถั่วเหลือง เมื่อนำไปใช้ในสภาพไร่ ที่มักพบการแข่งขันเข้าสร้างปมกับเชื้อแบรดดีไรโซเบียมท้องถิ่น ที่ไม่มีความสามารถในการตรึงไฮโดรเจนกลับเข้ามาใช้ในเซลล์ (Hup^-)

มหาวิทยาลัยเทคโนโลยีสุรนารี

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2557

ลายมือชื่อนักศึกษา _____
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ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

NARONGRIT SAKUNPON : IMPROVEMENT OF BRADYRHIZOBIAL INOCULANT EFFICIENCY BY USING H_2-OXIDIZING PGPR. THESIS ADVISOR : ASSIST. PROF. PANLADA TITTABUTR, Ph.D., 77 PP.

SOYBEAN/*BRADYRHIZOBIUM*/HYDROGENASE UPTAKE/PGPR

Bradyrhizobial inoculants play an important role in legume production. High amount of effective *Bradyrhizobium* should be inoculated onto the seed to compete with indigenous bradyrhizobia in order to ensure the successful nodulation. This study found that most indigenous bradyrhizobia in Thai soil exhibit low nitrogenase activity, while they have high ability to compete in the nodulation with the commercial strain. From this study, 69% of isolated indigenous bradyrhizobia lacked hydrogenase uptake ability (Hup^-). Thus, high amount of H_2 which is the by-product of biological nitrogen fixation would be released from nodules. It was found that H_2 did not have a direct effect on soybean growth and nitrogenase activity of *Bradyrhizobium* containing hydrogenase uptake ability (Hup^+). On the other hand, H_2 released from Hup^- nodule would benefit plant growth promoting rhizobacteria (PGPR) that contain uptake hydrogen (H_2) (Hup^+ PGPR) as extra energy and support soybean growth. Therefore, the overall objectives of this study were to select effective Hup^+ PGPR and determine their efficiency when they were co-inoculated with effective *Bradyrhizobium diazoefficiens* USDA110 on soybean growth. PGPR were selected on the basis of their ability to uptake H_2, indole-3-acetic acid (IAA), and 1-aminocyclopropane-1-carboxylate (ACC) deaminase production. The efficiency of the selected PGPR on soybean growth promotion was evaluated *in vitro* under non-symbiosis and co-inoculated with Hup^+ or Hup^- bradyrhizobia. The results

demonstrated that most of PGPR could promote soybean growth both under H_2 and air treated conditions. H39 (*B. megaterium*) and 2H17 (*Bacillus* sp.) were two Hup+ PGPR isolates found to be able to significantly increase plant growth under H_2 treated condition. In addition, the level of IAA production of isolate H39 was increased under H_2 treated condition. These results indicated that isolate H39 could uptake H_2 and potentially promote soybean growth in the presence of H_2 gas. Although co-inoculation of *B. diazoefficiens* USDA110 with isolate H39 could not promote plant growth, co-inoculation of isolate H39 with *Bradyrhizobium* sp. PC-5 Hup^- (indigenous bradyrhizobium) increased growth of soybean. The same result was also found under the competitive condition between USDA110 and PC-5. Therefore, Hup^+ PGPR isolate H39 can be applied as co-inoculant PGPR with bradyrhizobial inoculant to increase the efficiency of inoculum and promote soybean growth under natural competition in the fields.

มหาวิทยาลัยเทคโนโลยีสุรนารี

School of Biotechnology

Academic Year 2014

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Narongrit Sakunpon

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LIST OF ABBREVIATIONS

°C	=	Degree Celsius
cm^3	=	Centimeter
DNA	=	Deoxynucleotide
dNTP	=	Deoxynucleotide 5’ triphosphate
et al.	=	And others
g	=	Gram
h	=	Hour
l	=	Liter
mg	=	Milligram
mm	=	Millimeter
min	=	Minute
µl	=	Microliter
ml	=	Milliliter
mM	=	Millimolar
µM	=	Micromolar
ng	=	Nanogram
nm	=	Nanometer
PCR	=	Polymerase chain reaction
ρmol	=	Picomole
ppm	=	Parts per million
RNA	=	Ribonucleic acid

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LIST OF ABBREVIATIONS (Continued)

rRNA	=	Ribosomal RNA
rpm	=	Revolutions per minute
s	=	Second
µg	=	Microgram
µl	=	Microliter
V/V	=	Volume per volume
W/V	=	Weight per volume

มหาวิทยาลัยเทคโนโลยีสุรนารี

CHAPTER I

INTRODUCTION

1.1 Significant of this study

Soybean (*Glycine max*) is an important legume crop in many counties around the world since it is used for food and oil productions. Hence, to promote soybean growth and increase the yields, the efficiency of bradyrhizobial inoculant should be developed. Normally, *Bradyrhizobium* can form nodules with soybean plant prior to convert atmospheric nitrogen (N_2) into ammonia (NH_3) by nitrogenase enzyme through the biological nitrogen fixation. Interestingly, an amount of H_2 is produced as an obligate by-product from this biological reaction and related to the activity of nitrogenase enzyme (Somasegaran & Hoben, 1994). The energy loss in termed of H_2 in this reaction takes about 35% in overall biological nitrogen fixation (BNF) (Hunt & Layzell, 1993). However, some rhizobia have ability to recover H_2 diffused from nitrogenase activity inside the nodule and produce some energy to support nitrogen fixation. The key enzyme for H_2 uptake reaction is hydrogenase uptake (Hup^+), which presence only in some rhizobia. Although *B. diazoefficiens* USDA110 has been widely used as a commercial inoculant strain for soybean production and contain hydrogenase uptake activity (Hup^+) (Caldwell, 1969; Cregan & Keyser, 1986), this inoculant some time failed to nodulate soybean due to the nodulation competition from indigenous soil bradyrhizobia and resulted in reducing the efficiency of inoculant (Aung, Tittabutr, Boonkerd, Herridge, & Teaumroong, 2013). Most of indigenous bradyrhizobia lack of hydrogenase uptake (Hup^-) enzyme or have low

hydrogen uptake activity (Annan, Golding, Zhao, & Dong, 2012; Baginsky, Brito, Imperial, Ruiz-Argüeso, & Palacios, 2005). Hence, high amount of H_2 released from Hup^- nodule is lost to rhizospheric soil (Uratsu, Keyser, Weber, & Lim, 1982). However, Popelier, Liessens, and Verstraete (1985) found H_2 diffused to soil from soybean-Hup^- nodules was able to increase the rhizobacterial population. These rhizobacteria can use hydrogen as an electron donor in the energy-yielding process via the function of enzyme NiFe-hydrogenase. This situation was also found in pigeon pea and alfalfa rhizospheric soil (Cunningham, Kapulnik, & Phillips, 1986; La Favre & Focht, 1983). In addition, these bacteria have ability to promote plants growth (Maimaiti et al., 2007) recognized as plant growth promoting rhizobacteria (PGPR). Thus, H_2 released from nodule formed by Hup^- bradyrhizobia could indirectly benefit to plant by stimulate Hup^+ rhizobacteria that contain plant growth promoting properties.

Therefore, the application of PGPR containing Hup^+ character together with commercial bradyrhizobial inoculant may promote efficiency of inoculant when using in the soybean field presented high amount of indigenous Hup^- bradyrhizobial. The overall goal of this study is to obtain the PGPR containing hydrogenase uptake (Hup^+) ability to enhance the efficiency of commercial bradyrhizobial inoculant.

1.2 Research objectives

1.2.1 To investigate the influence of H_2 on plant growth and bradyrhizobial nitrogenase activity.

1.2.2 To isolate the efficient PGPR containing hydrogenase enzyme to be used as co-inoculant.

1.2.3 To investigate the effect of co-inoculation between *Bradyrhizobium* and Hup^+ PGPR on plant growth promotion.

CHAPTER II

LITERLATURE REVIEWS

2.1 Role of bradyrhizobia for soybean production

Soybean is an important legume crop in many counties around the world because soybean is used for food and oil production. Soybean is a subtropical plant, native to southeastern Asia, and has been a dietary staple in Asian countries for at least 5,000 years. It also provides the high amount of protein, substantial amounts of fat, carbohydrates, dietary fiber, vitamins and minerals (Table 2.1). Hence, increasing in soybean production is important to produce more food for consumption when increasing populations increased. To support soybean production, the symbiotic interaction between plant and bradyrhizobium is involved in order to reduce the cost of using chemical nitrogen fertilizer. *Bradyrhizobium* is a Gram negative soil bacteria, which plays a very important role in agriculture via symbiotic biological nitrogen fixation (BNF) reaction. They can induce nitrogen-fixing nodules on the roots of soybean *(Glycine max)* and perform symbiotic interaction with plant. This bacterial symbiosis in soybean has a great benefit in reducing the need for excessive synthetic chemical nitrogen fertilizer by generating nitrogen in form of ammonia (NH_3) through nitrogenase enzyme and transferring to plant directly. *Bradyrhizobium* plays a special role in the nitrogen cycle of agroecosystems. Several bradyrhizobia were applied for inoculation with soybean to promote and increase the yields, such as bradyrhizobia strains USDA110, 123, 138 (Jawson, Franzluebbers, & Berg, 1989),

B. elkanii USDA76 (Rumjanek, Dobert, Van Berkum, & Triplett, 1993), *B. japonicum* THA6 (Prakamhang et al., 2015), *B. japonicum* CB1809 (Aung et al., 2013), *B. elkanii* SEMIA 566, SEMIA 586, SEMIA 587, SEMIA 5019, SEMIA 5039, SEMIA 5079 and SEMIA 5080 (Ferreira & Hungria, 2002). The *B. diazoefficiens* USDA110 are commercially used in rhizobial inoculants production for soybean in Thailand (Prakamhang et al., 2015). Inoculation of soybean with brarhizobial inoculants is a common practice in most of the soybean growing in Thailand. However, field soybean inoculation with *bradyrhizobial inoculant* has often been unsuccessful because of several problems, such as poor survival of bradyrhizobial cell on legume seed (Fred, Baldwin, & McCoy, 1932), inappropriate soil and temperature (Parker & Harris, 1977). Highly alkaline soils are often associated with high salinity could reduce nitrogen fixation (Bordeleau & Prévost, 1994), while low soil temperature (RunEI & TisdalB, 1921) and indigenous bradyrhizobia strains that compete with the inoculant caused in reducing nodule formation on the host plants (Toro, 1996).

Moreover, soybean also has beneficial role in crop rotation because of their symbiosis with bradyrhizobia can contribute large amounts of nitrogen into the soil.

In nodulation process, the communications between the plant and bradyrhizobia are very important. In general, legume plant releases many compounds called flavonoids from their roots, these specific flavonoids induced the production of nod factors from the specific rhizobia (Phillips, 1992). Then, the amount of biochemical and morphological changes in roots was induced when the nod factor is accepted by the plant roots. The specific bradyrhizobia enter to the root cells through a structure called an infection thread. The bacterial cell grows through the root hair into the basal part of the epidermis cells. Finally, bacteria move into the root cortex that surrounds the bacterial cells by a plant-derived membrane prior to differentiate

into bacteroids (Alexander, 1977; Sahgal & Johri, 2003). The nitrogen fixing bacteria use nitrogenase enzyme to catalyze the N-fixation process that convert N_2 to ammonia, these compounds are available to host plants. The symbiosis bacteria with soybean in plant crop could reduce dependence of farmers on expensive chemical fertilizers and improves soil and water quality (Graham & Vance, 2003). However, the ability to form an effective symbiosis with the hosts is the main criterion used in selection of *Bradyrhizobium* strains for legume inoculation. The inoculation may not always lead to improved nodulation or enhanced N_2-fixation because of the presence of indigenous soil rhizobia which are more competitive than the inoculant strain (Roughley, Blowes, & Hurridge, 1976). Therefore, the *Rhizobium* strain selected for inoculants should not only have high N_2-fixation rates, but also be able to compete with the indigenous soil rhizobia populations

Table 2.1 The nutrient components of soybean seeds.

Nutrients	**per 100 g**	**Unit**
Water	3.81	g
Energy	441	kcal
Protein	34.8	g
Total lipid (fat)	21.86	g
Carbohydrate	33.67	g
Fiber	9.7	g
Sugars	7.61	g

Table 2.1 (Continued).

Nutrients	per 100 g	Unit
Minerals		
- Calcium, Ca	188	mg
- Iron, Fe	5.82	mg
- Magnesium, Mg	369	mg
- Phosphorus, P	476	mg
- Potassium, K	2041	mg
- Sodium, Na	12	mg
- Zinc, Zn	3.58	mg
Vitamins		
- Thiamin	0.412	mg
- Riboflavin	0.941	mg
- Niacin	3.286	mg
- Vitamin B-6	0.351	mg
- Folate, DFE	277	mg
-Vitamin A, RAE	6	µg
- Vitamin A, IU	122	IU
- Vitamin E (alpha-tocopherol)	1.98	mg
- Vitamin K (phylloquinone)	71	µg
Lipids		
- Fatty acids, total saturated	3.162	g
- Fatty acids, total monounsaturated	4.829	g
- Fatty acids, total polyunsaturated	12.341	g

2.2 Nitrogenase and nitrogen fixation reaction

The diazotrophic bacteria including bradyrhizobia fix atmospheric nitrogen gas into plant usable form using enzyme nitrogenase, which reduce N_2 to NH_3. The

equation given below represents the nitrogen fixation reaction. It involves one mole of N_2 to produce two moles of NH_3

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

Nitrogenase consists of two metalloproteins components (Fig 2.1), the Fe-protein is used for ATP hydrolysis and electron transfer, while the MoFe-protein with the MoFe-cofactor provides the active site for substrate reduction (Rees et al., 2005). Moreover, some nitrogenase enzymes in other bacteria contain the active site protein in form of iron-vanadium (FeV) protein or iron-iron (FeFe) protein (Reiher, Salomon, Sellmann, & Hess, 2001). The reactions occur when N_2 is bound to the enzyme complex, which required both electron donor and ATP for enzymatic reduction of N_2. The electrons are transferred from the Fe-protein to the active site protein during the reaction. In addition, eight electrons are required in the reduction of the substrate N_2 (dinitrogen), while ATP is hydrolyzed to ADP with the stoichiometry of two ATP for each electron (Erickson et al., 1999). From the equation above, the reduction of N_2 to NH_3 is a very expensive process energetically. It requires high amount of ATP molecules and electrons to reduce a single molecule of N_2. This reaction process needs to use all 8 electrons, which 6 electrons are used to reduce N_2 to NH_3, while 2 electrons move to H_2 production. Therefore, the H_2 from this reaction is a wasteful by-product for the plant.

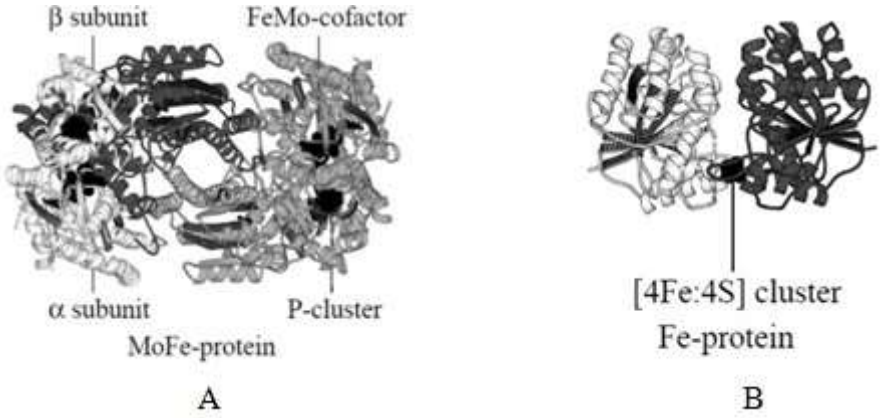

Figure 2.1 Ribbons diagrams of the polypeptide folds for A) the nitrogenase MoFe-protein and B) Fe-protein. The alpha and beta subunits on the left of the MoFe-protein are represented by the light and dark shading, respectively, while the metalloclusters are depicted by the dark space-filling models on the right side of the MoFe-protein. The two subunits of the Fe-protein are denoted by light and dark shadings, with the 4Fe : 4S cluster at the dimer interface designated as a space filling model (Rees et al., 2005).

2.3 Bacterial hydrogen uptake (Hup^+)

In the BNF, H_2 is an obligate by-product of the nitrogenase reaction. The energy lost in form of H_2 accounts approximately 25-35% of the total energy, while the remaining energy is used to generate NH_3 (Hunt & Layzell, 1993). It is revealed that 25-35% of all energy used for BNF were lost through H_2 release from N_2 fixing reaction. In a legume field, the H_2 exposure rate of soil adjacent to N_2 fixing nodules has been calculated to be in the range of 30-250 nmoles cm^{-3} h^{-1} (Dong & Layzell, 2001). However, some bacteria contain hydrogenase enzyme which is able to oxidize and use molecular hydrogen as an electron donor for produce the energy. These bacteria are called H_2-oxidizing bacteria or Hup^+ bacteria (Phelps & Wilson, 1941).

The advantages of hydrogenase enzyme benefit to N-fixing bacteria in the prevention of nitrogenase reduction inhibition by remove H_2 during N_2 fixation (Dixon, 1972) and hydrogenase act as O_2 remover, which remove the O_2 near the O_2-sensitive nitrogenase (La Favre & Focht, 1983). Hydrogenase is separated into three types according to their active sites, including the [NiFe]-, [FeFe]-, and [Fe]-hydrogenase enzymes (Fig 2.2). The key type enzyme is membrane bound NiFe- uptake hydrogenase. The common structural composition of [NiFe]-hydrogenases is a large subunit, HoxG, that contains the NiFe active site; and a small subunit, HoxK, that contains several FeS clusters. The functional NiFe-hydrogenase catalyzes the reversible cleavage of H_2 into protons (H^+) and electrons (e^-) then electrons are transferred to small subunit of hydrogenase enzyme (Fontecilla-Camps, Volbeda, Cavazza, & Nicolet, 2007; Vignais & Colbeau, 2004) (Fig 2.3). The Hup^+ bacterial species are presented in all subclasses, including alpha, beta, and gamma of Proteobacteria (Friedrich & Schwartz, 1993; Lechner & Conrad, 1997), and most of them belong to Gram negative bacteria (Aragno & Schlegel, 1981).

There are two groups of hydrogen oxidizing bacteria have to be considered. The first group is the bacteria that can use hydrogen under aerobic conditions, such as chemolithoautotrophic aerobic hydrogen-oxidizing bacteria (e.g. *Alcaligenes eutrophus*). They are able to use hydrogen for aerobic respiration and the reduction of carbon dioxide (Schink & Schlegel, 1978). These groups of bacteria also oxidize hydrogen under aerobic conditions, but without autotrophic CO_2 fixation (Aragno & Schlegel, 1981). The second group is the bacteria that can utilize hydrogen only under anaerobic conditions (Schink & Schlegel, 1978). These bacterial groups have the ability to utilize organic compounds provided by plant roots into rhizosphere soil

(Kloepper, 1994; Tilak et al., 2005; Zablotowicz, Tipping, Lifshitz, & Kloepper, 1991). The Hup^+ bacteria can also promote plant growth via many mechanisms, therefore some of them are also recognized as plant growth promoting rhizobacteria (PGPR). For example, *Variovorax paradoxus* was isolated from H_2 treated soil have ability to oxidize H_2 as well as could stimulate root elongation of spring wheat seedling (Maimaiti et al., 2007).

