แบรดดีไรโซเบียมเอนโดไฟท์ในข้าว และศักยภาพในการปลูกข้าว และพืชตระกลูถั่วแบบหมุนเวียน

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RICE ENDOPHYTIC BRADYRHIZOBIA AND THEIR POTENTIALS FOR RICE-LEGUME CROP ROTATION

Teerana Greetatorn

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RICE ENDOPHYTIC BRADYRHIZOBIA AND THEIR POTENTIALS FOR RICE-LEGUME CROP ROTATION

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

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แบคทีเรียในสกุลแบรดดีไรโซเบียม สามารถอาศัยอยู่แบบพึ่งพาอาศัยซึ่งกันและกันกับพืชตระกูลถั่ว และเป็นเอนโดไฟท์ในพืชที่ไม่ใช่พืชตระกูลถั่ว เช่น ข้าว ดังนั้น จึงมีความเป็นไปได้ว่าแบรดดีไรโซเบียมเอนโดไฟท์ในข้าว สามารถนำไปใช้ในระบบการปลูกข้าว และพืชตระกูลถั่วแบบหมุนเวียนได้ แบรดดีไรโซเบียมเอนโดไฟท์ถูกคัดเลือกมาจากเนื้อเยื่อส่วนต่าง ๆ ของข้าว (*Oryza sativa* L.) โดยเชื้อทั้ง 8 สายพันธุ์มีความสามารถในการผลิต IAA, เอนไซม์ ACC deaminase และ nitrogenase เมื่อทำการทดสอบประเภทของปุ๋ยไนโตรเจนร่วมกับแบคทีเรียแต่ละสายพันธุ์ที่มีผลต่อการเจริญของข้าว พบว่าเกิดผลกระทบทั้งเชิงลบ และบวกจากการใช้แหล่งของไนโตรเจนและแบรดดีไรโซเบียมเอนโดไฟท์ที่แตกต่างกัน น้ำหนักแห้งของข้าวเพิ่มขึ้นเมื่อได้รับโพแทสเซียมไนเตรท หรือแอมโมเนียมไนเตรท ร่วมกับเชื้อสายพันธุ์ที่ส่งเสริมการเจริญเติบโตของข้าว โดยเฉพาะอย่างยิ่งสายพันธุ์ SUTN9-2 นอกจากนี้ผลของการใช้ปุ๋ยไนโตรเจนร่วมกับเชื้อแบรดดีไรโซเบียม สามารถเพิ่มการสะสมไนโตรเจนในต้นข้าวได้สูงกว่าการไม่ใส่เชื้อ หรือไม่มีปุ๋ยไนโตรเจน เชื้อสายพันธุ์ที่ยับยั้งการเจริญเติบโตของข้าว (SUT-PR48, SUT-PR64 และ ORS285) พบว่ามีการผลิตไนตริกออกไซด์ (NO) ในรากข้าวเมื่อตรวจสอบด้วยวิธีการย้อม DAF FM-DA ผลเหล่านี้สอดคล้องกับผลการแสดงออกของยีนที่ตอบสนองต่อการผลิตไนตริกออกไซด์ ได้แก่ ยีน *nirK* และ *norB* เมื่อใช้เทคนิค RT-PCR การใส่เชื้อสายพันธุ์ SUT-PR48 กับไนเตร ท พบว่าเพิ่มระดับการแสดงออกของยีน *nirK* สูงกว่ายีน *norB* ในทางตรงกันข้าม จากการใส่เชื้อสายพันธุ์ SUTN9-2 พบว่าระดับการแสดงออกของยีน *nirK* ต่ำกว่ายีน *norB* แสดงให้เห็นว่าเชื้อสายพันธุ์ SUT-PR48 มีการสะสมไนตริกออกไซด์มากกว่าเชื้อสายพันธุ์ SUTN9-2 ซึ่งส่งผลให้เกิดผลกระทบเชิงลบต่อการเจริญเติบโตของข้าว นอกจากนี้ผลจากการสะสมไนโตรเจนก็มีความสอดคล้องกับการแสดงออกของยีนที่เกี่ยวข้องกับการตรึงไนโตรเจนด้วย (ยีน *nifH*) การแสดงออกของยีน *nifH* ของเชื้อสายพันธุ์ SUTN9-2 ถูกชักนำภายใต้เงื่อนไขการใส่ไนโตรเจนและเป็นเอนโดไฟท์ในข้าว เชื้อสายพันธุ์ SUTN9-2 จึงใช้เป็นหัวเชื้อไรโซเบียมสำหรับทดสอบกับถั่วเขียวในรูปแบบของตอซังข้าว ผลจากการทดลองแสดงให้เห็นว่าเชื้อสายพันธุ์ SUTN9-2 ยังคงมีชีวิตอยู่ในเนื้อเยื่อข้าวจนถึงฤดูเก็บเกี่ยวข้าว เมื่อทำการเก็บเกี่ยวข้าวได้ปลูกถั่วเขียวในกระถางเดียวกันกับข้าวที่เก็บเกี่ยวไปแล้วและใช้ตอซังข้าวที่มีเชื้อสายพันธุ์ SUTN9-2 เป็นหัวเชื้อในรูปตอซัง ผลการทดลองพบว่า