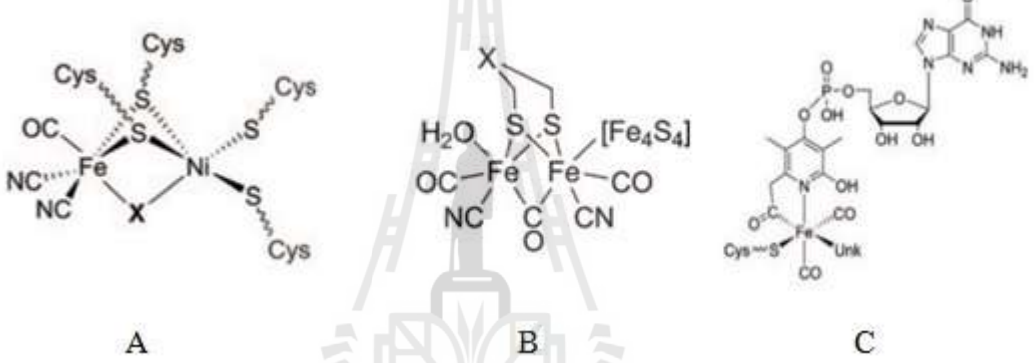

Figure 2.2 Schematic representations of A) the aerobically isolated, inactive form of the [NiFe]-hydrogenase, (X) HOO- for Ni-A and HO- for Ni-B; B) the active site of the [FeFe]-hydrogenase, (X) CH_2, NH, or O ; C) the [Fe]-hydrogenase, Unk, unknown ligand; this site appears to bind cyanide (Tard & Pickett, 2009).

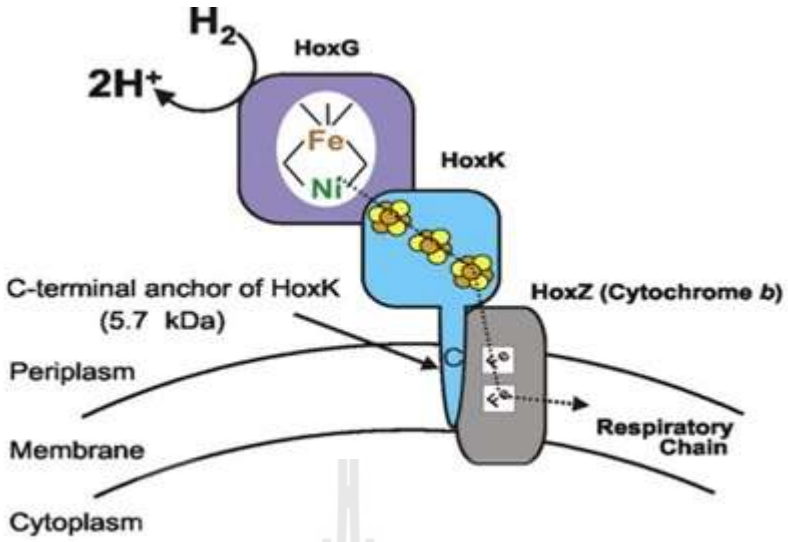

Figure 2.3 Models of membrane-bound NiFe-hydrogenase of *Ralstonia eutropha* (Schwarze, Kopczak, Rögner, & Lenz, 2010).

2.4 H_2-application with plant

Hydrogen is the colorless, odorless, tasteless and lightest abundant chemical element. It has been known as a reducing agent and generally regarded as a potential future energy source as an alternative to limited fossil fuel resources (Dutta, De, Chaudhuri, & Bhattacharya, 2005). Generally, H_2 gas was applied to therapeutic agent on various human diseases (Ohno, Ito, Ichihara, & Ito, 2012). Some reports found that H_2 could selectively reduce the hydroxyl radical (NOH) and peroxynitrite ($ONOO_2$), but it does not affect physiological reactive oxygen species (ROS). They suggested that this antioxidant activity could have preventive and therapeutic applications (Ohsawa et al., 2007). Moreover, H_2 has an important effect on plant physiological function, such as plays an important role in plant resistance to stress by increase the enzymes related with antioxidants**.** It has been reported that the H_2 application promote plant growth, and the winter rye seeds treated with H_2 have a positive impact

on germination rapidly than seeds control (Renwick, Giumarro, & Siegel, 1964). Hydrogen can stimulate mung bean, rice (Zeng, Zhang, & Sun, 2013), and alfalfa (*Medicago sativa*) (Jin et al., 2013) seed germination. Dong et al. (2002) also found that the soil sample treated with H_2 increased H_2 uptake capacity higher than the air treated soil which lead to soil bacterial community changes and contributes to plant growth. Furthermore, H_2 treated soil was used as fertilizer with soybean, barley, and spring wheat. The result showed that H_2 treated soil promoted plant growth more than controlling plant (Dong, Wu, Kettlewell, Caldwell, & Layzell, 2003). The Hup^+ PGPR could be isolated from soil treated with H_2 and they have several abilities to promote plant growth. The root elongation of spring wheat seedlings was enhanced by 17 isolates of Hup^+ PGPR. Moreover, plant biomass of *Arabidopsis thaliana* was increased when inoculated by *V. paradoxus* (Hup^+) or *Burkholderia* (Hup^+) (Maimaiti et al., 2007). Thus, there is the possibility to use Hup^+ PGPR as inoculant to promote plant growth.

2.5 Roles of PGPR in crops production

Chemical fertilizers are important components of modern agriculture because they provide essential plant nutrients. However, overuse of chemical fertilizer has negative on environmental impacts. One potential way decrease unanticipated environmental impacts are inoculation with plant growth promoting rhizobacteria (PGPR). These bacteria exert beneficial effect on plant growth and developments. These groups of bacteria are of beneficial for uptake nutrients and promote plant growth on the basis of many mechanisms such as N_2-fixation, phytohormone production, solubilization of phosphate (P), ACC-deaminase enzyme, synthesis of

siderophore, and anti-phytopathogens. Some of plant growth promoting mechanisms is listed below.

2.5.1 N_2-fixation

Nitrogen fixing bacteria, such as *Rhizobium, Azotobacter, Bacillus, Pseudomonas, Azospirillum* and *Acetobacter* had been isolated from the rhizosphere of various crops (Reiher et al., 2001). However, the amount of nitrogen fixation by free-living PGPR is not as great as by symbiont rhizobia. Adesemoye et al, 2010 found that PGPR as *Bacillus amyloliquefaciens* and *Bacillus pumilis,* which can fix nitrogen have ability to increase plant N uptake from fertilizer via other mechanisms but not with their own nitrogen fixing capability. This indicated that plant growth promotion is caused by other factors more than nitrogen fixation. Therefore, the other factors such as phytohormones production, phosphate solubilization would be the key factors for plant growth promotion.

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2.5.2 Phytohormones

Plant hormones are signal molecules produced within the plant. Hormones such as auxin, cytokinin and gibberellin regulate cellular processes and development of plant growth. Not only plant can produce phytohormones but also numerous plant associated beneficial bacteria or PGPR can also produce these plant hormones. *Azospirillum brasilense*, one of the most studied PGPR has shown an improve of plant growth development by the production of auxin, cytokinin and gibberellin. Using this bacterium as inoculant with plants can change the morphology of plants such as an increase in root surface area through root hair stimulation, which enhance mineral uptake (Steenhoudt & Vanderleyden, 2000). Phytohormones produced by

Azospirillum promoted epidermal-cell differentiation in root hairs that increased the number of potential sites for rhizobial infection (Yahalom, Dovrat, Okon, & Czosnek, 1991) leading to forming more nodules. In addition, the rhizobacterium *Bacillus amyloliquefaciens* FZB42 could produce the indole-3-acetic acid (IAA) effects plant growth promotion (Idris, Iglesias, Talon, & Borriss, 2007). The IAA produced by PGPR increase root length, root surface area and the number of root tips, leading to stimulated uptake of nitrate and phosphorous (Adesemoye, Torbert, & Kloepper, 2008; Gyaneshwar, Kumar, Parekh, & Poole, 2002; Mantelin & Touraine, 2004). The IAA is strongly associated with the positive response by the plant (Spaepen, Das, Luyten, Michiels, & Vanderleyden, 2009). In addition, PGPR including the strains in the genera *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Azotobacter*, *Bacillus*, *Beijeriakia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Rhizobium*, *Seratia*, etc. were also reported as phytohormone producer (Lucy, Reed, & Glick, 2004).

2.5.3 Phosphate solubilizing

Phosphorus (P) is one of the major essential macronutrients for plant growth development, which plays an important role in plant metabolism by providing energy required for metabolic processes (Duff, Sarath, & Plaxton, 1994). It is applied to soil in the form of phosphate fertilizers, but the phosphorus is most limiting element in soil due to it is nonavailable form. Therefore, the P-solubilization ability of the bacteria is considered to be one of the most important role associated with plant phosphate nutrition. Phosphate solubilizing bacteria (PSB) are a group of beneficial bacteria that can produce organic acids needed to convert the insoluble phosphate into

soluble forms (Kpomblekou-a & Tabatabai, 1994; Vessey, 2003). It was also widely recognized that soil inoculation with phosphate solubilizing bacteria (PSB) can improve solubilization of fixed soil phosphates resulting in higher crop yields (Chen et al., 2006).

2.5.4 1-aminocyclopropane-1-carboxylate (ACC) - deaminase enzyme

Although, ethylene is an important growth hormone of plants, which is mediates a wide range of different plant response and developmental processes, the high concentration of ethylene can inhibit plant growth. For example, after seed germination, ethylene concentration remains high in root elongation resulted in symbiotic nodulation in legume plants by rhizobia is inhibited (Ma, Penrose, & Glick, 2002). The 1-aminocyclopropane-1-carboxylate (ACC) is a precursor of ethylene production. Therefore, reducing ACC concentration is one of the effective ways to reduce ethylene production in plant roots (Yang & Hoffman, 1984). Stress factors have been reported to stimulate ethylene production and affect on growth, survival and crop yields. The limitations and excesses of the main abiotic factors (temperature, humidity, light intensity, the supply of water and minerals, and CO_2), and of other stress factors in particular situations (e.g. pests, pathogens, or pollutants) can induce high level of stress ethylene production. The plant inoculated with ACC deaminase containing bacteria has shown to decrease the levels of ethylene, which promoted root elongation and changes better in the growth and development of plants (Glick, Penrose, & Li, 1998). For example, the bacterium *V. paradoxus* strain producing ACC deaminase could reduce the concentration of ACC in Indian mustard and rape (*Brassica napus* var. *oleifera* L.) (Belimov et al., 2001).

2.5.5 Siderphore

Iron is one of the major limiting factors and essential nutrients of microbial and plant lives. Hence, bacteria produce siderophores for its acquisition to grow under low iron stress. The important role of these compounds is to change the insoluble form of iron in soil to soluble forms that has positive effects on plant growth (Vessey, 2003) . Previous studies have reported that the siderophore-producing bacteria were inoculated to plants such as chickpea and soybean seeds, it showed an increased seed germination, plant growth, and yield (Kumar & Dube, 1992). Moreover, some strains of *Burkholderia* (*B. vietnamiensis* TW 75) could inhibit phytopathogenic fungi and produce an efficient siderophore that increased the plant growth (Van, Berge, Ke, Balandreau, & Heulin, 2000).

2.5.6 Biological control

Biological control is a bioeffector-method of controlling pests including insects, mites, weeds, and plant diseases using living organisms. PGPR can also be used for suppression of phytopathogens through many mechanisms such as antibiotics, fungal wall-lysing enzymes, or hydrogen cyanide productions which suppress the growth of fungal pathogens. The synthesis of molecules is involved in the antagonistic interactions and disease suppression, such as the antibiotic 2, 4-diacetylphoroglucinal (2,4-DAPG), pyoluteocin, biocides such as HCN (Costa et al., 2007). The biofilms of *B. subtilis* is a complex process includes the secretion of surfactin which is a lipopeptide antimicrobial agent that cause suppression of *Pseudomonas syringae* in *Arabidopsis* roots (Bais, Fall, & Vivanco, 2004). One of the applications of *B. subtilis* RB14 was quoted as an effective bacteria to suppress the

pathogen *Rhizoctonia* on tomato root (Asaka & Shoda, 1996). J. Zhang, Howell, and Starr (1996) also reported that *Fusarium* colonization in cotton roots was also inhibited by *Gliocladium virens* and *Bacillus* strains.

2.6 Application of bradyrhizobia and Hup$^+$ PGPR with soybean

Soybeans inoculated with *B. japonicum* contaianing uptake hydrogenase (Hup$^+$) system significantly increase the ability to fix nitrogen and produced greater yields than plants inoculated without hydrogenase strains (Hup$^-$ bradyrhizobia) (Albrecht et al., 1979). Using this strain of *B. japonicum* Hup$^+$ as an inoculant could increase nitrogen fixation efficiency in soybean. The advantage of Hup$^+$ bradyrhizobia could lead to a higher ability of nitrogen fixation (Maier & Triplett, 1996). However, some of bradyrhizobia do not have uptake hydrogenase, therefore H_2 is evolved into the soil. The H_2 lost from the nodules to the soil is a wasteful process (Eisbrenner and Evans, 1983). Therefore, from these data of H_2 evolutionary perspective, the symbiosis with Hup$^+$ bradyrhizobia should have more advantages on legume growth than Hup$^-$ bradyrhizobia. In the soil, most of bradyrhizobial strains lack of hydrogenase uptake enzyme system (Hup$^-$), or low activity of the uptake hydrogenase (Schubert & Evans, 1976), therefore large amounts of H_2 can diffuse out of the bacterial cell into the soil. The calculation of symbiotic bradyrhizobia in soybean field produces hydrogen emission rates about 5000 L H_2 day^{-1} hectare^{-1} at a peak growth (Dong & Layzell, 2001). The release of H_2 into the soil may be beneficial to plant growth and yield. Most H_2 released from legume nodules is absorbed by soil (Dong & Layzell, 2001; La Favre & Focht, 1983). Moreover, the H_2 treated soils or soil adjacent Hup$^-$ nodule of soybean able to change bacterial

community structures (Y. Zhang, He, & Dong, 2009). Some of them are Hup^+ PGPR that may be used to enhance plant growth. However, the application of Hup^+ PGPR with soybean bradyrhizobium as co-inoculant has never been reported. Therefore, the co-inoculation of Hup^+ PGPR with bradyrhizobium on soybean fields may an alternative way to enhance the plant growth and production by taking advantage from H_2 released from biological nitrogen fixation that can support Hup^+ PGPR to promote plant growth.

CHAPTER III

MATERIAL AND METHODS

3.1 Isolations of bradyrhizobia from soybean root nodules

The root nodules of soybean (*Glycine max*) grown at different provinces in Thailand including Chiangmai (18°26'15.9"N/98°41'19.9"E; 19°17'191"N/99°11' 12.7"E), Lampang (19°08'55.0"N/99°37'20.9"E), Phrae (17°51'15.5"N/99°40'36.6"E) and Chaiyaphum (16°29'55.9"N/10°14'53.01"E) were collected. The collected nodules were surface sterilized by immersion in 70% ethanol for 30 s and then immersed in 3% sodium hypochlorite solution for 3 min. The nodules were rinsed at least five times in sterilized water. Each root nodule was cut and the bacterial cells inside a nodule was streaked onto Yeast Extract Mannitol (YEM) agar medium (Mannitol, 10.0 g, K_2HPO_4, 0.5 g; $MgSO_4.7H_2O$, 0.2 g, NaCl, 0.1 g, yeast extract, 0.5 g; agar, 15 g, with 25 mg congo red in 1 L water and adjusted pH to 6.8) (Somasegaran & Hoben, 1994) and incubated at 28 ^oC for 5-7 days. Each isolate was re-inoculated onto soybean seed for authentication.

3.2 Genomic DNA extraction and BOX-PCR amplification

Bradyrhizobial isolates were cultured in YEM liquid medium. After cultivation for 5 days on a rotary shaker at 160 rpm at 28 °C, total genomic DNA from each bradyrhizobial isolate was prepared as described by Hartmann and Amarger (1991). DNA fingerprints were obtained by using a BOXA1R primer

(5’ CTACGGCAAGGCGACGCTGAC 3’) to investigate the repetition of the strains (Versalovic, Schneider, De Bruijn, & Lupski, 1994). The PCR reaction contained 100 ng of DNA template, 10 pmol of primer, 2.5 µM of dNTP, 25 mM of $MgCl_2$, 1× PCR buffer, and 2.5 U Taq, DNA polymerase in total volume of 25 µl. The PCR reaction condition was used as follows; 95°C for 5 min 1 cycle, 94°C for 1 s, 53°C for 1 min, 65°C for 8 min 35 cycles and finally 65°C for 16 min 1 cycle (Versalovic et al., 1994). PCR products were separated on 2% agarose gel for 35 min as 100 volt and stained with ethidium bromide. The isolated strains showing different DNA fingerprint patterns were selected for further study.

3.3 Hydrogenase uptake (Hup) phenotype of bradyrhizobia

The Methylene Blue Reduction (MBR) assay was used to determine hydrogenase uptake ability of all isolated strains (Haugland, Hanus, Cantrell, & Evans, 1983). Bradyrhizobia colonies were grown on YEM agar plates, then re-streaked onto the hydrogen uptake medium (HUM) agar containing the following in 1 liter of distilled water; 150 mg, $NaH_2PO_4.H_2O$; 150 mg, $CaCl_2.2H_2O$; 250 mg, $MgSO_4.7H_2O$; 28 mg, Fe-EDTA; 10 mg, $MnSO_4.H_2O$; 3 mg, H_3BO_3; 2 mg, $ZnSO_4.7H_2O$; 0.25 mg, $NaMoO_4.2H_2O$; 0.04 mg, $CuSO_4.5H_2O$; 0.025 mg, $CoC1_2.6H_2O$; 0.78 mg, KI; 100 mg, inositol; 10 mg, thiamine hydrochloride; 1 mg, nicotinic acid; 1 mg, pyridoxal HCl; 0.5 g, sucrose; 1.0 g, L-arabinose; 0.5 g, sodium gluconate; 0.5 g, sodium glutamate; 0.1 g, yeast extract, and 12 g of agar.

The colonies were transferred to upper side of filter disk and placed on plate of Repaske agar medium containing the following chemical substances dissolved in 1 liter of KH_2PO_4 buffer (pH 6.8); 0.018 M, NH_4Cl; 6×10^{-5} M, CaC_{l2}; 0.012 M, $NaHCO_3$; 0.001 M, K_2SO_4; 0.02 M, $MgCl_2$; 2×10^{-5} M, $Fe(NH_4)_2(SO_4)_2$; 5×10^{-7} M,

$NiCl_2$; 4×10^{-7} M, $CuCl_2$; 2×10^{-7} M, $CrCl_3$; 2×10^{-7} M, $CoCl_2$; 1×10^{-7} M, $ZnC1_2$; 2×10^{-6} M, $MnC1_2$; 2×10^{-7} M, Na_2MoO_4; 1 µg, thiaminehydrochloride; 1 µg, nicotinic acid and 1.5 µg, biotin (Maier et al., 1978). The colonies on disks were incubated in 5% (v/v) hydrogen gas mixed with air to induce hydrogenase expression. Filter disks were transferred to new plate containing 0.8 ml of a solution consist of 200 mM iodoacetic acid, 200 mM malonic acid, 10 mM methylene blue, 50 mM KH_2PO_4, and 2.5 mM $MgCl_2$ and adjusted to pH 5.6 with KOH. After 15 min, the filter dish was removed from the solution and incubated in air for 45 min; this allowed the mixing of dye solution with bacterial colonies. Then, filter disks were placed in the sealed screening tray flushed with H_2 at a flow rate of 150 ml min^{-1} for 1 to 3 h (Haugland et al., 1983). The colonies with hydrogenase uptake ability (Hup^+) showed blue colony, while white colony indicated no hydrogenase uptake ability (Hup^-).