เชื้อสายพันธุ์ SUTN9-2 ที่อาศัยอยู่ในตอซังสามารถสร้างปมได้ในถั่วเขียว ทั้งนี้ ยืนยันโดยผลการทดลองจากการใช้ยีนติดตาม GUS ดังนั้นวิธีการใช้ตอซังข้าวที่มีแบรดดีไรโซเบียมเอนโดไฟท์สามารถใช้เป็นหัวเชื้อในระบบการปลูกข้าว และพืชตระกูลถั่วแบบหมุนเวียนได้

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2557 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

TEERANA GREETATORN : RICE ENDOPHYTIC BRADYRHIZOBIA AND THEIR POTENTIALS FOR RICE-LEGUME CROP ROTATION. THESIS ADVISOR : PROF. NEUNG TEAUMROONG, Dr.rer.nat., 88 PP.

ENDOPHYTIC BRADYRHIZOBIA/RICE/MUNG BEAN/ RICE-LEGUME CROP ROTATION

Bradyrhizobium encompasses a variety of bacteria that can live in symbiotic and endophytic associations with leguminous plants and non-leguminous plants such as rice. Therefore, it can be expected that rice endophytic bradyrhizobia can be applied in the rice-legumes crop rotation system. In this study, endophytic bradyrhizobial strains were isolated from various rice (*Oryza sativa* L.) tissues. The selected 8 endophytic bradyrhizobial strains had the capability of IAA, ACC deaminase and nitrogenase enzymes productions. The rice growth promotion showed both of negative and positive impacts by supplying different nitrogen sources and inoculation of endophytic bradyrhizobia. The rice biomass could be enhanced when supplied with KNO_3 or NH_4NO_3 and inoculated with positive rice growth promotion strains especially, SUTN9-2. In addition, the effect of nitrogen fertilizer and bradyrhizobial inoculation could increase nitrogen accumulation in rice plant higher than that of uninoculation or without nitrogen fertilizer. The strains which suppressed rice growth (SUT-PR48, SUT-PR64 and ORS285) were also found to produce nitric oxide (NO) in the rice root when detected with DAF-FM DA staining method. These results were accordance with the expression of genes involved in NO production including *nirK* and *norB* genes when RT-PCR technique was conducted. The

inoculation of SUT-PR48 with nitrate enhanced the expression level of *nirK* gene better than that of *norB* gene. In contrast, the expression level of *nirK* gene in inoculation of SUTN9-2 was lower than that of *norB* gene. These results taken together suggested that SUT-PR48 possibly accumulated NO more than SUTN9-2 did, resulting in the negative effect on rice growth. Furthermore, the results from nitrogen accumulation were also in accordance with the expression of nitrogen fixation gene (*nifH* gene). The expression of *nifH* genes of SUTN9-2 was induced under nitrogen treatment in endophytic association with rice. The strain SUTN9-2 was selected and then used as rhizobium inoculum for mung bean in the form of rice stubble. The results revealed that SUTN9-2 still persisted in rice tissues until harvest. After harvesting the rice, the mung bean was planted to the same pot for 3 weeks. The results showed nodulation of GUS-tagging bradyrhizobia SUTN9-2 in mung bean. Therefore, it is possible that rice stubble can be used as inoculum in the rice-legume crop rotation system.