3.4 Characterization of Hup^- *Bradyrhizobium*

3.4.1 Nitrogenase activity

Leonard's jar assembly using plastic jar (250 cm^3) was filled with vermiculite and provided N-free plant nutrient solution (Somasegaran & Hoben, 1994). The whole apparatus was sterilized (30 min at 121°C) prior to transplantation of seedlings. The seeds of soybean (*Glycine max* (L.) Merrill) cv. Chiang Mai 60 were surface sterilized in 95% ethanol for 30 s, then immersed in 3% sodium hypochlorite for 5 min. The seeds were rinsed at least five times in sterilized water and pre-germinated on a sterilized moist tissue paper for 3 days at room temperature. Uniformly germinated seeds of soybean were grown in Leonard's jars under aseptic condition. Each isolate of Hup^- bradyrhizobium was used as inoculant (10^8 cells ml^{-1}

seed^{-1}) to determine the ability of N_2-fixation. This experiment was conducted as three replicates. Plants were grown under controlled light of approximately 400 μE cm^{-3} (16 h day/8 h night) at 25 ^{o}C. At 30 days after planting, plant roots with nodules were cut and transferred into 60 ml test tube, seal with rubber then injected 10 % (v/v) acetylene (C_2H_2) into the tube after removed air at the same volume. The roots were incubated for 1 hour before determination the activity of nitrogenase by using acetylene reduction assay (ARA). Ethylene produced by nitrogenase enzyme was measured by using gas chromatograph (GC) with a flame ionization detector and PE-Alumina column equipped, 50 m × 0.32 mm × 0.25 mm (Perkin Elmer, USA) (Prakamhang, Minamisawa, Teamtaisong, Boonkerd, & Teaumroong, 2009). The activity of the enzyme was calculated as μmol of ethylene forming h^{-1} g of nodule^{-1}.

3.4.2 Competitiveness ability

To construct of *gus*-marked *B. diazoefficiens* strain USDA 110, two bacterial strains, *Escherichia coli* S17-1 donor strain (harboring plasmid pCAM120, *Tn5* fusion with *gus*-gene) which is resistant to 20 μg/ml of both Streptomycin and Spectinomycin, and recipient *B. diazoefficiens* strain USDA 110 which is resistant to Gentamycin (20 μg/ml), were grown to stationary phase in Luria-Bertani broth (LB) (Sambrook, Fritsch, & Maniatis, 1989) and yeast-extract-mannitol (YEM) broth for overnight and seven days, at 37°C and 28±2°C, respectively. The method for biparental mating was that of (Krause, Doerfel, & Göttfert, 2002). Blue forming colonies on HEPES-MES HM solid media (Cole & Elkan, 1973) containing Streptomycin (200 μg/ml), Gentamycin (30 μg/ml) and X-gluc (5-Bromo-4-chloro-3-indolyl-beta-D-glucoside) (20 μg/ml) were selected as transconjugants. 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 competition nodulation ability of each Hup$^-$ bradyrhizobial strain was examined by competed with *gus*-marked *B. diazoefficiens* USDA110 (Hup$^+$) constructed by Aung et al. (2013). All bradyrhizobial strains were prepared at 10^8 cells ml^{-1} in sterilized 0.85% NaCl solution. Each Hup$^-$ *Bradyrhizobium* strain was mixed with *B. diazoefficiens* USDA110 in a ratio of 3:2 before inoculating onto each germinated seed at concentration of 10^8 cells/seed. The plants were grown under controlled condition as mentioned above. At 30 days after inoculation, the soybean nodules were collected from the root to detect the presence of inoculated *gus*-marked bradyrhizobia in each nodule. The nodules were cut in half and immersed in a microtiter plate containing the gus-assay solution (40 µl of 20 mg ml^{-1} of X-Gluc in N, N-Dimethylformamide, 20 mg SDS, 2 ml methanol, 0.2 ml of 1 M sodium phosphate buffer and 7.76 ml distilled water), and applied with vacuum for 2 h before incubation for overnight at 28°C (Aung et al., 2013). The soybean inoculated with wild type of *B. diazoefficiens* USDA110 was used as control. The competitiveness ability on nodulation was calculated as a percentage of nodule occupancy.

3.5 Investigation the influence of the H_2 gas on plant growth and bradyrhizobial nitrogenase activity

Germinated soybean seeds were transferred to sterilized Leonard's jar (250 cm^3) containing vermiculite and treated with synthetic H_2 (500 ppm H_2 in an artificial air mixture of 360 ppm CO_2 and 21% O_2, balanced with N_2) at a flow rate of 12 ml min^{-1} to give approximately 250 nmol H_2 cm^{-3} h^{-1}, while the plant treated with

air at the same flow rate was used as a control (Osborne, Peoples, & Janssen, 2010). The plants were treated with H_2 gas directly at root zone and watered with plant nutrients solution supplemented with KNO_3 (0.05%) (Somasegaran & Hoben, 1994). The light/dark cycle and plant growth condition were conducted following the previous experiment. The data of root, shoot, and plant dry weigh were collected at 30 days after planting. To investigate the effect of H_2 on nitrogenase activity, *B. diazoefficiens* USDA110 (Hup^+) was cultured in YEM medium for 5 days and the cells were washed twice with sterilized 0.85% NaCl solution, and re-suspended in 15 ml of the same solution before inoculating 10^8 cells onto germinated seed. Inoculated plants were grown in H_2 treated condition as indicated above and watered with N-free nutrients solution (Somasegaran & Hoben, 1994). The data of nitrogenase activity were analyzed comparing with air treated condition and nodule numbers (mature nodules), root, shoot, and plant dry weight were collected at 30 days after planting.

3.6 Isolation of PGPR containing hydrogenase uptake enzyme

3.6.1 Isolation of soybean rhizospheric bacteria

The isolation procedure used in this study was modified from the protocol of Maimaiti (2007). The rhizospheric soil of soybean collected from the same fields where collected the soybean nodules were mixed and treated with 0.3% (v/v) H_2 gas in air at 25°C for 30 days. Each soil sample (100 mg) was serially diluted from 10^{-3}-10^{-10}, and 100 µl of each dilution were spread on the mineral salt agar medium (MSA) (in 1 liter contained (2.0 g, $NaNO_3$; 1.2 g, K_2HPO_4; 0.5 g, $MgSO_4$; 0.5 g, KCl; 0.14 g, KH_2PO_4; 0.02 g, Yeast Extract; 0.01 g, $Fe_2(SO_4)_3$. H_2O; Agar, 15 g; pH 7.2) supplemented with cycloheximide (10 mg l^{-1}) (Schlegel & Meyer, 1985) in

duplicates. The MSA plates were incubated for 21 days at 25 ± 2°C under 0.3% (v/v) H_2 gas in air (Dong & Layzell, 2001). Different morphologies of colonies were collected. The DNA fingerprints of isolated strains were obtained by using a BOXA1R to investigate the repetition of the strains as described previously. The isolated strains showing different DNA fingerprint patterns were selected for further studies.

3.6.2 Detection hydrogenase uptake (Hup) ability of rhizospheric bacteria

The rhizobacterial isolates were grown on nutrient agar (NA) plates (beef extract, 3 g; peptone, 5 g; agar, 15 g in 1 liter of distilled water) for 18 h at 25 ±2 °C approximately 0.3% (v/v) H_2 gas in air. The bacterial colonies were transferred to upper side of filter disk, then immersed in plate containing a methylene blue solution as the same method described above.

3.6.3 Characterization of plant growth promoting properties

3.6.3.1 Nitrogenase activity in free-living

The Hup^+ rhizobacterial strains were cultured in LG medium broth (Hardy, Holsten, Jackson, & Burns, 1968) without nitrogen source for 2 days at 28 °C, then removed air in tubes 5% of volume in the headspace and replaced by acetylene with the same removed volume. The samples were incubated at 28 °C for 24 h. Then, the amount of ethylene was measured to investigate the nitrogenase activity by using gas chromatograph (GC) as described above. Total protein concentration of the cell suspension was determined according to Bradford (1976). The activity of nitrogenase enzyme was calculated as nmol of ethylene forming day^{-1} mg $protein^{-1}$.

3.6.3.2 Indole-3-acitic acid (IAA) production

The colorimetrical method was used to determine the production of indole-3-acitic acid (IAA). Each bacterial isolate was grown in LG medium broth supplemented with tryptophan 2 g L^{-1} for 1 days at 28 °C (Fukuhara, Minakawa, Akao, & Minamisawa, 1994). The culture was centrifuged at 12,000 rpm for 15 min, then 1 ml supernatant was mixed with 2 ml of Salkowski's reagent and incubated under dark for 30 min then measured the absorbance with spectrophotometer at 530 nm (Costacurta, Mazzafera, & Rosato, 2006). The known amounts of pure indole-3-acitic acid were used as standard. The IAA production was calculated as μM mg of $protein^{-1}$.

3.6.3.3 Putative ACC-deaminase activity

Each bacterial isolate was cultured in LG broth (N-free) medium at 28 °C for 2 days with shaking at 200 rpm until cell reached the early stationary phase. The cells were collected by centrifugation at 5,000 rpm for 5 min and washed twice with minimal medium (1.36 g, KH_2PO_4; 2.13 g, Na_2HPO_4; 0.2 g, $MgSO_4.7H_2O$; 0.7 g, $CaCl_2$ $.2H_2O$; 0.2 g, $FeSO_4.7H_2O$; 0.04, $CuSO_4.5H_2O$; 0.02 g, $MnSO_4.H_2O$; 0.02 g, $ZnSO_4$ $.7H_2O$; 0.003 g, H_3BO_3; 0.007 g, $CoCl_2.6H_2O$; 0.004 g, $Na_2MoO_4.2H_2O$; glucose, 10 g in 1 liter of distilled water). Cell pellets were suspended in 15 ml minimal medium, then drop 100 μl on minimal medium agar supplemented with 5 mM of 1-aminocyclopropane-1-carboxylate (ACC) as a nitrogen source and further incubated at 28 °C for 5 days (Penrose & Glick, 2003). The bacterial colonies duplicated on this medium were assumed as putative ACC-deaminase producing strains.

3.7 The influence of PGPR on soybean growth under H_2 treated condition

Soybeans were grown in Leonard's jar (250 cm^3) containing sterilized vermiculite, and watered with plant nutrients solution supplemented with 0.03% (w/v) KNO_3. One ml of each selected Hup^+ PGPR isolate (10^8 cells ml^{-1}) was used to inoculate on to a germinated soybean seed, and treated with hydrogen gas as described above. The data of root, shoot and plant dry weight were collected at 30 days after planting under the controlled condition as described above and compared with un-inoculated plant in the same condition.

3.8 The effect of co-inoculation between Hup^+ or Hup^- bradyrhizobia with Hup^+ PGPR on plant growth

The *B. diazoefficiens* USDA110 Hup^+ (10^8 cells ml^{-1}) or *Bradyrhizobium* sp. PC-5 Hup^- (10^8 cells ml^{-1}) were mixed with each selected Hup^+ PGPR (10^8 cells ml^{-1}) before inoculating onto soybean seed grown in sterilized vermiculite. Plants were watered with the N-free plant nutrient solution under light /dark condition as mented above. The nitrogenase activity was measured and the data of nodule numbers (mature nodules), nodule, root, shoot, and plant dry weight were collected at 30 days after planting.

3.9 Investigation plant growth promotion by Hup^+ PGPR under competitive condition

To mimic the competitive nodulation condition in the field, Hup^+ bradyrhizobia were grown in Yeast Extract Mannitol (YEM) medium, while selected

PGPR strain was grown in nutrients broth (NB) to obtain the cell concentration at 10^8 cells ml^{-1}. Single and co-inoculation were separated as following treatments.

(1) only *gus*-marked *B. diazoefficiens* USDA110 Hup^+

(2) only *Bradyrhizobium* sp. PC-5 Hup^-

(3) USDA110 (*gus*) + H39 (1:1)

(4) PC-5 + H39 (1:1)

(5) PC-5 + USDA110 (*gus*) (1:1)

(6) PC-5 + USDA110 (*gus*) + H39 (1:1:1)

(7) PC-5 + USDA110 (*gus*) (3:1)

(8) PC-5 + USDA110 (*gus*) + H39 (3:1:1)

The germinated soybean seeds were grown on sterilized vermiculite and watered with N-free plant nutrient solution under controlled condition as mented above. At 30 days after inoculation, the nitrogenase activity, and competitive nodulation were determined. The soybean inoculated with wild type of *B. diazoefficiens* USDA110 was used a control. The data of nodule numbers (mature nodules), root, shoot and plant dry weights were collected.

3.10 16S rRNA gene analysis

The Hup^- bradyrhizobium isolate PC-5 and Hup^+ PGPR isolate H39 were identified by cloning and sequencing of the 16S rRNA gene. The chromosomal DNA were extracted (Manassila, Nuntagij, Kotepong, Boonkerd, & Teaumroong, 2007) and used as a DNA template in PCR reactions. 16S rRNA gene was amplified by using the primers pair (fD1; 5’ AGAGTTTGATCCTGGCTCAG 3’ : rP2; 5’ ACGGCTAC CTTGTTACGACTT 3’) (Weisburg, Barns, Pelletier, & Lane, 1991). The PCR

products were purified using the QIA quick PCR purification kit (Qiagen, Hilden, Germany). The amplicons were ligated into the pTG19-T vector and then further transformed into *Escherchia coli* DH5∞ competent cells, following the manufacturer's protocol. Cells were grown overnight at 37 °C on petridish plates containing 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) (Sigma–Aldrich) supplemented with 100 μg ml^{-1} ampicillin (Sigma-Aldrich). White colonies (transformants) were picked randomly from the plates for colony PCR using the M13F and M13R primers (Bio Basic Canada Inc.). Twenty-five microliter of PCR reactions containing 0.1 U μl^{-1} GoTaq® DNA Polymerase (Promega), 1× PCR buffer and 1.5 mM $MgCl_2$ supplied with the enzyme, 0.2 mM dNTPs, 0.2 μM of each primer were performed using an Bio-Rad T100 TM Thermal Cycler under the following reaction conditions: 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 s, 55 °C for 1 s, and 72 °C for 1 s, and a final extension step at 72 °C for 7 min. PCR products were evaluated by running a small volume of product in 1% agarose gel. DNA sequencing was performed by MACROGEN company (Korea). The DNA sequences were generated and compared with the known DNA sequences obtained from the NCBI database.

3.11 The statistical analysis

Data from each experiment were submitted to analysis of variance (ANOVA), and a Duncan's multiple range test at $p \leq 0.05$ was used as a multiple comparison procedure by SPSS® software for WINDOWSTM, version 14.0 (Chicago, IL).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Hydrogenase uptake (Hup) phenotype of isolated indigenous bradyrhizobia in Thai soils and characterization of Hup$^-$ bradyrhizibia

Sixty two strains of bradyrhizobia were isolated from 12 soybean fields located in 4 provinces of Thailand. It was found that 69% of isolated bradyrhizobia lacked hydrogenase activity. Thus, most of indigenous bradyrhizobia in Thai soils were Hup$^-$ stains, this result matches to those observed in several studies indicating the most of *Bradyrhizobium japonicum* (previously named *Rhizobium japonicum*) isolates were Hup$^-$ strain (Schubert & Evans, 1976), and most of the nodules from several types of legume plant lacked hydrogenase uptake enzyme and unable to uptake hydrogen (Annan et al., 2012).

Some isolated Hup$^-$ bradyrhizobia phenotypes were found in several locations as indicated by DNA fingerprinting patterns of each isolated bacterium. Most of Hup$^-$ bradyrhizobia have N_2 fixation efficiency around 1-4 folds lower than that of commercial strain *B. diazoefficiens* USDA110, which performed nitrogenase activity around 15.86 µmole C_2H_4 h^{-1} g nodules DW^{-1} (Table 4.1). Some Hup$^-$ bradyrhizobia were selected based on their highly present as indigenous bradyrhizobia found in several locations, containing low nitrogenase activity, but performing good nodulation. The nodulation competitive ability of these bradyrhizobia as compared

with the commercial strain, USDA110. The result showed that most of them could compete for nodulation better than commercial inoculant (Table 4.2). The competitive nodulation ability of a *Bradyrhizobium* is one of the important properties to have a successful nodulation in the soil fields. The results in this study indicated that inoculation of commercial strain USDA110 could have high possibility failing to nodulate soybean in the field. Thus, nodules formed by Hup^- indigenous bradyrhizobia would be more than nodules formed by Hup^+ inoculated strain. These situations revealed that high amount of H_2 gas released from nitrogen fixation through nitrogenase activity would be in high level when soybeans were grown in the field condition. H_2 from Hup^- nodules released to soil may affect soybean growth, nitrogenase activity of inoculated bradyrhizobium, and rhizospheric bacterial community. To verify the effect of H_2 released from Hup^- nodule, bradyrhizobium isolate PC-5 was used as representatives of Hup^- indigenous brarhizobia for further experiments due to its high nodulation competitiveness ability when compared with commercial strain USDA110.

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Table 4.1 Characterization of isolated Hup$^-$ bradyrhizobia.

Box-PCR pattern no.	Isolates found	*Lacation	**Nitrogenase activity (nmole of C_2H_4 mg protein^{-1} day^{-1})	Nodules Number	Plant DW (g)
B. diazoefficiens USDA110 Hup$^+$			15.86 ± 1.72 ^{a}	50 ± 5 $^{c-h}$	1.45 ± 0.21 ^{a}
1	CC1-1	CC1	4.86 ± 0.98 $^{g-o}$	42 ± 0 $^{f-i}$	1.09 ± 0.12 $^{b-d}$
2	CC1-3	CC1	8.64 ± 3.57 $^{c-i}$	47 ± 3 $^{d-i}$	0.83 ± 0.15 $^{d-i}$
3	CC1-5	CC1	4.91 ± 3.07 $^{g-o}$	48 ± 18 $^{d-i}$	0.95 ± 0.18 $^{c-h}$
4	CC1-6	CC1	12.59 ± 2.62 $^{a-c}$	46 ± 2 $^{e-i}$	0.93 ± 0.09 $^{c-h}$
5	CC1-16	CC1	1.84 ± 2.07 $^{i-o}$	70 ± 2 $^{a-e}$	1.40 ± 0.40 ^{ab}
6	CC2-7	CC2	6.32 ± 1.02 $^{e-m}$	50 ± 0 $^{c-h}$	0.94 ± 0.18 $^{c-h}$
7	CC2-8	CC2	6.77 ± 0.14 $^{e-m}$	47 ± 4 $^{d-i}$	0.84 ± 0.09 $^{c-i}$
8	CC2-13	CC2, WL3	6.30 ± 2.27 $^{e-m}$	64 ± 1 $^{a-g}$	0.91 ± 0.20 $^{c-i}$
9	CC2-15	CC2, WP	5.58 ± 1.77 $^{f-o}$	64 ± 13 $^{a-g}$	0.74 ± 0.06 $^{d-i}$
10	CC3-4	CC3	2.01 ± 1.12 $^{i-o}$	47 ± 16 $^{d-i}$	0.82 ± 0.15 $^{d-i}$
11	CC3-7	CC3	0.35 ± 0.27 ^{o}	84 ± 21 ^{a}	0.64 ± 0.23 $^{f-i}$

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Table 4.1 (Continued).

Box-PCR pattern no.	Isolates found	*Lacation	**Nitrogenase activity (nmole of C_2H_4 mg protein^{-1} day^{-1})	Nodules Number	Plant DW (g)
12	CC3-10	CC3, JC1	4.05 ± 0.40 $^{i-o}$	78 ± 17 $^{a-b}$	0.88 ± 0.12 $^{c-i}$
13	CC3-14	CC3	7.25 ± 3.53 $^{d-k}$	57 ± 10 $^{b-g}$	0.86 ± 0.15 $^{c-i}$
14	JC1-4	JC1	8.51 ± 2.59 $^{c-i}$	45 ± 19 $^{e-i}$	0.86 ± 0.14 $^{c-i}$
15	JC1-5	JC1	9.55 ± 4.12 $^{b-h}$	50 ± 7 $^{c-h}$	0.73 ± 0.11 $^{d-i}$
16	JC1-8	JC1	8.54 ± 3.06 $^{c-i}$	60 ± 5 $^{b-g}$	0.64 ± 0.07 $^{f-i}$
17	JC1-15	WP	5.74 ± 4.11 $^{f-n}$	62 ± 14 $^{a-g}$	0.69 ± 0.03 $^{d-i}$
18	JC2-4	JC2	6.98 ± 3.16 $^{e-m}$	52 ± 14 $^{c-h}$	1.03 ± 0.22 $^{b-f}$
19	JC2-9	JC2	14.35 ± 4.83 ^{ab}	30 ± 6 $^{h-i}$	1.05 ± 0.37 $^{b-f}$
20	JC2-12	JC2	6.55 ± 1.62 $^{e-m}$	45 ± 16 $^{e-i}$	0.67 ± 0.04 $^{e-i}$
21	JC2-13	JC2	7.53 ± 1.84 $^{c-k}$	43 ± 11 $^{f-i}$	0.97 ± 0.16 $^{c-h}$
22	KC2-1	KC2	4.50 ± 0.68 $^{h-o}$	51 ± 14 $^{c-h}$	0.81 ± 0.25 $^{d-i}$
23	KC2-12	KC2	6.12 ± 0.67 $^{e-m}$	55 ± 13 $^{b-h}$	0.85 ± 0.20 $^{c-i}$

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Table 4.1 (Continued).

Box-PCR pattern no.	Isolates found	*Lacation	**Nitrogenase activity (nmole of C_2H_4 mg protein^{-1} day^{-1})	Nodules Number	Plant DW (g)
24	KC3-1	KC3	2.84 ± 3.23 $^{j-o}$	38 ± 11 $^{g-i}$	0.56 ± 0.07 $^{h-i}$
25	KC4-3	KC4	0.85 ± 0.11 $^{n-o}$	66 ± 6 $^{a-f}$	0.72 ± 0.04 $^{d-i}$
26	ML-8	ML	4.40 ± 0.61 $^{h-o}$	47 ± 1 $^{d-i}$	0.68 ± 0.10 $^{d-i}$
27	ML-9	ML	10.60 ± 2.64 $^{b-e}$	60 ± 10 $^{a-g}$	0.97 ± 0.18 $^{c-h}$
28	ML-10	ML	6.92 ± 0.16 $^{e-m}$	55 ± 4 $^{b-h}$	1.06 ± 0.22 $^{b-e}$
29	ML-12	ML	11.27 ± 1.27 $^{a-e}$	52 ± 12 $^{c-h}$	0.79 ± 0.07 $^{d-i}$
30	ML-13	ML	7.35 ± 1.76 $^{d-k}$	50 ± 9 $^{c-h}$	0.85 ± 0.18 $^{c-i}$
31	PC-2	PC	3.84 ± 2.09 $^{i-o}$	53 ± 16 $^{b-h}$	0.71 ± 0.02 $^{d-i}$
32	PC-3	PC	8.56 ± 5.85 $^{c-i}$	74 ± 21 $^{a-c}$	0.98 ± 0.28 $^{c-g}$
33	PC-5	CC2, CC3, ML, WL3, PC	5.07 ± 2.38 $^{g-o}$	80 ± 8 ^{a}	1.24 ± 0.05 $^{a-c}$
34	PC-6	PC	6.20 ± 2.33 $^{e-m}$	46 ± 1 $^{e-i}$	1.04 ± 0.38 $^{b-f}$

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Table 4.1 (Continued).

Box-PCR pattern no.	Isolates found	*Lacation	**Nitrogenase activity (nmole of C_2H_4 mg protein^{-1} day^{-1})	Nodules Number	Plant DW (g)
35	PC-7	PC	5.71 ± 1.97 $^{f-n}$	53 ± 13 $^{b-h}$	0.94 ± 0.25 $^{c-h}$
36	PC-8	PC	8.00 ± 2.58 $^{c-j}$	47 ± 8 $^{d-i}$	0.93 ± 0.29 $^{c-h}$
37	PC-11	PC	9.84 ± 4.25 $^{b-g}$	44 ± 13 $^{e-i}$	0.72 ± 0.06 $^{d-i}$
38	WL3-9	WL3	10.96 ± 1.38 $^{b-f}$	49 ± 8 $^{c-h}$	0.69 ± 0.15 $^{d-i}$
39	WL3-13	WL3	12.16 ± 5.70 $^{a-d}$	56 ± 28 $^{c-h}$	1.01 ± 0.46 $^{c-f}$
40	WP6	WP	5.14 ± 1.45 $^{g-o}$	48 ± 10 $^{c-i}$	0.96 ± 0.15 $^{c-h}$
41	WP-8	WP	5.63 ± 1.01 $^{f-n}$	72 ± 22 $^{a-d}$	0.67 ± 0.17 $^{e-i}$
42	WP-12	WP	2.72 ± 2.17 $^{k-o}$	23 ± 8 ^{i}	0.71 ± 0.14 $^{d-i}$
43	WP-14	WP	1.72 ± 1.03 $^{m-o}$	48 ± 19 $^{d-i}$	0.51 ± 0.08 ^{i}

*CC; Chiang Dow, Chiang Mai. JC; Jomthong, Chiang Mai. KC; Khon San, Chaiyaphum. ML; Muang, Lampang. PC; Phrao, Chiang Mai. WL; Wang Nua, Lampang. WP; Wang Chin, Phrae, and number after location indicated different area in the same location.

**Different letters in the same column indicated a significant difference among treatments ($P \leq 0.05$).

Table 4.2 Competitive nodulation of some Hup^- bradyrhizobial with USDA110 Hup^+.

Isolates	**Competitiveness ability (%) compared to USDA110 (ratio 1:1)**
CC1-16	80
CC2-8	60
CC2-13	63
CC2-15	36
CC3-10	32
JC1-8	71
JC1-15	74
JC2-4	58
KC2-1	67
KC4-3	39
ML-8	78
ML-9	37
ML-10	55
PC-2	72
PC-5	82
PC-6	64
PC-8	80

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4.2 The influence of the H_2 gas on plant growth and nitrogenase activity

The soybean grown under hydrogen with completed plant nutrients treatments did not show any significant difference in plant dry weight when compared to soybean treated with air. This result revealed that hydrogen gas (H_2) did not have any influence on soybean growth (Fig 4.1 and 4.3A).

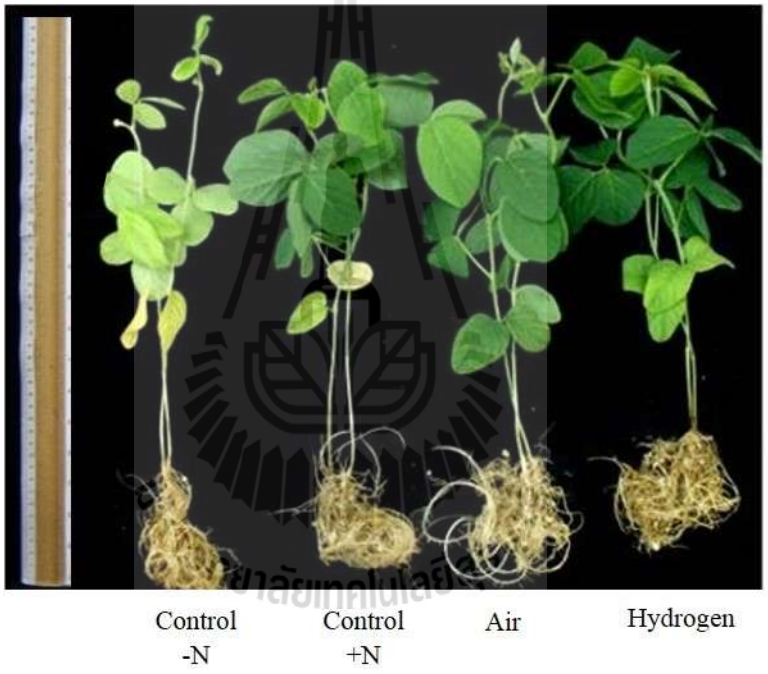

Figure 4.1 The growth of soybean in completed plant nutrient solution when treated with air or H_2. Data were collected at 30 days after planting.

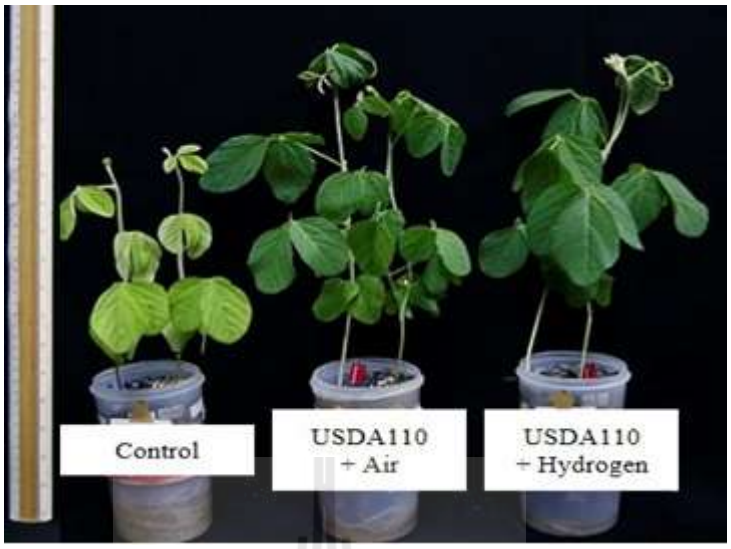

Figure 4.2 The growth of soybean when inoculated with *B. diazoefficiens* USDA110 under air or H_2 treated conditions. Data were collected at 30 days after planting.

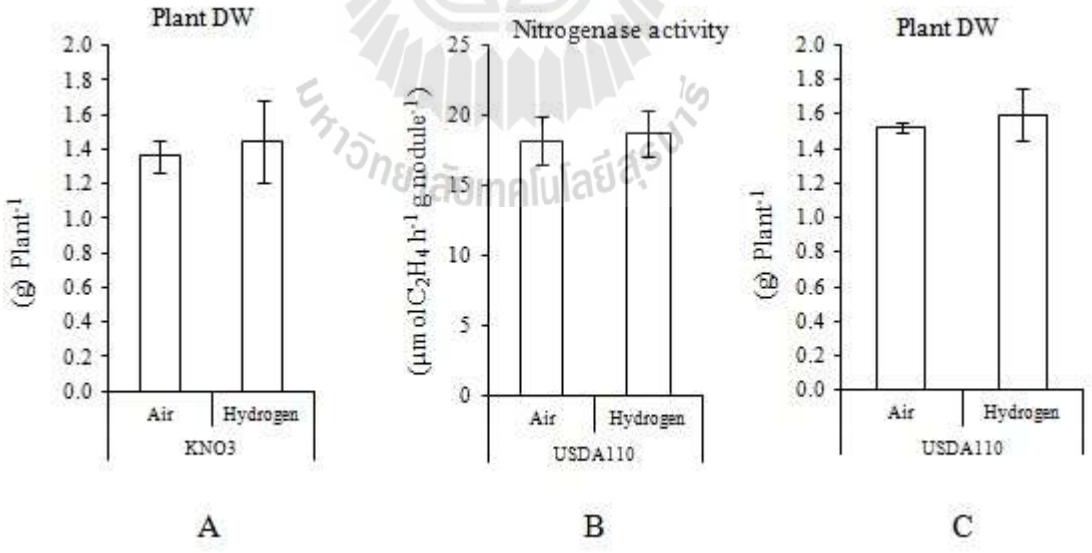

Figure 4.3 Effect of H_2 on (A) plant growth under completed plant nutrients condition, and (B-C) the nitrogenase activity and plant dry weight of soybean when inoculated with *B. diazoefficiens* USDA110 under H_2 or air treated conditions.

The exogenous H_2 gas was also treated to soybean inoculated with *B. diazoefficiens* USDA110 (Hup^+), which was able to uptake H_2 gas and recover some energy back for nitrogen fixation reaction (Schubert, Jennings, & Evans, 1978). The results showed that exogenous H_2 gas could not promote nitrogenase activity of USDA110 (Hup^+) (Fig 4.3B) and the growth of soybean (Fig 4.2 and 4.3C). Thus, it could be implied that hydrogen gas released from nitrogenase activity into soil did not support directly on plant growth and nitrogenase activity of USDA110 Hup^+ bradyrhizobial inoculant. Nevertheless, it has been reported that the nitrogenase activity of some of rhizobial bacteroids was increased under H_2 treated condition (Nelson & Salminen, 1982). On the other hand, the available hydrogen gas in the soil may benefit to other rhizospheric bacteria. Therefore, it is interesting to isolate the rhizospheric bacteria that can uptake H_2 as their energy source and having plant growth promoting activities. This Hup^+ PGPR can be used as co-inoculation with bradyrhizobium to support the growth of soybean in the field which may contain high number of Hup^- indigenouse bradyrhizobia.

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4.3 Isolation of Hup^+ rhizobacteria and their plant growth promoting properties

Soybean rhizospheric bacteria 20 isolates were selected and found 10 isolates having uptake hydrogenase (Hup^+) enzyme or hydrogen uptake ability. The plant growth promoting properties such as N_2 fixation ability, IAA production, and ACC-deaminase activity of isolated Hup^+ rhizobacteria strains were summarized in Table 4.3. Although, most of Hup^+ rhizobacteria had N_2 fixation ability, the activity still lower than that of Rhizobium-legume symbiosis (Shanmugam, O'gara, Andersen, &

Valentine, 1978). The mechanism mostly explain the direct effects of plant growth promoting bacteria on plants is the production of phytohormones, including auxins such as indole acetic acid or IAA (Barazani & Friedman, 1999; Joseph, Ranjan Patra, & Lawrence, 2012; Lawongsa, Boonkerd, Wongkaew, O'Gara, & Teaumroong, 2008; Patten & Glick, 1996, 2002). The results showed that all of isolated rhizobacteria produced plant hormone indole acetic acid (IAA) in different levels. Although, isolate H3 produced highest IAA, PGPR produced high amount of IAA did not always show the highest capability of promoting soybean biomass when co-inoculated with *Bradyrhizobium* (Prakamhang et al., 2015). On the opposite way, it has been reported that the lower amount of IAA produced by *B. subtilis* 101 could promote more tomato biomass than higher amount of IAA produced from *Azospirillum brasilense* Sp245 (Felici et al., 2008). Thus, the low level of IAA production produced from PGPR might play an important role for soybean growth promotion. The reduction of ethylene in the plant through ACC-deaminase activity is another mode of action for PGPR (Vacheron et al., 2013). The rhizobacteria containing ACC-deaminase activity has been reported to promote nodulation (Ma, Guinel, & Glick, 2003) and plant growth under stress condition (Saleem, Arshad, Hussain, & Bhatti, 2007). The ACC-deaminase can cleave the plant ethylene precursor ACC, and thereby lower the level of ethylene in a developing or stressed plant (Glick et al., 1998; Jacobson, Pasternak, & Glick, 1994). Inoculation rhizobacterial strains containing ACC-deaminase activity also significantly promoted root, shoot, and other growth contributing parameters of wheat under salinity condition (Zahir, Ghani, Naveed, Nadeem, & Asghar, 2009). Thus, this Hup^+ PGPR may be able to co-inoculate with bradyrhizobia to increase root

elongation and finally promote plant growth under normal or stress conditions, which often occurred in the field.

Some bacterial isolates were identified at species level based on the partial sequence analysis of 16S rRNA gene. The bradyrhizobial isolate PC-5 (accession number LC005452) was closely related (99% identity) to *Bradyrhizobium* sp. SEMIA 5021 (accession number FJ390905.1), while rhizobacterial isolate H39 (accession number LC005453) was closely related (99% identity) to *Bacillus megaterium* strain HDYM-24 (accession number EF428248.2).

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Table 4.3 Characterization of plant growth promoting properties of isolated Hup^+ rhizobacteria.

Hup^+ PGPR isolates	**Nitrogenase activity (nmole of C_2H_4 mg protein^{-1} day^{-1})**	**IAA (µM mg protein^{-1})**	**ACC-deaminase activity test***
H3	0.120 ± 0.105 ^{bcd}	2.392 ± 0.146 ^{a}	-
H22	0.047 ± 0.004 ^{d}	1.498 ± 0.412 ^{b}	-
H23	Nd	0.295 ± 0.151 ^{d}	-
H39	0.094 ± 0.012 ^{cd}	0.507 ± 0.032 ^{c}	+
H55	0.061 ± 0.017 ^{d}	0.040 ± 0.003 ^{d}	+
2H6	0.228 ± 0.013 ^{a}	0.109 ± 0.077 ^{d}	+
2H10	0.065 ± 0.030 ^{cd}	0.107 ± 0.010 ^{d}	-
2H11	0.173 ± 0.011 ^{ab}	0.087 ± 0.005 ^{d}	+
2H16	0.135 ± 0.041 ^{bc}	0.210 ± 0.004 ^{d}	+
2H17	0.096 ± 0.007 ^{cd}	0.128 ± 0.008 ^{d}	+

*Growth on minimal agar medium containing ACC (+, can grow; -, cannot grow) nd; not detected. Different letters in the same column indicate a significant different among treatments ($P \leq 0.05$).

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4.4 Soybean plant growth promotion by Hup^+ PGPR under H_2 treated condition

To investigate the effect of H_2 and Hup^+ PGPR on soybean growth without symbiosis with *Bradyrhizobium*, the minimum effective level of nitrogen required for soybean growth was determined. The germinated seeds of soybean were grown in vermiculite and watered with N-free plant nutrient solution (Haugland et al., 1983) supplemented with different concentrations of KNO_3 as 0.01, 0.02, 0.03, 0.04 and 0.05% (w/v). After 30 days of planting, plants were dried and investigated the plant dry weight. Finally, the KNO_3 at 0.03% was selected at the minimum requirement amount of nitrogen that could support plant growth better than control (Fig 4.4 and 4.5). Then, soybean grown under H_2-treated condition was conducted and the results are presented in Fig 4.6 and 4.7. The growth of soybean increased when inoculated with some Hup^+ PGPR under both H_2 treated and non-H_2 treated conditions. The soybean growth was significantly increased when inoculated with isolates 2H17 and H39. Interestingly, soybean growth promotion by these Hup^+ PGPR was higher in H_2 treated more than that of air treated conditions. Plants inoculated with PGPR isolate H39 under H_2 treated condition were found to increase both of shoot and root dry weights higher than that of non-H_2 treated condition (Fig 4.7A and B). Therefore, this result clearly showed that the H_2 gas contributed the Hup^+ PGPR to promote plant growth. It could be possible that PGPR isolate H39 containing hydrogenase uptake (Hup^+) derived chemical energy from H_2 and use it for cell metabolisms (Baginsky, Brito, Imperial, Palacios, & Ruiz-Argüeso, 2002), leading to increase PGPR properties to promote the root and shoot of soybean.