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Academic Year 2014

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CHAPTER I

INTRODUCTION

1.1 Significant of this study

The nitrogen-fixing root nodule formation is the result of symbiosis relationship between rhizobia and their legume host plants. Rhizobia are able to infect the specific leguminous host roots and form nodule through complexed interaction between plant and microbe (Spaink, 2000). It is now well established that in addition to symbiotic association with legumes, rhizobia may occur as an endophyte (colonize in intercellular spaces) of root in nonlegumes such as rice *Oryza sativa* L. (Biswas et al., 2000), *O. breviligulata* (Chaintreuil et al., 2000), *O. sativa* L. cv. Pelde (Perrine et al., 2001), wheat (*Triticum aestivum*) (Hilali et al., 2001), sugarcane (*Saccharum officinarum*) and maize (*Zea mays*) (Bhattacharjee et al., 2008) and can promote theirs growth and productivity. The genus *Bradyrhizobium* encompasses a variety of bacteria that can live in symbiotic and endophytic associations with legumes and non-legumes. In addition, most of species in genus *Bradyrhizobium* were recognized on oligotrophic soil bacteria that is able to grow in extra-low nutrient environments (Hattori, 1984). These features suggest that *Bradyrhizobium* is able to adapt to a wide range of environments, probably including low-nutrient conditions, with multiple survival strategies in soil and rhizosphere.

Actually legumes are suitable rotational crops with rice and can be planted before or after harvesting of rice season (International institute of rural reconstruction,

1990**)**. Especially mung bean (*Vigna radiata*), pre-rice mung bean significantly increased rice dry weight. In addition, growing mung bean before rice provides the advantage of marketable grain of mung bean (Polthanee et al., 2012). Therefore, if we can select endophytic bradyrhizobia that can nodulate mung bean as well as can establish in the rice (*O. sativa* L. cv. Pathum thani 1), these are the first observations demonstrate that it is possible that rice stubble can be used as inoculum in field grown legumes to reduce the use of bacterial inoculum. Moreover, cost of rice production can be reduced and also additional values as profit from mung bean production can be contribute to the farmers.

Therefore, the aims of the present study were to obtain the effective strain of endophytic bradyrhizobia for rice and investigate the infection, growth promotion and the persistence of endophytic bradyrhizobia in rice. The selected endophytic bradyrhizobia would be able to use in the system of grown legumes using rice stubble as inoculum.

1.2 Research hypotheses

1.2.1 Some of endophytic bradyrhizobia may promote the rice growth.

1.2.2 Endophytic bradyrhizobia can persist in rice stubble and can be used as inoculum on legume plant.

1.3 Research objectives

1.3.1 To find the effect of inoculated of selected bradyrhizobia on rice plant growth

1.3.2 To investigate the persistence of the selected bradyrhizobia in the rice plant on field grown legumes using rice stubble as inoculums.

CHAPTER II

LITERATURES REVIEW

2.1 Bacteria and their root colonization

The rhizosphere environment is relatively rich in nutrients released by the roots, mainly organic carbon substances. Roots secrete both high- and low-molecular weight compounds, which may contain free amino acids, proteins, carbohydrates, alcohols, vitamins, or hormones (Hawes and Pueppke, 1986). These root exudates functions not only serve as nutrients for microorganisms in the rhizosphere but also participate in nodulation and colonization of soil microbes with legume plants and cereals as signal molecules in plant–microbe interactions (Bacilio-Jiménez et al., 2003; Sugiyama and Yazaki, 2012) (Table 2.1). The root cracks are recognized as the main 'hot spots' for bacterial colonization (Sørensen and Sessitsch, 2007).. Generally, the first stage of bacterial infection is colonization: the establishment of the bacteria at the appropriate portal of entry. Bacteria normally colonize host tissues that are in contact with the external environment. Sites of entry in plant hosts include the root cracks, wounds caused by microbial or nematode phytopathogens, and the stomata found in leaf tissue. Bacteria that infect these regions have usually developed tissue adherence mechanisms and some ability to overcome or withstand the constant pressure of the host defenses at the surface (Todar, 2006).