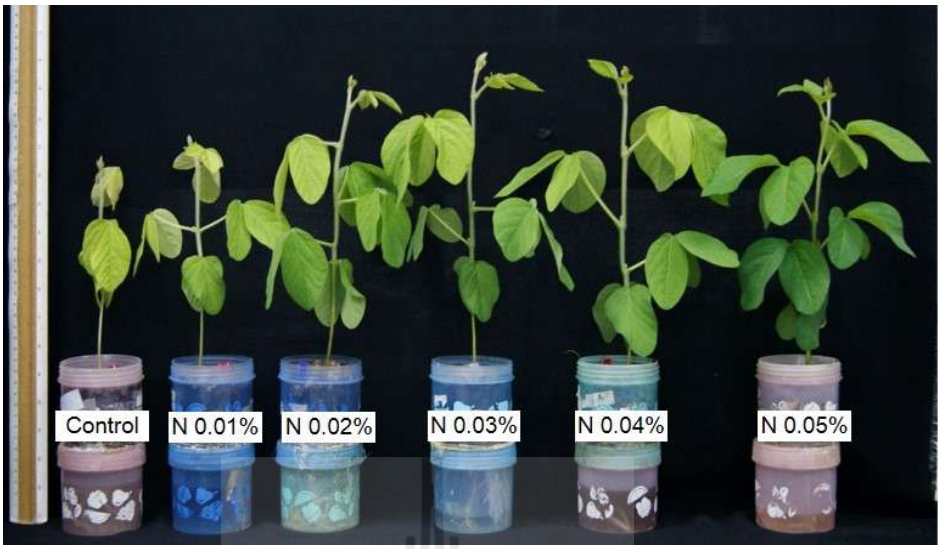

Figure 4.4 Soybean growth under N-free plant nutrient solution supplemented with different concentrations of KNO_3. Data were collected at 30 days after planting.

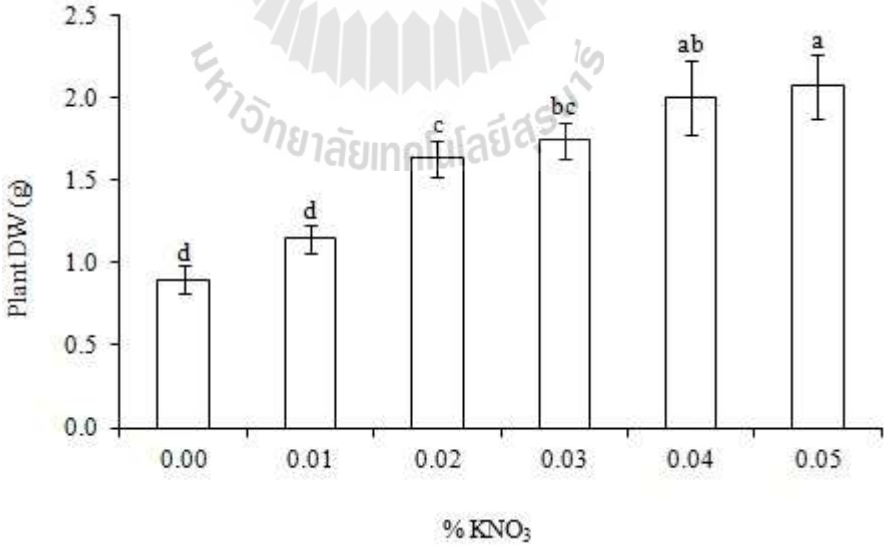

Figure 4.5 Plant dry weight of soybean grown under N-free plant nutrient solution supplemented with different concentrations of KNO_3.

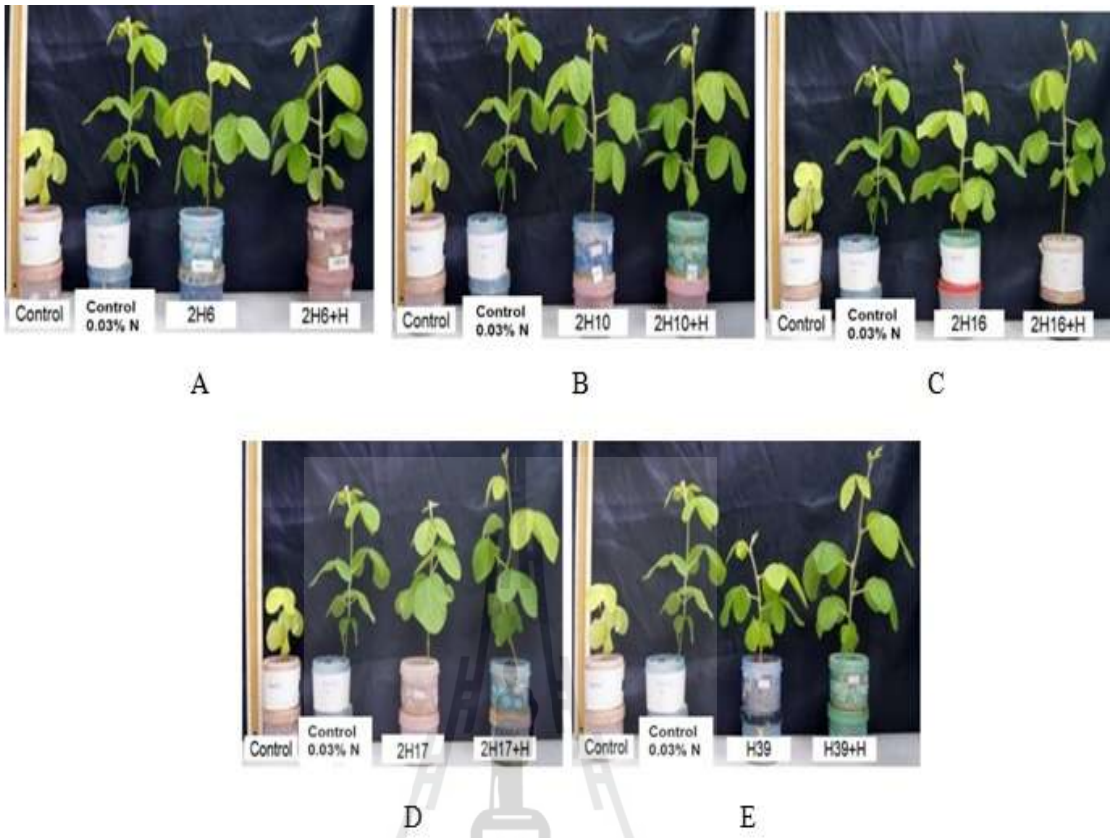

Figure 4.6 The growth of soybean in N-free plant nutrient solution supplemented with 0.03% KNO_3 and inoculated with Hup^+ PGPR isolates 2H6 (A) 2H10 (B) 2H16 (C) 2H17 (D) and H39 (E) under H_2 treated and non-H_2 treated conditions.

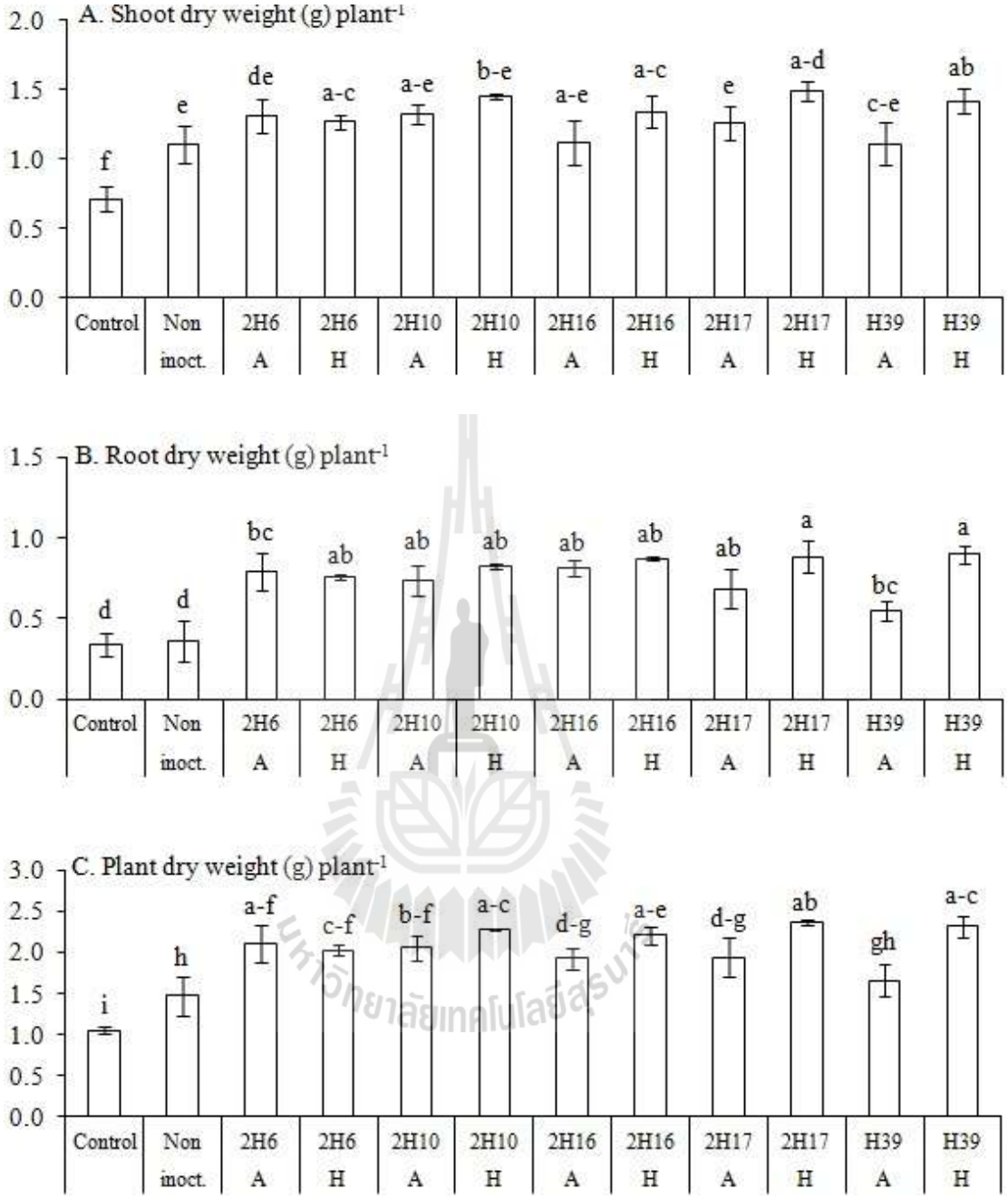

Figure 4.7 Shoot dry weight (A), root dry weight (B), plant dry weight (C) of soybean grown in N-free plant nutrient solution supplemented with 0.03% KNO_3 and inoculated with Hup^+ PGPR isolates 2H6, 2H10, 2H16, 2H17 and H39 under H_2 treated (H) and air treated (A) conditions.

4.5 The effect of different H_2 concentrations on nitrogenase activity and IAA production in selected Hup^+ PGPR

Since Hup^+ PGPR isolate H39 obviously showed soybean plant growth promotion under H_2 treated condition, it was intrigued to investigate whether the nitrogenase activity and IAA production in H39 response to different concentrations of H_2 gas. The experiments were performed *in vitro* under H_2 treated condition. The nitrogenase activity of this bacterium tended to decrease when the concentration of H_2 was increased and significantly decreased at H_2 concentration 50,000 ppm (Fig 4.8A). It is possible that H_2 act as inhibitor of nitrogenase activity (Guth & Burris, 1983). Nevertheless, the IAA production of this isolate at H_2 concentration of 500 ppm was significantly increased when compared with non-H_2 treated condition, while increasing the H_2 concentration to 5,000 and 50,000 ppm reduced the IAA production (Fig 4.8B). To my knowledge, this is the first report showing the influence of H_2 on IAA production. Moreover, the IAA production of PGPR isolate H39 was also determined when grown in the medium with and without-tryptophan as precursor. The result showed that H_2 could not induce IAA production in isolate H39 when absent tryptophan as precursor (Fig 4.9). It could be assumed that PGPR isolate H39 produce IAA only through tryptophan dependent path way, which is activated by H_2 gas. Therefore, it is interesting to investigate whether IAA produced by Hup^+ PGPR play an important role on plant growth promotion.

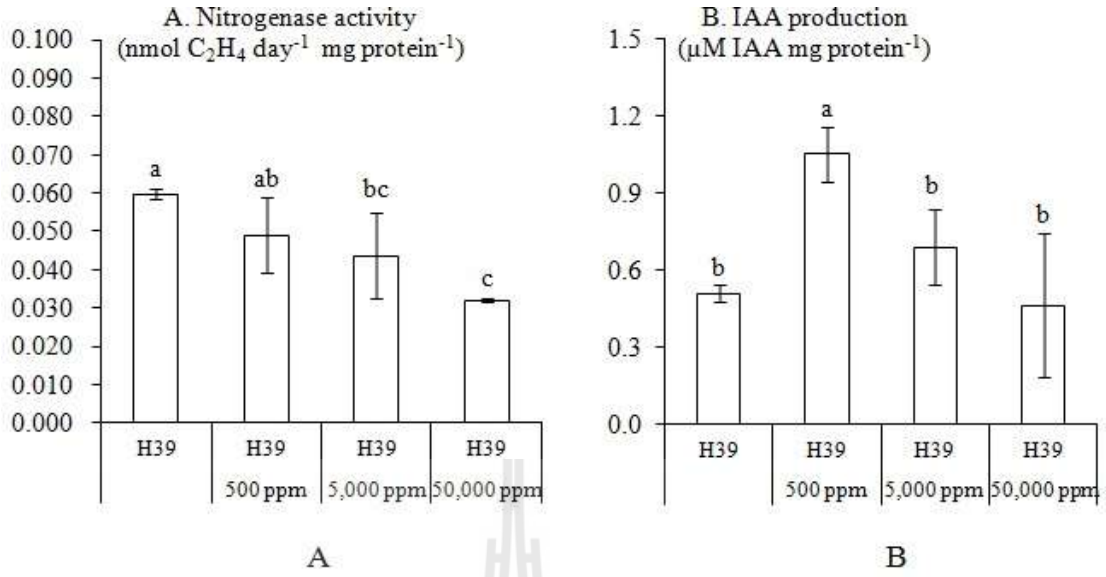

Figure 4.8 The effect of different H_2 concentrations on (A) nitrogenase activity (B) IAA production of PGPR H39 grown under H_2 treated condition.

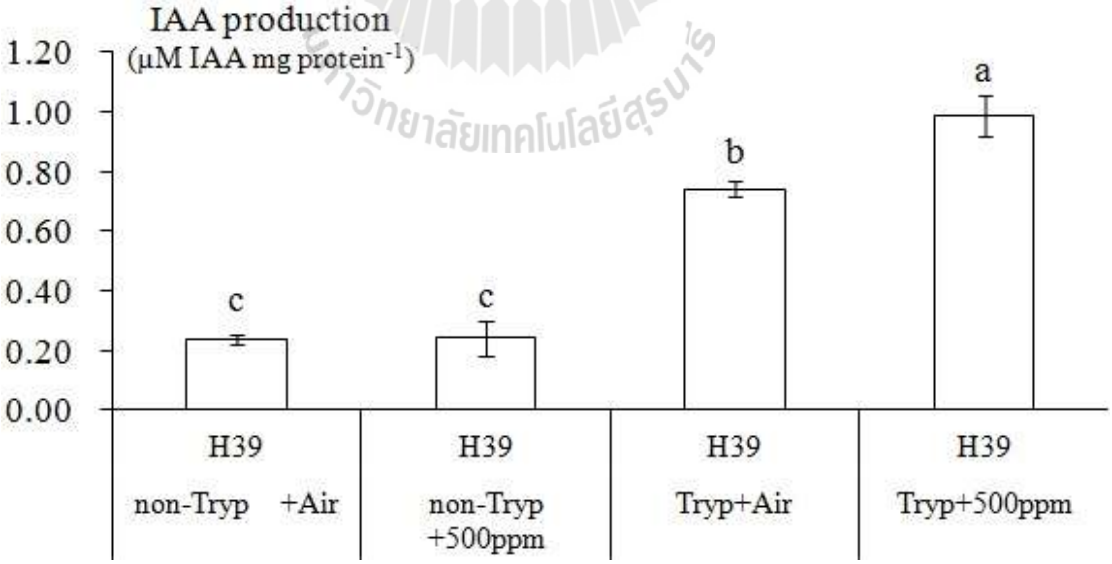

Figure 4.9 The effect of H_2 on IAA production of PGPR isolate H39 in the medium with and without tryptophan as precursor.

4.6 The effect of different IAA concentrations on soybean growth

The Hup^+ PGPR isolate H39 produced IAA in the range of 0.51-1.05 µM mg $protein^{-1}$ under air and H_2 treated conditions, respectively (Fig 4.8B). To investigate the role of IAA on plant growth, the different IAA concentrations were applied to soybean and the data of shoot, root, and total plant dry weight were determined at 30 days after planting. It was found that the IAA concentration in range of 0.2-0.6 µM did not support soybean growth when compared with non-IAA treated plant. Whereas, IAA concentration in the range of 0.8-1 µM could increase the shoot dry weight, led to promote the total plant dry weight better than that of control N (Fig 4.10 and 4.11A, C). Thus, in this case, IAA produced from isolate H39 may be one of the PGPR properties that support soybean growth.

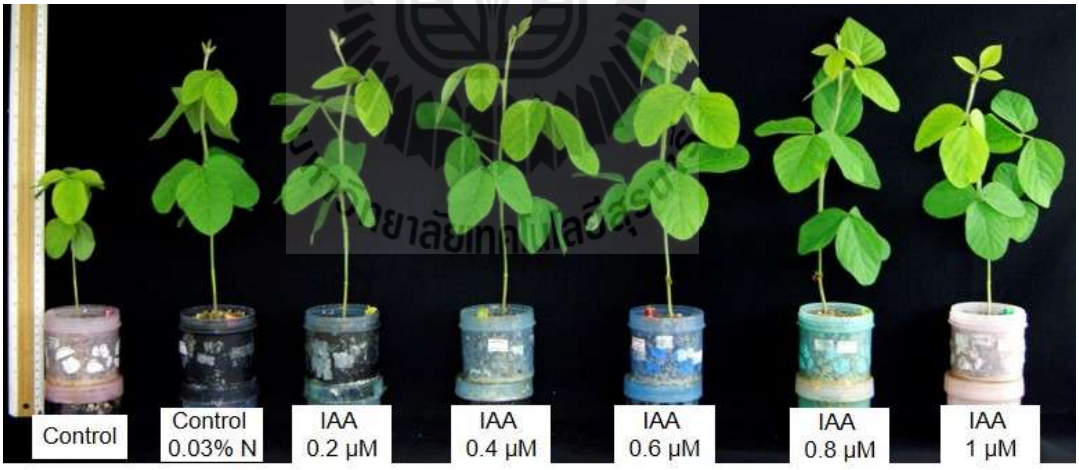

Figure 4.10 The growth of soybean in N-free plant nutrient solution supplemented with 0.03% KNO_3 and different IAA concentrations.