Table 2.1 Nodulation and colonization-inducing compounds of root exudates of legumes and rice plants.

Plant	**Compounds**	**Reference**
Alfalfa (*Medicago sativa* L.)	luteolin 7,40-dihydroxyflavone 7,40-dihydroxyflavanone 4,40-dihydroxy-20-methoxychalcon chrysoeriol trigonelline stachydrine	Peters et al., (1986) Maxwell et al., (1989) Hartwig et al., (1990) Phillips et al., (1992)
Cowpea (*Vigna unguiculata*)	daidzein genistein coumestrol	Kanu and Dakora, (2012)
Soybean (*Glycine max*)	daidzein genistein coumestrol	Kosslak et al., (1987)
Mung bean (*Vigna radiata*)	flavonoid naringenin	Jain et al., (1991)
Rice (*Oryza sativa)*	histidine, proline, valine, alanine, and glycine glucose, arabinose, mannose, galactose, and glucuronic acid.	Bacilio-Jiménez et al., (2003)

The population density of microorganisms, especially bacteria, is considerably higher in the rhizosphere than in the bulk soil and its microbial communities differ from those outside the influence of the roots (Hardarson and Broughton, 2003). Bacteria are common inhabitants of both the surfaces and the internal tissues of most plants. Plant-associated bacteria isolated from rhizoplane and phylloplane surfaces are known as epiphytes (Andrews and Harris, 2000) whereas those isolated from the interior tissues, which they inhabit without causing harm to the host, are called endophytes (Azevedo et al., 2000; Petrini et al., 1989). *Epiphytic* and *endophytic bacteria* are characterized by the colonization of surface and inner tissues of plants, respectively. However, in addition to these definitions is the separation of endophytes according to their essentiality in niche occupations (Maheshwari, 2007). In that case, the endophytic community is divided into passenger endophytes, (red cells) (Figure 2.1) i.e. bacteria that eventually invade internal plant tissues by stochastic events and are often restricted to the root cortex tissue. Opportunistic endophytes (blue cells) (Figure 2.1) show particular root colonization characteristics (e.g. a chemotactic response, which enables them to colonize the rhizoplane and then invade the internal plant tissues through cracks formed at the sites of lateral root emergence and root tips). True endophytes or competent endophytes (yellow cells) (Figure 2.1) are proposed to have all properties of opportunistic endophytes and in addition, be well adapted to the plant environment. They are capable of invading specific plant tissue, such as vascular tissue, spreading throughout the plant and, by manipulating plant metabolism, maintaining a harmonious balance with the plant host, even when they are present in high (Hardoim et al., 2008).

Endophytic and epiphytic bacteria can contribute to the health, growth and development of plants. Plant growth promotion by endophytic and epiphytic bacteria

may have diverse effects on host plant development and result either from indirect effects such as the biocontrol of soilborne diseases through competition for nutrients, siderophore-mediated competition for iron, antibiosis or the induction of systemic resistance in the plant host, or from direct effects such as the production of phytohormones or by providing the host plant with fixed nitrogen or the solubilization of soil phosphorus and iron via their effects on root morphology and physiology. (Glick, 1995; Kinkel et al., 2000; Shishido et al., 1999; Sturz et al., 2000).

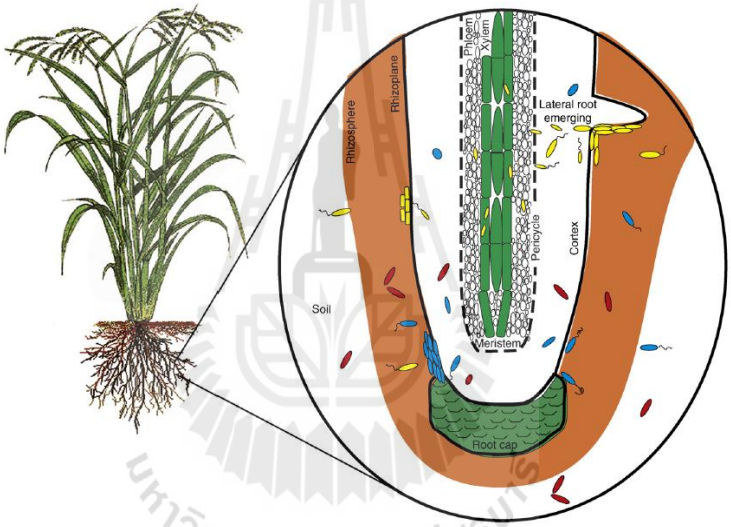

Figure 2.1 Types of endophytes and their root colonization process (Hardoim et al., 2008)

2.2 Mechanism of endophytic bacteria entering to the plants

In general, endophytes have been defined as bacteria that are able to colonize living plant tissues without harming the plant or gaining benefit other than securing residency (Kado, 1992). The rhizosphere is assumed to be the main source of endophytic colonizers (de Bruijn, 2013). The mechanisms by which endophytic bacteria enter to a plant's interior generally starts with their establishment in the

rhizosphere. Following rhizosphere colonization, bacteria attach to the rhizoplane, i. e. the root surface. The attachment of bacterial cells to the root is a crucial step for subsequent endophytic establishment. Several bacterial surface components can be involved in this process (Malfanova, 2013). A previous study reported that type IV pili of *Azoarcus* sp. BH72 (Dörr et al., 1998), LPS (lipopolysaccharide) of *Herbaspirillum seropedicae* (Balsanelli et al., 2010) and EPS (exopolysaccharide) of *Gluconacetobacter diazotrophicus* (Meneses et al., 2011) are required for attachment to the root surfaces and necessary for rhizoplane and endosphere colonization.

The preferable sites of bacterial attachment and subsequent entry are the apical root zone with the thin-walled surface root layer such as the cell elongation and the root hair zone (zone of active penetration) and the basal root zone with small cracks caused by the emergence of lateral roots (zone of passive penetration) (Zachow et al., 2010) (Figure 2.2). For active penetration, endophytic bacteria have to be well-equipped with cellulolytic enzymes which hydrolyze the plant's exodermal cell (Compant et al., 2005; Reinhold-Hurek and Hurek, 1998). Bacterial cell-wall degrading enzymes are also known to be involved in the elicitation of defense pathways in plants (Norman-Setterblad et al., 2000). However, this is not the case for endophytes, endophytic bacteria must be able to escape the plant immune response or even reduce it to some extent. For passive penetration, by entering a plant through natural cracks at the region where the lateral roots appear, bacteria remain "invisible" for the plant's immune system. This mode of entry (often combined with active penetration) has been suggested in rice (*Oryza sativa* L.) such as for *Azoarcus* sp. BH72 (Reinhold-Hurek and Hurek, 1998) and *Herbaspirillum seropedicae* Z67 (James et al., 2002).