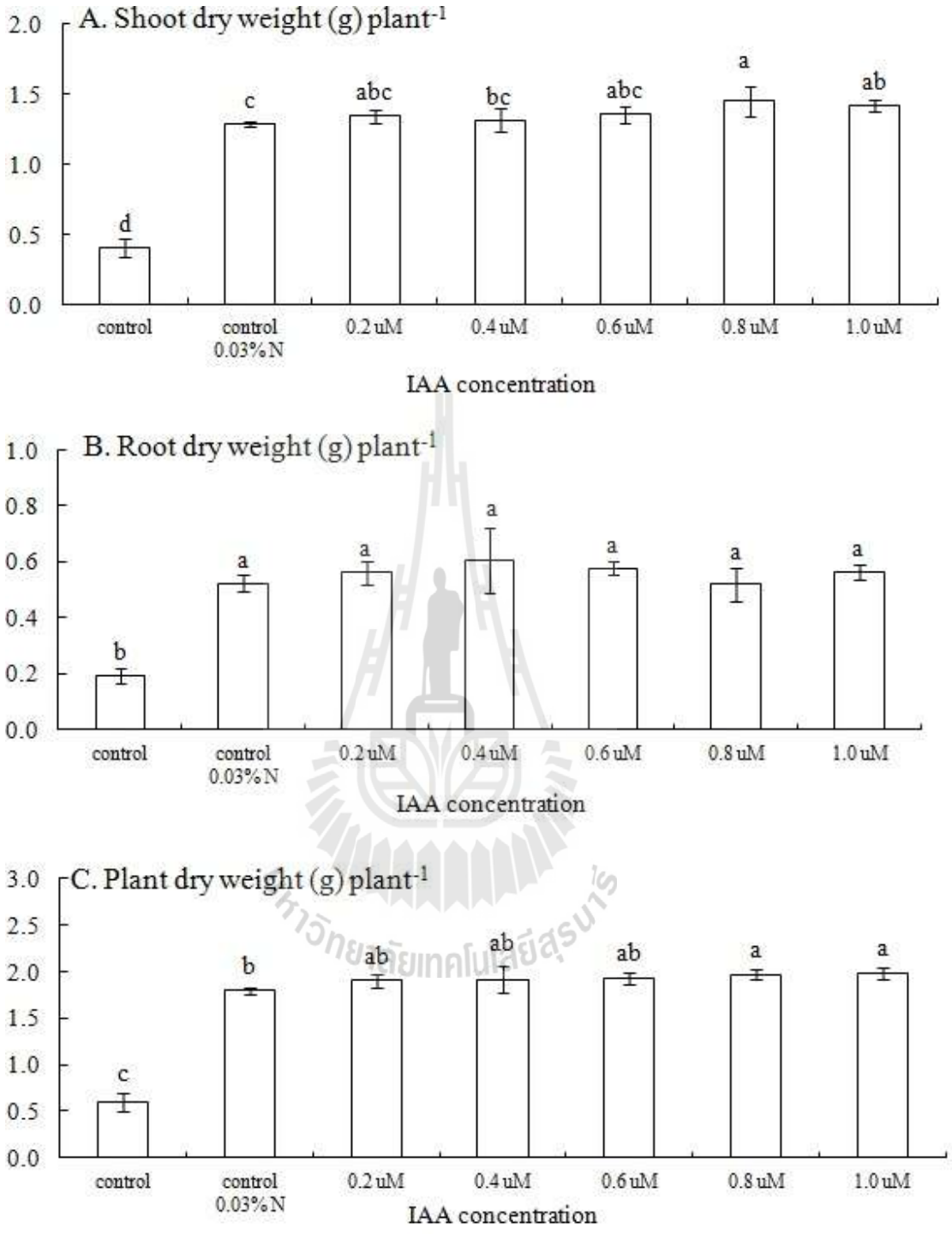

Figure 4.11 Shoot dry weight (A), root dry weight (B), plant dry weight (C) of soybean grown in N-free plant nutrient solution supplemented with 0.03% KNO_3 and different IAA concentrations.

4.7 The effect of co-inoculation between Hup$^+$ or Hup$^-$ bradyrhizobia with Hup$^+$ PGPR on plant growth

In order to evaluate the plant growth promotion effects induced by different Hup$^+$ PGPR (2H6, 2H10, 2H16, 2H17, H39), the nitrogenase activity and plant growth were investigated when co-inoculation *B. diazoefficiens* USDA110 Hup$^+$ or *Bradyrhizobium* sp. PC-5 Hup$^-$ with different Hup$^+$ PGPR strains in N-free plant nutrient solution. It was found that the dry weight of plant co-inoculated Hup$^+$ PGPR strains with USDA110 was not significantly different from plant inoculated with Hup$^+$ USDA110 alone (Fig 4.12 and 4.13A). It could be possible that the amount of H_2 released from nitrogen fixation reaction of Hup$^+$ bradyrhizobial may not be enough to support PGPR and lead to promote soybean growth, since co-inoculation with *Bradyrhizobium* containing hydrogenase (Hup$^+$) could uptake H_2 back to the cells. Although the Hup$^+$ PGPR was found to promote plant grown in minimum N source under both H_2 treated and air treated conditions, the Hup$^+$ USDA110 performed high nitrogenase activity and provided high amount of N to host plant. Therefore, the plant growth promotion properties by PGPR may not be obviously observed. However, co-inoculation of Hup$^+$ USDA110 with some Hup$^+$ PGPR strains, such as H39 significantly increased nodule number and nodule dry weight (Fig 4.13B and C), while co-inoculation with Hup$^+$ PGPR strain 2H6 significantly increased nitrogenase activity (Fig 4.13D).

The *Bradyrhizobium* sp. PC-5 was used as a representative of native Hup$^-$ *Bradyrhizobium* to investigate the effect of co-inoculation with Hup$^+$ PGPR on soybean growth. The results showed that all Hup$^+$ PGPR co-inoculated with Hup$^-$ *Bradyrhizobium* sp. PC-5 increased soybean growth and plant dry weight when

compared to the single inoculation of Hup$^-$ PC-5 (Fig 4.14 and 4.15A). However, co-inoculation with Hup$^+$ PGPR strains did not enhance the nodule number, while the nodule dry weigh was significantly increased when plants were co-inoculated Hup$^-$ PC-5 with Hup$^+$ PGPR strain H39 (Fig 4.15B and C). Similar results were also found in co-inoculation of USDA110 with H39. The nitrogenase activity obtained from co-inoculations of PC-5 Hup$^-$ and PGPR (2H6, 2H16, 2H17, H39) treatments were significantly increased and higher than that of single inoculation (Fig 4.15D). Therefore, the H_2 diffused from nodule Hup$^-$ may activate Hup$^+$ PGPR isolates 2H17 and H39 enabling promote the soybean growth with the same as results H_2 treated to plant under minimum N condition. All results demonstrated that Hup$^+$ PGPR isolate H39 could be used for co-inoculation with USDA110 when presented high competition from indigenous bradyrhizobia.

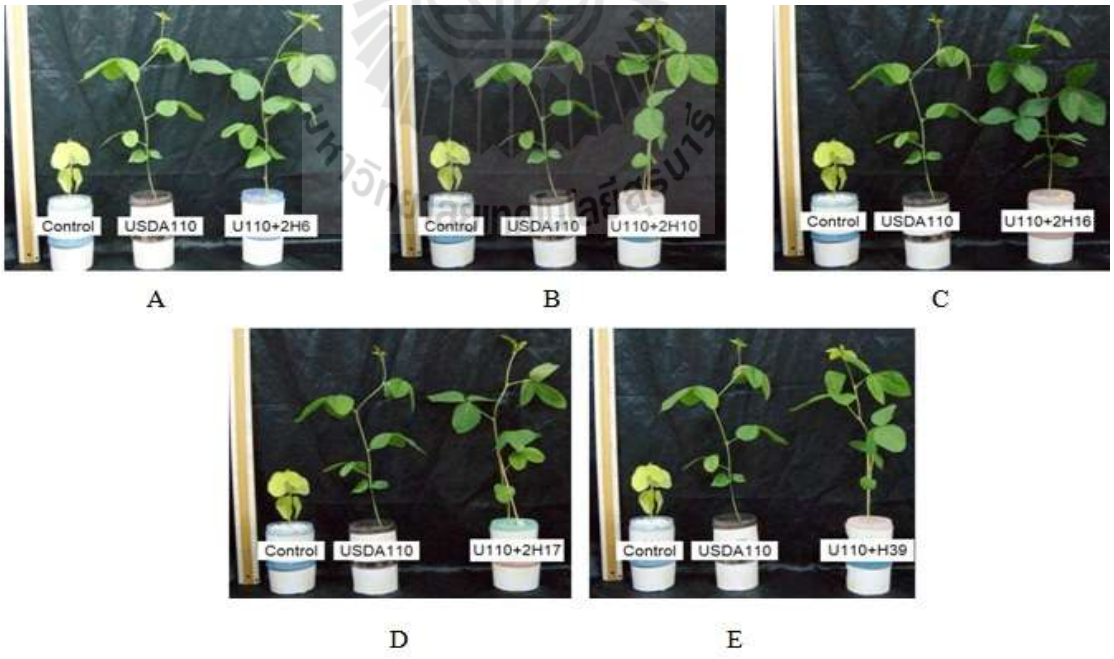

Figure 4.12 Soybean growths when co-inoculation between *B. diazoefficiencs* USDA110 Hup$^+$ and Hup$^+$ PGPR isolates 2H6 (A) 2H10 (B) 2H16 (C) 2H17 (D) and H39 (E) respectively.

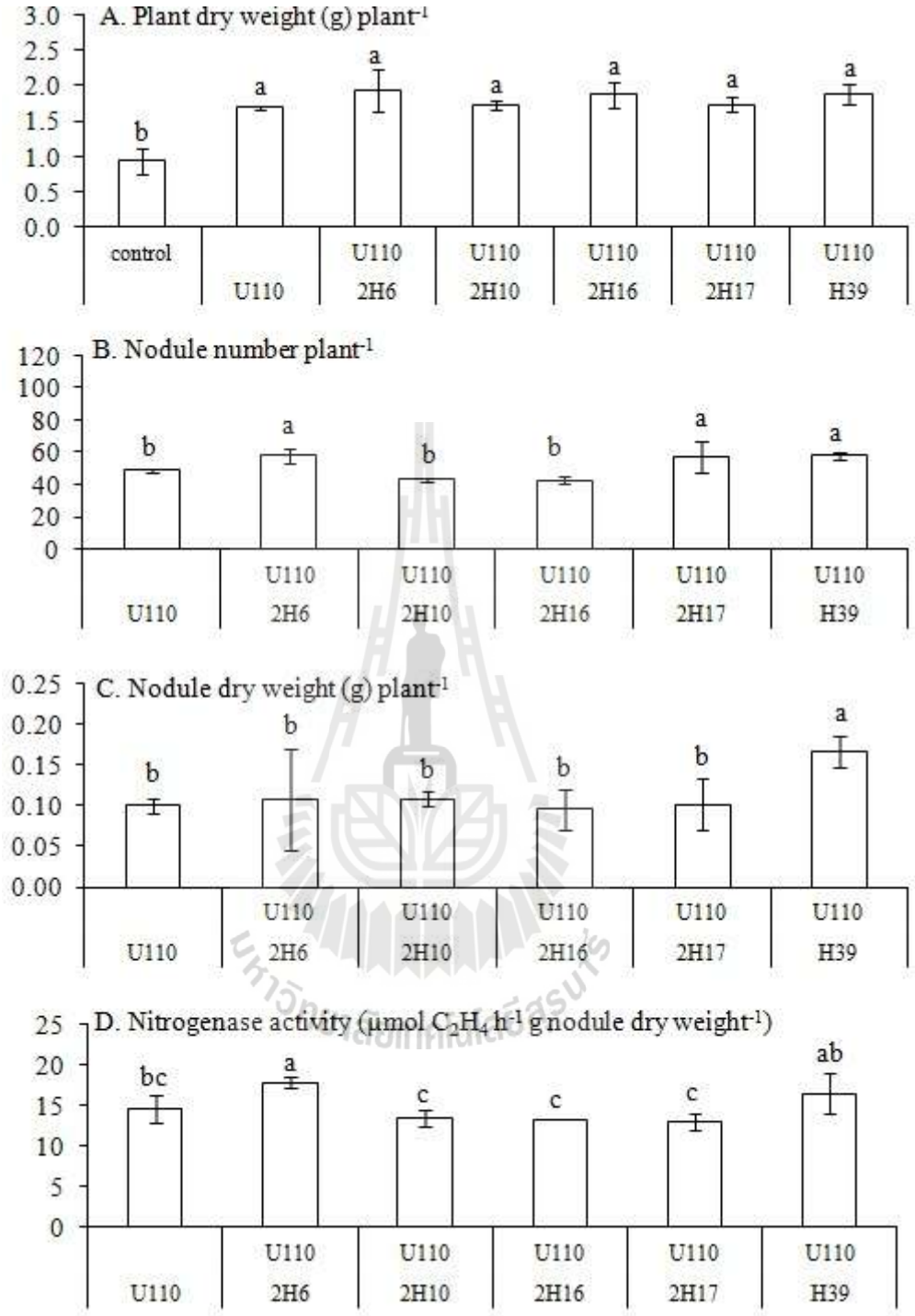

Figure 4.13 Plant dry weight (A), nodule number (B), nodule dry weight (C), and nitrogenase activity (D) of soybean co-inoculated *B. diazoefficiencs* USDA110 Hup^+ with Hup^+ PGPR isolates 2H6, 2H10, 2H16, 2H17 and H39.

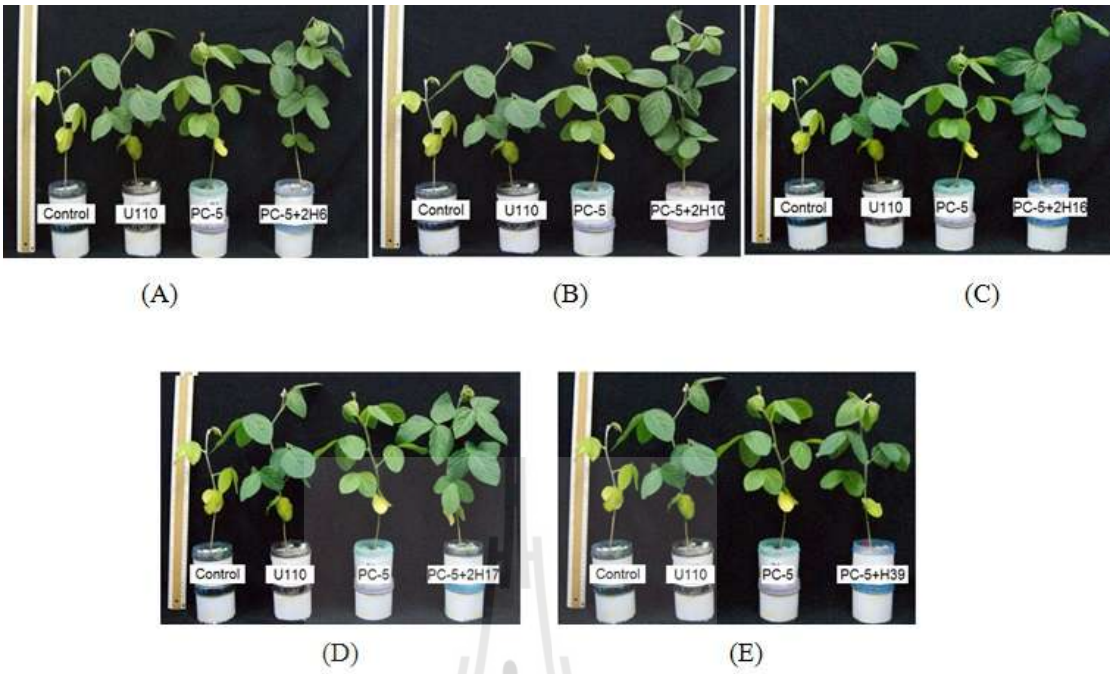

Figure 4.14 Soybean growth when co-inoculation between *Bradyrhizobium* sp. PC-5 Hup^- with Hup^+ PGPR isolates 2H6 (A) 2H10 (B) 2H16 (C) 2H17 (D) and H39 (E), respectively.

4.8 Investigation of plant growth promotion by Hup^+ PGPR under competitive condition

The experiments were conducted to evaluate the properties of PGPR isolate H39 in order to increase the efficiency of inoculant USDA110 in the presence of Hup^- PC-5 to promote soybean growth. Therefore, the several competitive nodulation conditions were used to imitate the competitive situation occurred in the field and investigated on the basis of nodule occupancy.

Single inoculation of USDA110 and co-inoculation with H39 (USDA110 + H39) did not show any difference in term of plant dry weight. However, number of soybean nodules obtained from co-inoculation USDA110 with H39 (USDA110 +

H39) was higher than that of USDA110 single inoculation. The single inoculation of PC-5 obtained significantly lower plant dry weight than that of USDA110, but the data were not significantly different when compared with the USDA110 + H39 treatment. Even though, the nitrogenase activity of USDA110 was significantly higher than PC-5 (Fig 4.15D), the PC-5 produced more nodule number (~80 nodules) as per plant than USDA110 (~50 nodules). Hence, the nitrogen content available to plant may not be different between treatments. Co-inoculation of PC-5 with H39 (PC-5 + H39) promoted the plant growth better than that of PC-5 single inoculation (Fig 4.14 and 4.15A).

The competitive condition between USDA110: PC-5 (1:1) was significantly increased in plant dry weight when compared to plant inoculated with PC-5 alone. The nodule occupancy of USDA110 and PC-5 was 28% and 72%, respectively. This result indicated that the nitrogen provided from USDA110 and PC-5 nodules supported plant growth in the similar level when inoculated with USDA110 alone. However, the plant dry weight was significantly increased when co-inoculation Hup^+ PGPR isolate H39 with bradyrhizobia under competitive condition of USDA110 : PC-5 (1:1). It could be possible that soybean nodules formed by some Hup^+ bradyrhizobia act as a nitrogen fixer, while some nodules formed by Hup^- bradyrhizobia released the H_2 to support Hup^+ PGPR and rendering promote soybean growth. The nodule occupancy of USDA110 was lower in the treatment condition of USDA110 : PC-5 (1:3), since the number of PC-5 increased 3-folds over USDA110. The nodule occupancy of USDA110 and PC-5 was 12% and 88%, respectively, which was not significantly different when co-inoculated with H39. Under high competition conditions, the plant dry weight tended to decrease and co-inoculation of

bradyrhizobia with H39 did not significantly enhance plant growth. This result indicated that nitrogen fixation by USDA110 is required to promote plant growth, while the PGPR properties produced by H39 did not have much influence under this condition. However, the analysis of soybean nodule dry weights revealed that inoculation of H39 resulted in a significant increase in the nodule biomass (Fig 4.16 and 4.17). Dong et al. (2003) performed the H_2 treated soil at a rate and duration similar to the amount of H_2 presence in soil adjacent to legume nodules before planted. They found that the soil treated with H_2 could enhance plant growth of spring wheat, canola, barley and soybean (nonsymbiotic) when compared with the un-treated soils or with the soils pretreated with air. Hence, releasing of H_2 from Hup^- nodules into soil may be beneficial to soil indigenous bacteria, which can improve plant growth by providing plant available nutrients, increasing the uptake of mineral nutrients, and protecting plants against pests and diseases (Glick, 1995). It has been shown that the H_2 released from the Hup^- nodules is uptaken and oxidized by native bacteria in the soil around nodules (Dong & Layzell, 2001). Maimaiti et al. (2007) found that 17 isolates of Hup^+ PGPR isolated from soil adjacent to Hup^- nodule and H_2 treated soil promoted roots elongation of spring wheat seedling and increased the plant biomass of *Arabidopsis thaliana*. Therefore, using Hup^+ PGPR co-inoculation with effective bradyrhizobium could enhance the efficiency of soybean inoculant when applied in the field which contains Hup^- bradyrhizobia compete for nodulation.