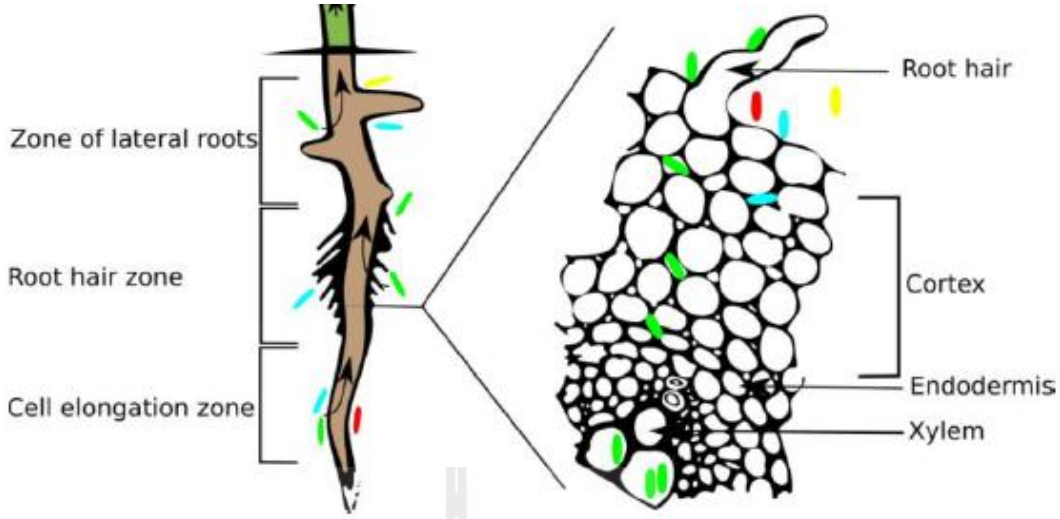

Figure 2.2 The colonization by endophytic bacteria. Bacteria can enter a plant at several root zones as indicated above. Endophytes can either remain at the site of entry (indicated inblue) or move deeper inside and occupy the intercellular space of the cortex and xylem vessels (indicated in green). Red and yellow represent rhizospheric bacteria which are unable to colonize inner plant tissues (Malfanova, 2013).

For nodulation in legume plants by rhizobia, legume plants establish symbiotic interactions with rhizobia to obtain several nutrients such as nitrogen. In these interactions, flavonoids and root exudates serve as signal molecules to establish the symbiotic interactions (Sugiyama and Yazaki, 2012). Flavonoids released by legume roots trigger the synthesis of rhizobial lipochitooligosaccharides signaling molecules, Nod factors (NFs), which induce root hair curling, NFs activate nodule organogenesis in the roots by stimulating the division of cortical cells. Infection threads extend through root hairs towards the cortical cells of the root. Infection threads ramify in nodule primordia (which are formed by dividing cortical cells) (Deakin and Broughton, 2009). The nodulation mechanism is highly specific, because it involves

the nod factors, plant phytohormones, including auxin, cytokinin, and ethylene, which are signal molecules required for plant physiological activities and root development, they are regulate nodulation and nitrogen fixation in the legume-rhizobium symbiosis (Nagata and Suzuki, 2014).

2.3 Bradyrhizobia

The Rhizobiaceae are a family of proteobacteria, including many species of rhizobia, as well as the genus *Rhizobium, Bradyrhizobium, Mesorhizobium, Sinorhizobium, Azorhizobium, Allorhizobium* and new genus *Neorhizobium* known as rhizobia (Mousavi et al., 2014). Many species of the Rhizobiaceae are diazotrophs, they are able to fix atmospheric nitrogen and are symbiotic with plant roots especially in legume plants. Biological nitrogen fixation (BNF), this reaction is occurred by the activity of nitrogenase enzyme, which convert atmospheric nitrogen in to ammonia and can be used as nitrogen source for plant (Deacon, 1997). The reaction is shown below:

$$N_2 + 8\,H^+ + 8\,e^- + 16ATP \xrightarrow{\text{Nitrogenase}} 2NH_3 + H_2 + 16ADP + 16Pi$$

nitrogen (atmospheric) — ammonia — phosphate (inorganic)

Bradyrhizobia are Gram-negative bacilli (rod shaped) soil bacteria that can form nodules on host plants. They also have symbiotic relationships with leguminous plant species, which cannot live without these bacteria's essential nitrogen-fixing processes. Bradyhizobia can be found in the roots, or rhizosphere, where they cause the formation of nodules. In nodules, the rhizobia bacteriods use carbon and energy

from the plant in the form of dicarboxylic acids. Recent studies have suggested that the bacteroids do more than just provide the plant with ammonium (through nitrogen fixation). It was showed that a more complex amino-acid cycle is needed for *Rhizobium* to successfully fix nitrogen. However, the bacteria must provide the plant with ammonium in order to obtain the amino acids (Stothard et al., 2005).

2.3.1 Bradyrhizobia and legume symbiosis

Bradyhizobia normally live in the soil and can exist without a host plant. However, *Bradyrhizobium* has a symbiotic relationship with legumes and or more specifically to infect the specific leguminous host roots (Zahran, 1999). When legume plants encounter low nitrogen conditions and want to form a symbiotic relationship with rhizobia they release flavinoids into the soil. Rhizobia respond by releasing nodulation factor (nod factor), which stimulates nodule formation in plant roots. Rhizobia then form an infection thread, which is an intercellular tube that penetrates the root cells of the host plant. Infection triggers rapid cell division in the root cells and forming a nodule of tissue (Burrows, 1963) (Figure 2.3).

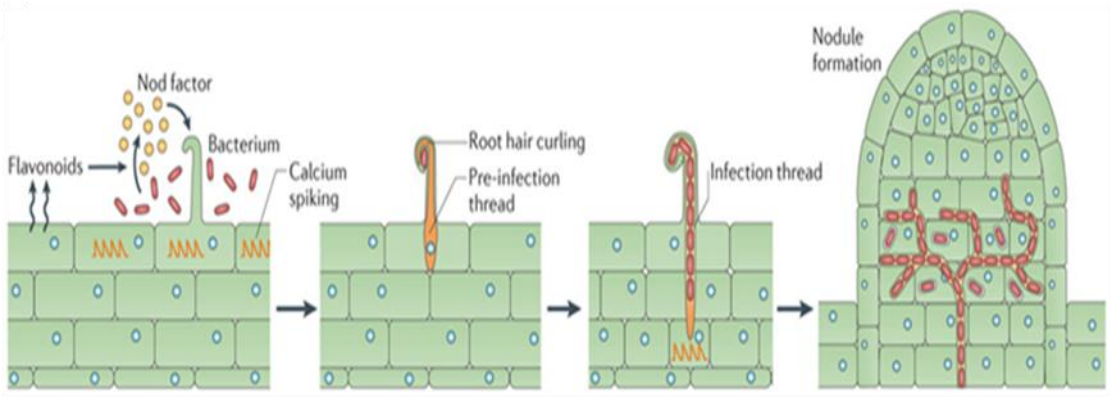

Figure 2.3 Stages in the biology of the nodulation process (Oldroyd, 2013).

However, it has been recently shown that some photosynthetic bradyrhizob (ORS278 and BTAi1 strains) lack the canonical *nodABC* genes required for the synthesis of nod factors (NF) (Giraud et al., 2007). This finding indicated that some rhizobia use an alternative Nod-independent process to form a symbiotic interaction with a legume plant, especially, *Aeschynomene* host species via cracks in the epidermis created by emerging lateral roots (Goormachtig et al., 2004). This mode of invasion by "crack-entry" in the absence of infection threads has been described in *Arachis Hypogeal* (Boogerd and Rossum, 1997).

Bradyhizobium-legume symbioses are the primary source of fixed nitrogen in land-based systems (Tate, 1995) and can provide well over half of the biological source of fixed nitrogen. Atmospheric N_2 fixed symbiotically by the association between *Rhizobium* species and legumes represents a renewable source of N for agriculture (Peoples et al., 1995). For example, symbiotic system between rhizobia and many legumes such as peanut, pigeon pea, chickpea, soybean and mung bean (Table 2.2) (Cocking, 2003).

2.3.2 Bradyrhizobia as nitrogen-fixing oligotrophic bacteria

Oligotrophic bacteria are ubiquitous in the environment and have been isolated from soil (Hattori and hattori, 1980; Hattori, 1984), rivers (Yanagita et al., 1978), lakes (Lango, 1987) oceans (Deming, 1986) and tap water lacking organic substances (Jaeggi and Schmidt-Lorenz, 1990). Some oligotrophic isolates can even grow in distilled water (Favero et al., 1971; Suwa and Hattori, 1984). Two different types of oligotrophs can be distinguished. Those oligotrophsthat can grow on only a low concentration of carbon are called obligate oligotrophs (Fry, 1990; Ishida and

Kadota, 1981). While for those are able to grow at both low and high concentrations of organic substances are called facultative oligotrophs (Ishida et al., 1982).