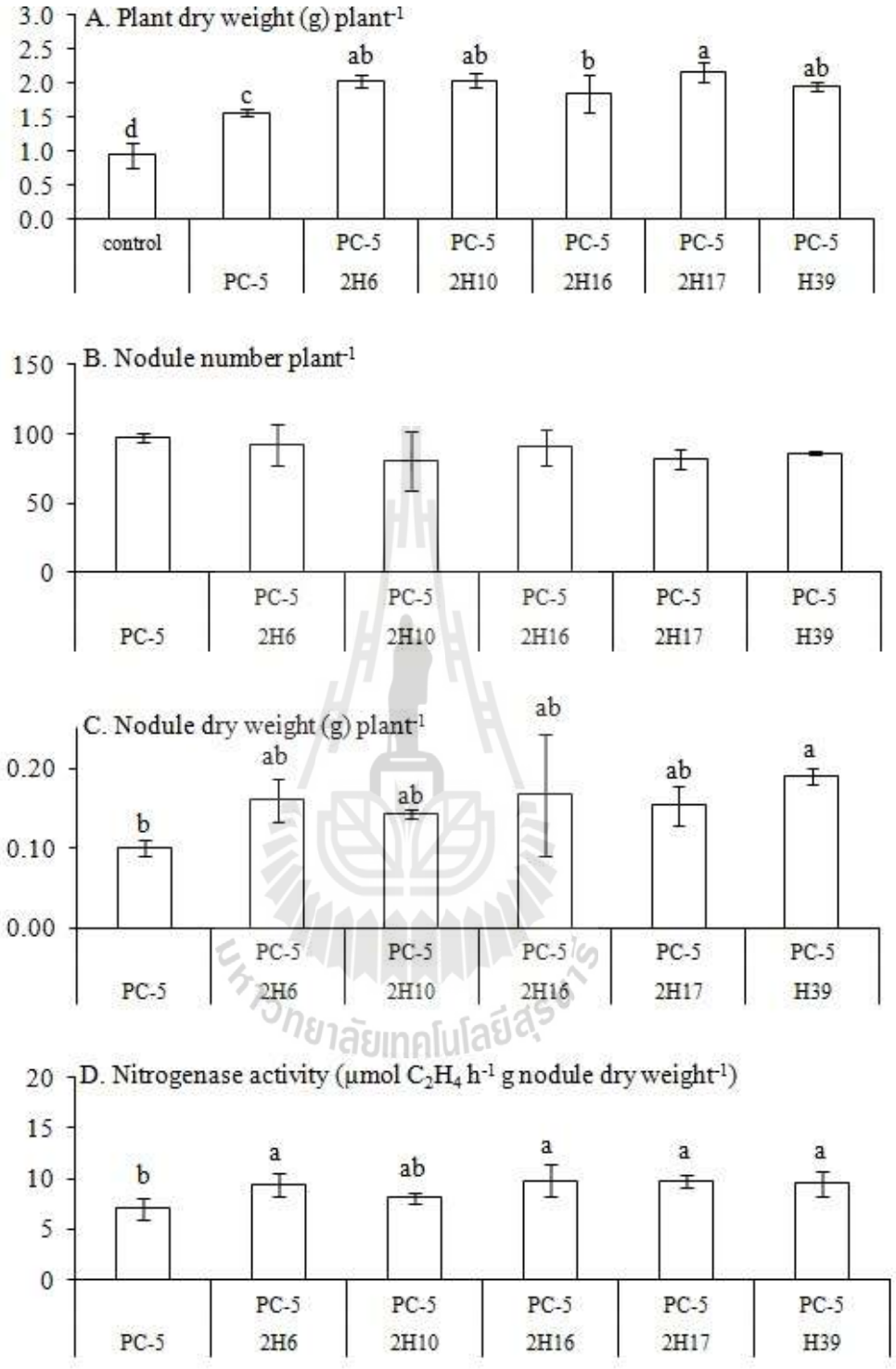

Figure 4.15 Plant dry weight (A), nodule number (B), nodule dry weight (C), and nitrogenase activity (D) of soybean co-inoculated between *Bradyrhizobium* sp. PC-5 Hup^- with Hup^+ PGPR isolates 2H6, 2H10, 2H16, 2H17 and H39, respectively.

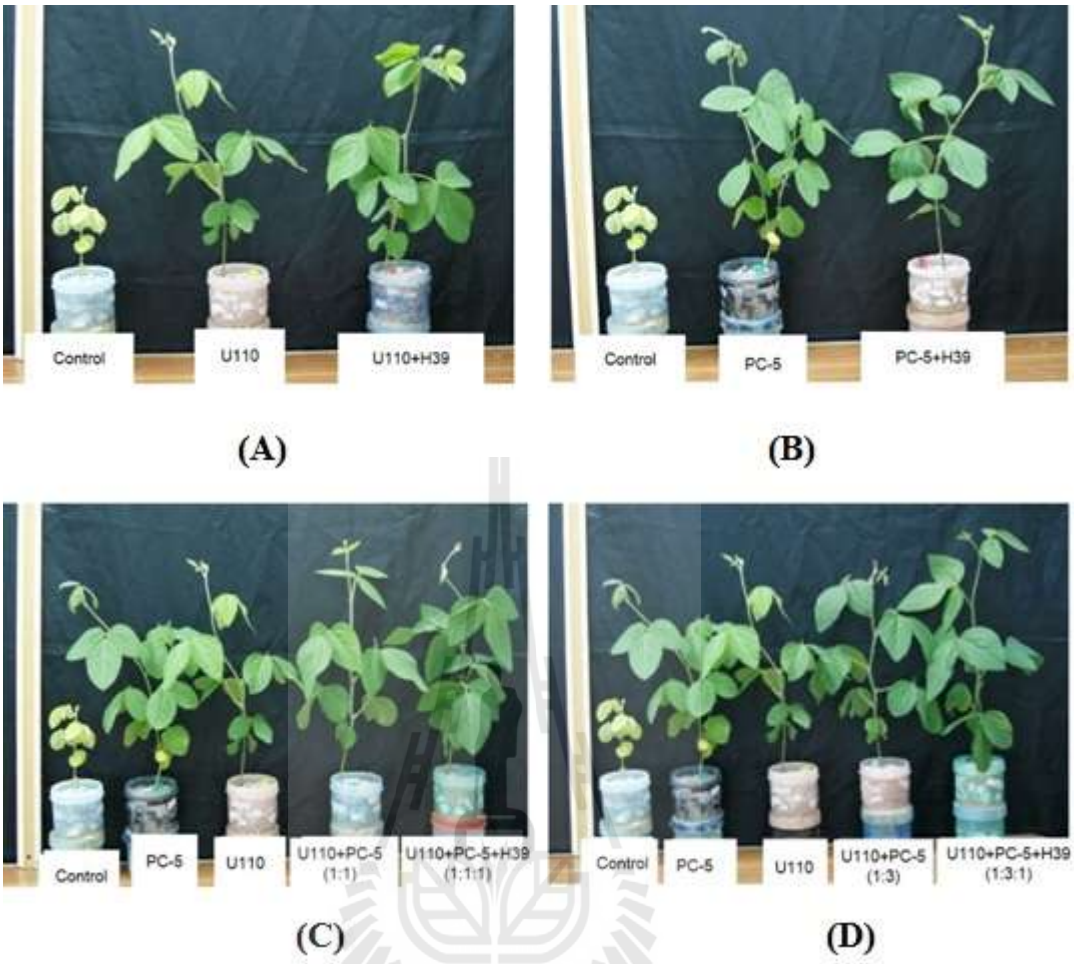

Figure 4.16 Soybean growth promotion by Hup^{+} PGPR isolate H39 with *B. diazoefficiencs* USDA110 (A) or *Bradyrhizobium* sp. PC-5 (B) under normal and competitive condition as ratio 1:1 (C), 1:3 (D).

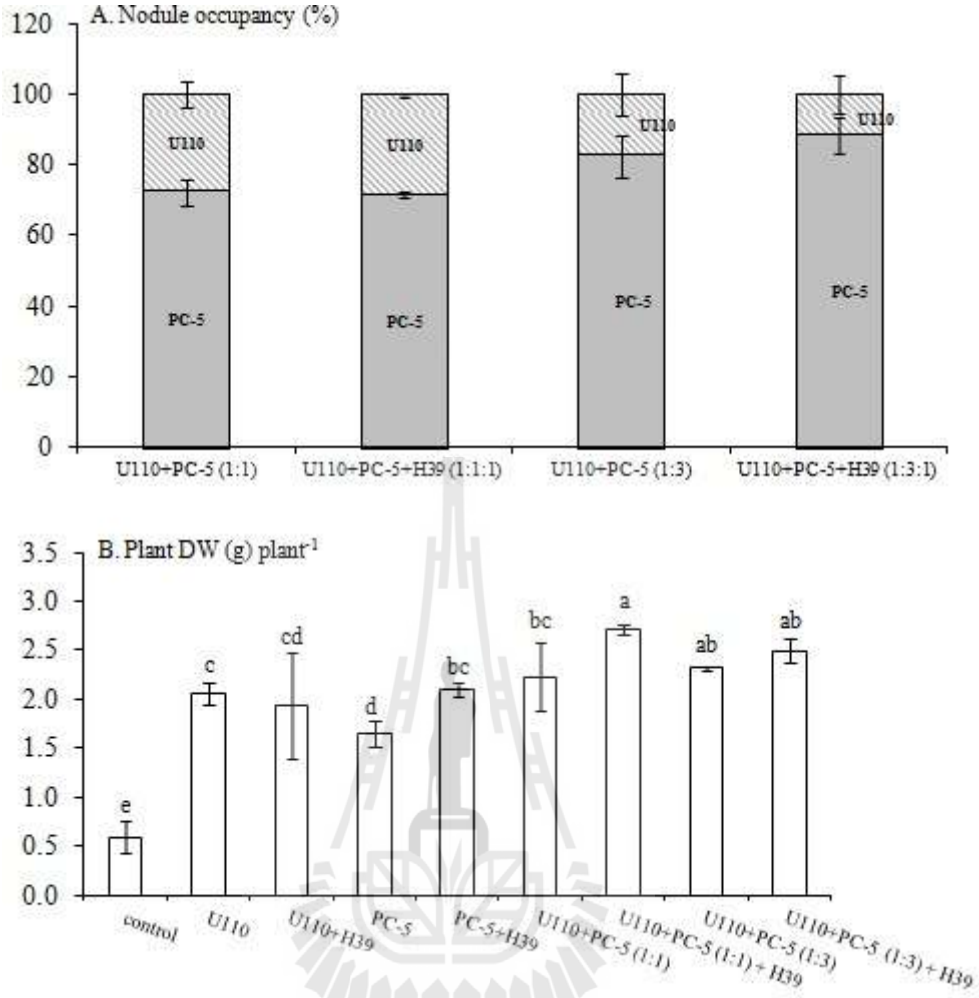

Figure 4.17 Nodule occupancy (%) of *B. diazoefficiencs* USDA110 and *Bradyrhizobium* sp. PC-5 (A), plant dry weight (B), nodule number (C), nodule dry weight (D) and nitrogenase activity (E) of soybean growth promotion by Hup^+ PGPR isolate H39 under normal and competitive condition between USDA110 with PC-5 as ratio 1:1 (C), 1:3 (D).

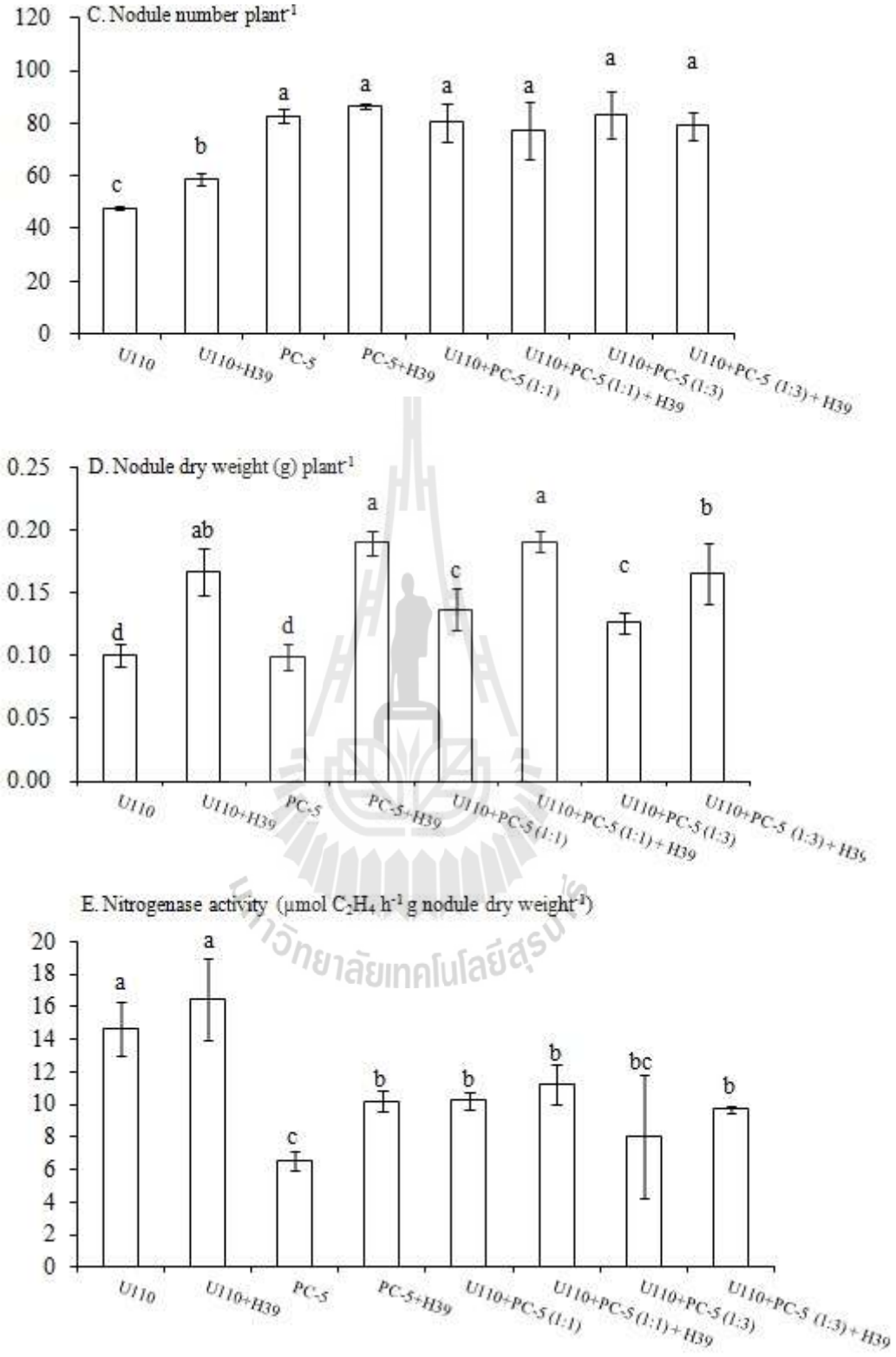

Figure 4.17 (Continued).

CHAPTER V

CONCLUSION

Most of the isolated bradyrhizobia in Thailand are Hup^- strain and the hydrogen gas released from nitrogenase of Hup^- bradyrhizobia could not directly promote nitrogenase activity and soybean growth. However, the Hup^+ PGPR could be isolated from rhizospheric soil treated with H_2 and contain plant growth promoting properties. All Hup^+ PGPR promoted plant growth and 2 isolates (2H17 and H39) significantly increased soybean growth under H_2 treated. The H_2 gas could induce IAA production of Hup^+ PGPR isolate H39. Co-inoculation of H39 with USDA110 under competitive condition with Hup^- *Bradyrhizobium* PC-5 promoted plant growth. Therefore, the selected Hup^+ PGPR H39 are prominent bacteria that can be applied for co-inoculant formulation to promote and increase yields of soybean in the presence of Hup^- bradyrhizobia as indigenous bacteria in the soil.

REFERENCES

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REFERENCES

Adesemoye, A., Torbert, H., & Kloepper, J. (2008). Enhanced plant nutrient use efficiency with PGPR and AMF in an integrated nutrient management system. **Canadian Journal of Microbiology**, 54(10): 876-886.

Albrecht, S. L., Maier, R. J., Hanus, F. J., Russell, S. A., Emerich, D. W., & Evans, H. J. (1979). Hydrogenase in *Rhizobium japonicum* increases nitrogen fixation by nodulated soybeans. **Science**, 203(4386): 1255-1257.

Alexander, M. (1977). **Introduction to soil microbiology**: John Wiley & Sons.

Annan, H., Golding, A. L., Zhao, Y., & Dong, Z. (2012). Choice of hydrogen uptake (Hup) status in legume-rhizobia symbioses. **Ecology and Evolution**, 2(9): 2285-2290.

Aragno, M., & Schlegel, H. G. (1981). The hydrogen-oxidizing bacteria **The Prokaryotes** (pp. 865-893): Springer.

Asaka, O., & Shoda, M. (1996). Biocontrol of *Rhizoctonia solani* damping-off of tomato with *Bacillus subtilis* RB14. **Applied and Environmental Microbiology**, 62(11): 4081-4085.

Aung, T. T., Tittabutr, P., Boonkerd, N., Herridge, D., & Teaumroong, N. (2013). Co-inoculation effects of *Bradyrhizobium japonicum* and *Azospirillum* sp. on competitive nodulation and rhizosphere eubacterial community structures of soybean under rhizobia-established soil conditions. **African Journal of Biotechnology**, 12: 2850-2862.

Baginsky, C., Brito, B., Imperial, J., Palacios, J.-M., & Ruiz-Argüeso, T. (2002). Diversity and evolution of hydrogenase systems in rhizobia. **Applied and Environmental Microbiology**, 68(10): 4915-4924.

Baginsky, C., Brito, B., Imperial, J., Ruiz-Argüeso, T., & Palacios, J. M. (2005). Symbiotic hydrogenase activity in *Bradyrhizobium* sp.(Vigna) increases nitrogen content in *Vigna unguiculata* plants. **Applied and Environmental Microbiology**, 71(11): 7536-7538.

Bais, H. P., Fall, R., & Vivanco, J. M. (2004). Biocontrol of *Bacillus subtilis* against infection of Arabidopsis roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. **Plant Physiology**, 134(1): 307-319.

Barazani, O., & Friedman, J. (1999). Is IAA the major root growth factor secreted from plant-growth-mediating bacteria? **Journal of Chemical Ecology**, 25(10): 2397-2406.

Belimov, A. A., Safronova, V. I., Sergeyeva, T. A., Egorova, T. N., Matveyeva, V. A., Tsyganov, V. E., Borisov, A. Y., Tikhonovich, I. A., Kluge, C., Preisfeld, A. Dietz, K. J., & Stepanok, W. (2001). Characterization of plant growth promoting rhizobacteria isolated from polluted soils and containing 1-aminocyclopropane-1-carboxylate deaminase. **Canadian Journal of Microbiology**, 47(7): 642-652.

Bordeleau, L., & Prévost, D. (1994). Nodulation and nitrogen fixation in extreme environments **Symbiotic Nitrogen Fixation** (pp. 115-125): Springer.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Analytical Biochemistry**, 72(1): 248-254.

Caldwell, B. (1969). Initial competition of root-nodule bacteria on soybeans in a field environment. **Agronomy Journal**, 61(5): 813-815.

Chen, Y., Rekha, P., Arun, A., Shen, F., Lai, W.-A., & Young, C. (2006). Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. **Applied Soil Ecology**, 34(1): 33-41.

Cole, M. A., & Elkan, G. H. (1973). Transmissible resistance to penicillin G, neomycin, and chloramphenicol in *Rhizobium japonicum*. **Antimicrobial Agents and Chemotherapy**, 4(3): 248-253.

Costa, R., Gomes, N., Krögerrecklenfort, E., Opelt, K., Berg, G., & Smalla, K. (2007). Pseudomonas community structure and antagonistic potential in the rhizosphere: insights gained by combining phylogenetic and functional gene-based analyses. **Environmental Microbiology**, 9(9): 2260-2273.

Costacurta, A., Mazzafera, P., & Rosato, Y. B. (2006). Indole-3-acetic acid biosynthesis by Xanthomonas axonopodis pv. citri is increased in the presence of plant leaf extracts. **FEMS Microbiology Letters**, 159(2): 215-220.

Cregan, P., & Keyser, H. (1986). Host restriction of nodulation by *Bradyrhizobium japonicum* strain USDA 123 in soybean. **Crop Science**, 26(5): 911-916.

Cunningham, S. D., Kapulnik, Y., & Phillips, D. A. (1986). Distribution of hydrogen-metabolizing bacteria in alfalfa field soil. **Applied and Environmental Microbiology**, 52(5): 1091-1095.

Dixon, R. (1972). Hydrogenase in legume root nodule bacteroids: occurrence and properties. **Archiv für Mikrobiologie**, 85(3): 193-201.

Dong, Z., & Layzell, D. (2001). H_2 oxidation, O_2 uptake and CO_2 fixation in hydrogen treated soils. **Plant and Soil**, 229(1): 1-12.

Dong, Z., Layzell, D., Finan, T., O'Brian, M., Vassey, J., & Newton, W. (2002). Why do legume nodules evolve hydrogen gas? Paper presented at the Nitrogen fixation: global perspectives. **Proceedings of the 13th International Congress on Nitrogen Fixation, Hamilton, Ontario, Canada, 2-7 July 2001**.

Dong, Z., Wu, L., Kettlewell, B., Caldwell, C., & Layzell, D. (2003). Hydrogen fertilization of soils–is this a benefit of legumes in rotation? **Plant, Cell & Environment**, 26(11): 1875-1879.

Duff, S. M., Sarath, G., & Plaxton, W. C. (1994). The role of acid phosphatases in plant phosphorus metabolism. **Physiologia Plantarum**, 90(4): 791-800.

Dutta, D., De, D., Chaudhuri, S., & Bhattacharya, S. K. (2005). Hydrogen production by Cyanobacteria. **Microbial Cell Factories**, 4(1): 36.

Erickson, J. A., Nyborg, A. C., Johnson, J. L., Truscott, S. M., Gunn, A., Nordmeyer, F. R., & Watt, G. D. (1999). Enhanced efficiency of ATP hydrolysis during nitrogenase catalysis utilizing reductants that form the all-ferrous redox state of the Fe protein. **Biochemistry**, 38(43): 14279-14285.

Ferreira, M. C., & Hungria, M. (2002). Recovery of soybean inoculant strains from uncropped soils in Brazil. **Field Crops Research**, 79(2): 139-152.

Fontecilla-Camps, J. C., Volbeda, A., Cavazza, C., & Nicolet, Y. (2007). Structure/function relationships of [NiFe]-and [FeFe]-hydrogenases. **Chemical Reviews**, 107(10): 4273-4303.

Fred, E., Baldwin, I., & McCoy, E. (1932). Root Nodule Bacteria and Leguminous Plants. **University of Wisconsin Studies in Science, number 5**: University of Wisconsin Press, Madison.

Friedrich, B., & Schwartz, E. (1993). Molecular biology of hydrogen utilization in aerobic chemolithotrophs. **Annual Reviews in Microbiology**, 47(1): 351-383.

Fukuhara, H., Minakawa, Y., Akao, S., & Minamisawa, K. (1994). The involvement of indole-3-acetic acid produced by *Bradyrhizobium elkanii* in nodule formation. **Plant and Cell Physiology**, 35(8): 1261-1265.

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

Glick, B. R., Penrose, D. M., & Li, J. (1998). A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. **Journal of Theoretical Biology**, 190(1): 63-68.

Graham, P. H., & Vance, C. P. (2003). Legumes: importance and constraints to greater use. **Plant Physiology**, 131(3): 872-877.

Guth, J. H., & Burris, R. H. (1983). Inhibition of nitrogenase-catalyzed ammonia formation by hydrogen. **Biochemistry**, 22(22): 5111-5122.

Gyaneshwar, P., Kumar, G. N., Parekh, L., & Poole, P. (2002). Role of soil microorganisms in improving P nutrition of plants. **Food Security in Nutrient-Stressed Environments: Exploiting Plants' Genetic Capabilities** (pp. 133-143): Springer.

Hardy, R. W., Holsten, R., Jackson, E., & Burns, R. (1968). The acetylene-ethylene assay for N2 fixation: laboratory and field evaluation. **Plant Physiology**, 43(8): 1185-1207.

Hartmann, A., & Amarger, N. (1991). Genotypic diversity of an indigenous *Rhizobium meliloti* field population assessed by plasmid profiles, DNA fingerprinting, and insertion sequence typing. **Canadian Journal of Microbiology**, 37(8): 600-608.

Haugland, R. A., Hanus, F. J., Cantrell, M. A., & Evans, H. J. (1983). Rapid colony screening method for identifying hydrogenase activity in *Rhizobium japonicum*. **Applied and Environmental Microbiology**, 45(3): 892-897.

Hunt, S., & Layzell, D. B. (1993). Gas exchange of legume nodules and the regulation of nitrogenase activity. **Annual Review of Plant Biology**, 44(1): 483-511.

Idris, E. E., Iglesias, D. J., Talon, M., & Borriss, R. (2007). Tryptophan-dependent production of indole-3-acetic acid (IAA) affects level of plant growth promotion by *Bacillus amyloliquefaciens* FZB42. **Molecular Plant-Microbe Interactions**, 20(6): 619-626.

Jacobson, C. B., Pasternak, J., & Glick, B. R. (1994). Partial purification and characterization of 1-aminocyclopropane-1-carboxylate deaminase from the plant growth promoting rhizobacterium *Pseudomonas putida* GR12-2. **Canadian Journal of Microbiology**, 40(12): 1019-1025.

Jawson, M. D., Franzluebbers, A. J., & Berg, R. K. (1989). *Bradyrhizobium japonicum* survival in and soybean inoculation with fluid gels. **Applied and Environmental Microbiology**, 55(3): 617-622.

Jin, Q., Zhu, K., Cui, W., Xie, Y., Han, B., & Shen, W. (2013). Hydrogen gas acts as a novel bioactive molecule in enhancing plant tolerance to paraquat-induced oxidative stress via the modulation of heme oxygenase-1 signalling system. **Plant, Cell & Environment**, 36(5): 956-969.

Joseph, B., Ranjan Patra, R., & Lawrence, R. (2012). Characterization of plant growth promoting rhizobacteria associated with chickpea (*Cicer arietinum* L.). **International Journal of Plant Production**, 1(2): 141-152.

Kloepper, J. W. (1994). Plant growth-promoting rhizobacteria (other systems). **Azospirillum/Plant Associations**, 137-166.

Kpomblekou-a, K., & Tabatabai, M. (1994). Eefect of organic acids on release of phosphorus from phosphate rocks1. **Soil Science**, 158(6): 442.

Krause, A., Doerfel, A., & Göttfert, M. (2002). Mutational and transcriptional analysis of the type III secretion system of Bradyrhizobium japonicum. **Molecular Plant-Microbe Interactions**, 15(12): 1228-1235.

Kumar, B., & Dube, H. (1992). Seed bacterization with a *Pseudomonas fluorescent* for enhanced plant growth, yield and disease control. **Soil Biology and Biochemistry**, 24(6): 539-542.

La Favre, J., & Focht, D. (1983). Conservation in soil of H_2 liberated from N_2 fixation by Hup-nodules. **Applied and Environmental Microbiology**, 46(2): 304-311.

Lawongsa, P., Boonkerd, N., Wongkaew, S., O'Gara, F., & Teaumroong, N. (2008). Molecular and phenotypic characterization of potential plant growth-promoting *Pseudomonas* from rice and maize rhizospheres. **World Journal of Microbiology and Biotechnology**, 24(9): 1877-1884.

Lechner, S., & Conrad, R. (1997). Detection in soil of aerobic hydrogen-oxidizing bacteria related to *Alcaligenes eutrophus* by PCR and hybridization assays targeting the gene of the membrane-bound (NiFe) hydrogenase. **FEMS Microbiology Ecology**, 22(3): 193-206.

Lucy, M., Reed, E., & Glick, B. R. (2004). Applications of free living plant growth-promoting rhizobacteria. **Antonie van Leeuwenhoek**, 86(1): 1-25.

Ma, W., Guinel, F. C., & Glick, B. R. (2003). *Rhizobium leguminosarum* biovar viciae 1-aminocyclopropane-1-carboxylate deaminase promotes nodulation of pea plants. **Applied and Environmental Microbiology**, 69(8): 4396-4402.

Ma, W., Penrose, D. M., & Glick, B. R. (2002). Strategies used by rhizobia to lower plant ethylene levels and increase nodulation. **Canadian Journal of Microbiology**, 48(11): 947-954.

Maier, R. J., Campbell, N. E., Hanus, F. J., Simpson, F. B., Russell, S. A., & Evans, H. J. (1978). Expression of hydrogenase activity in free-living *Rhizobium japonicum*. **Proceedings of the National Academy of Sciences**, 75(7): 3258-3262.

Maier, R. J., & Triplett, E. W. (1996). Toward more productive, efficient, and competitive nitrogen-fixing symbiotic bacteria. **Critical Reviews in Plant Sciences**, 15(3): 191-234.

Maimaiti, J., Zhang, Y., Yang, J., Cen, Y. P., Layzell, D. B., Peoples, M., & Dong, Z. (2007). Isolation and characterization of hydrogen-oxidizing bacteria induced following exposure of soil to hydrogen gas and their impact on plant growth. **Environmental Microbiology**, 9(2): 435-444.

Manassila, M., Nuntagij, A., Kotepong, S., Boonkerd, N., & Teaumroong, N. (2007). Characterization and monitoring of selected rhizobial strains isolated from tree legumes in Thailand. **African Journal of Biotechnology**, 6(12).

Mantelin, S., & Touraine, B. (2004). Plant growth-promoting bacteria and nitrate availability: impacts on root development and nitrate uptake. **Journal of Experimental Botany**, 55(394): 27-34.

Nelson, L., & Salminen, S. (1982). Uptake hydrogenase activity and ATP formation in Rhizobium leguminosarum bacteroids. **Journal of Bacteriology**, 151(2): 989-995.

Ohno, K., Ito, M., Ichihara, M., & Ito, M. (2012). Molecular hydrogen as an emerging therapeutic medical gas for neurodegenerative and other diseases. **Oxidative Medicine and Cellular Longevity, 2012**.

Ohsawa, I., Ishikawa, M., Takahashi, K., Watanabe, M., Nishimaki, K., Yamagata, K., Katsura, K., Katayama, Y., Asoh, S., & Ohta, S. (2007). Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. **Nature Medicine**, 13(6): 688-694.

Osborne, C. A., Peoples, M. B., & Janssen, P. H. (2010). Detection of a reproducible, single-member shift in soil bacterial communities exposed to low levels of hydrogen. **Applied and Environmental Microbiology**, 76(5), 1471-1479.

Parker, M., & Harris, H. (1977). Yield and leaf nitrogen of nodulating and nonnodulating soybeans as affected by nitrogen and molybdenum. **Agronomy Journal**, 69(4): 551-554.

Patten, C. L., & Glick, B. R. (1996). Bacterial biosynthesis of indole-3-acetic acid. **Canadian Journal of Microbiology**, 42(3): 207-220.

Patten, C. L., & Glick, B. R. (2002). Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. **Applied and Environmental Microbiology**, 68(8): 3795-3801.

Penrose, D. M., & Glick, B. R. (2003). Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. **Physiologia Plantarum**, 118(1): 10-15.

Phelps, A., & Wilson, P. (1941). Occurrence of hydrogenase in nitrogen-fixing organisms. **Experimental Biology and Medicine**, 47(2): 473-476.

Phillips, D. A. (1992). Flavonoids: plant signals to soil microbes **Phenolic Metabolism in Plants** (pp. 201-231): Springer.

Popelier, F., Liessens, J., & Verstraete, W. (1985). Soil H_2-uptake in relation to soil properties and rhizobial H_2-production. **Plant and Soil**, 85(1): 85-96.

Prakamhang, J., Minamisawa, K., Teamtaisong, K., Boonkerd, N., & Teaumroong, N. (2009). The communities of endophytic diazotrophic bacteria in cultivated rice (*Oryza sativa* L.). **Applied Soil Ecology**, 42(2): 141-149.

Prakamhang, J., Tittabutr, P., Boonkerd, N., Teamtisong, K., Uchiumi, T., Abe, M., & Teaumroong, N. (2015). Proposed some interactions at molecular level of PGPR coinoculated with *Bradyrhizobium diazoefficiens* USDA110 and *B. japonicum* THA6 on soybean symbiosis and its potential of field application. **Applied Soil Ecology**, 85: 38-49.

Rees, D. C., Tezcan, F. A., Haynes, C. A., Walton, M. Y., Andrade, S., Einsle, O., & Howard, J. B. (2005). Structural basis of biological nitrogen fixation. **Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences**, 363(1829): 971-984.

Reiher, M., Salomon, O., Sellmann, D., & Hess, B. A. (2001). Dinuclear diazene iron and ruthenium complexes as models for studying nitrogenase activity. **Chemistry-A European Journal**, 7(23): 5195-5202.

Renwick, G., Giumarro, C., & Siegel, S. (1964). Hydrogen metabolism in higher plants. **Plant physiology**, 39(3): 303.

Roughley, R., Blowes, W., & Hurridge, D. (1976). Nodulation of *Trifolium subterraneum* by introduced rhizobia in competition with naturalized strains. **Soil Biology and Biochemistry**, 8(5): 403-407.

Rumjanek, N. G., Dobert, R., Van Berkum, P., & Triplett, E. (1993). Common soybean inoculant strains in Brazil are members of *Bradyrhizobium elkanii*. **Applied and Environmental Microbiology**, 59(12), 4371-4373.

RunEI, F., & TisdalB, W. (1921). Effect of soil temperature upon the development of nodules on the roots of certain legumes. **Journal of Agricultural Research**, 22: 17.

Sahgal, M., & Johri, B. (2003). The changing face of rhizobial systematics. **Current Science-Bangalore**, 84(1): 43-48.

Saleem, M., Arshad, M., Hussain, S., & Bhatti, A. S. (2007). Perspective of plant growth promoting rhizobacteria (PGPR) containing ACC deaminase in stress agriculture. **Journal of Industrial Microbiology and Biotechnology**, 34(10): 635-648.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). **Molecular Cloning** (Vol. 1): Cold spring harbor laboratory press New York.

Schink, B., & Schlegel, H.-G. (1978). Hydrogen metabolism in aerobic hydrogen-oxidizing bacteria. **Biochimie**, 60(3): 297-305.

Schlegel, H. G., & Meyer, M. (1985). Isolation of hydrogenase regulatory mutants of hydrogen-oxidizing bacteria by a colony-screening method. **Archives of Microbiology**, 141(4): 377-383.

Schubert, K. R., & Evans, H. J. (1976). Hydrogen evolution: a major factor affecting the efficiency of nitrogen fixation in nodulated symbionts. **Proceedings of the National Academy of Sciences**, 73(4): 1207-1211.

Schubert, K. R., Jennings, N. T., & Evans, H. J. (1978). Hydrogen reactions of nodulated leguminous plants II. Effects on dry matter accumulation and nitrogen fixation. **Plant Physiology**, 61(3): 398-401.

Schwarze, A., Kopczak, M. J., Rögner, M., & Lenz, O. (2010). Requirements for construction of a functional hybrid complex of photosystem I and [NiFe]-hydrogenase. **Applied and Environmental Microbiology**, 76(8), 2641-2651.

Shanmugam, K., O'gara, F., Andersen, K., & Valentine, R. (1978). Biological nitrogen fixation. **Annual Review of Plant Physiology**, 29(1): 263-276.

Somasegaran, P., & Hoben, H. J. (1994). **Handbook for rhizobia: methods in legume-Rhizobium technology**: Springer-Verlag New York Inc.

Spaepen, S., Das, F., Luyten, E., Michiels, J., & Vanderleyden, J. (2009). Indole-3-acetic acid-regulated genes in *Rhizobium etli* CNPAF512. **FEMS Microbiology Letters**, 291(2): 195-200.

Steenhoudt, O., & Vanderleyden, J. (2000). *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. **FEMS Microbiology Reviews**, 24(4): 487-506.

Tard, C., & Pickett, C. J. (2009). Structural and Functional Analogues of the Active Sites of the [Fe]-,[NiFe]-, and [FeFe]-Hydrogenases†. **Chemical Reviews**, 109(6): 2245-2274.

Tilak, K. V. B. R., Ranganayaki, N., Pal, K. K., De, R., Saxena, A. K., Shekhar Nautiyal, C., Mittal, S., Tripathi, A. K., & Johri, B N. (2005). Diversity of plant growth and soil health supporting bacteria. **Current Science**, 89(1): 136-150.

Toro, A. (1996). Nodulation competitiveness in the Rhizobium-legume symbiosis. **World Journal of Microbiology and Biotechnology**, 12(2), 157-162.

Uratsu, S., Keyser, H., Weber, D., & Lim, S. (1982). Hydrogen uptake (HUP) activity of *Rhizobium japonicum* from major US soybean production areas. **Crop Science**, 22(3): 600-602.

Vacheron, J., Desbrosses, G., Bouffaud, M. L., Touraine, B., Moënne-Loccoz, Y., Muller, D., Legendre, L., Wisniewski-Dyé, F., & Prigent-Combaret, C.

(2013). Plant growth-promoting rhizobacteria and root system functioning. **Frontiers in Plant Science**, 4.

Van, V. T., Berge, O., Ke, S. N., Balandreau, J., & Heulin, T. (2000). Repeated beneficial effects of rice inoculation with a strain of *Burkholderia vietnamiensison* early and late yield components in low fertility sulphate acid soils of Vietnam. **Plant and Soil**, 218(1-2): 273-284.

Versalovic, J., Schneider, M., De Bruijn, F. J., & Lupski, J. R. (1994). Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. **Methods in Molecular and Cellular Biology**, 5(1): 25-40.

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

Vignais, P., & Colbeau, A. (2004). Molecular biology of microbial hydrogenases. **Current Issues in Molecular Biology**, 6(2): 159-188.

Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. **Journal of Bacteriology**, 173(2): 697-703.

Yahalom, E., Dovrat, A., Okon, Y., & Czosnek, H. (1991). Effect of inoculation with *Azospirillum brasilense* strain Cd and *Rhizobium* on the root morphology of burr medic (*Medicago polymorpha* L.). **Israel Journal of Botany**, 40(2): 155-164.

Yang, S. F., & Hoffman, N. E. (1984). Ethylene biosynthesis and its regulation in higher plants. **Annual Review of Plant Physiology**, 35(1): 155-189.

Zablotowicz, R. M., Tipping, E. M., Lifshitz, R., & Kloepper, J. W. (1991). Plant growth promotion mediated by bacterial rhizosphere colonizers. **The rhizosphere and plant growth** (pp. 315-326): Springer.

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Zahir, Z. A., Ghani, U., Naveed, M., Nadeem, S. M., & Asghar, H. N. (2009). Comparative effectiveness of *Pseudomonas* and *Serratia* sp. containing ACC-deaminase for improving growth and yield of wheat (*Triticum aestivum* L.) under salt-stressed conditions. **Archives of Microbiology**, 191(5): 415-424.

Zeng, J., Zhang, M., & Sun, X. (2013). Molecular hydrogen is involved in phytohormone signaling and stress responses in plants. **PLOS ONE**, 8(8): e71038.

Zhang, J., Howell, C., & Starr, J. (1996). Suppression of Fusarium colonization of cotton roots and Fusarium wilt by seed treatments with *Gliocladium virens* and *Bacillus subtilis*. **Biocontrol Science and Technology**, 6(2): 175-188.

Zhang, Y., He, X., & Dong, Z. (2009). Effect of hydrogen on soil bacterial community structure in two soils as determined by terminal restriction fragment length polymorphism. **Plant and Soil**, 320(1-2), 295-305.

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