Bradyrhizobium oligotrophica (*Agromonas oligotrophicum*) S58 was nitrogen-fixing oligotrophic bacterium isolated from paddy field soil that is able to grow in extra-low-nutrient environments (Okubo et al., 2013). Also *Bradyrhizobium* sp. S23321 is an oligotrophic bacterium isolated from paddy field soil. Although S23321 is phylogenetically close to *B. diazoefficiens* (former; *japonicum*) (Delamuta et al., 2013) USDA110, a legume symbiont, it is unable to induce root nodules in siratro, a legume often used for testing Nod factor-dependent nodulation. S23321 contains a *nif* (nitrogen fixation) gene cluster; the organization, homology, and phylogeny of the genes in this cluster were more similar to those of photosynthetic bradyrhizobia strains ORS278 and BTAi1 than to those on the symbiosis island of USDA110. In addition, their genes in S23321 encoded a complete photosynthetic system, many ABC transporters for amino acids and oligopeptides, and two types (polar and lateral) of flagella, multiple respiratory chains, and a system for lignin monomer catabolism. These features suggested that S23321 was able into adapt to a wide range of environments, probably including low-nutrient conditions, with multiple survival strategies in soil and rhizosphere (Okubo et al., 2011). Thus, the characteristics of oligtroph may offer opportunities to use as inoculum for plants in low nutrients condition.

Table 2.2 Estimates of nitrogen fixed by rhizobia in different legume plants (Cocking, 2003).

Legume plants	Amount of N_2 fixed (kg/ha)
Peanut (*Arachis hypogaea*)	37–206/crop
Pigeon pea (*Cajanus cajan*)	7–235/crop
Chickpea (*Cicer arietinum*)	3–141/crop
Soybean (*Glycine max*)	0–450/crop
Garden pea (*Pisum sativum*)	17–244/crop
Faba bean (*Vicia faba*)	53–330/crop
Mung bean (*Vigna radiata*)	9–112/crop
Leucaena (*Leucaena leucocephala*)	100–300/year
Sesbania (*Sesbania rostrata*)	11–458/crop

Adapted from Cocking, (2003).

2.4 Rice production

Rice is the most important cereal crop and staple food for two-thirds of the world's population (Yanni and Dazzo, 2010). Rice is vital for the nutrition of much of the population in Asia, as well as in Latin America and the Caribbean and in Africa. It is central to the food security of over half the world population (Norman and Otoo, 2002). In the future, the world will need to produce more rice than today's global production to feed the world population (Maheshwari, 2007) and have resulted in large increases in rice production but require large amounts of nitrogen fertilizers.

2.4.1 Chemical fertilizer

Nitrogen is the most frequent limiting nutrient for rice production, which requires 1 kg of nitrogen to produce 15–20 kg of grain (Ladha and Reddy, 2003). Presently, chemical fertilizers are widely used for rice production. Consequently, rice production currently requiring large addition of chemical N fertilizers, which are not sustainable systems because they deplete nonrenewable natural resources and can intensify health hazards and environmental pollution (Ahmad et al., 2008). Therefore, endophytic rhizobia, known to directly supply biologically fixed nitrogen to the legume plant, may also have great potential to improve sustainable rice production (Chaintreuil et al., 2000).

2.4.2 Plant growth-promoting bacteria (PGPR)

Plant growth-promoting bacteria (PGPR) are a group of soil bacteria that colonize the roots of plants following inoculation onto seed and that enhance plant growth (Aziz et al., 2012), which may facilitate plant growth either indirectly or directly. There are several ways in which plant growth promoting bacteria can directly facilitate plant growth. They may fix atmospheric nitrogen and supply it to plants, although this is usually a minor component of the benefit that the bacterium provides to the plant; synthesize siderophores which can sequester iron from the soil and provide it to plant cells as a siderophore–iron complex which can be taken up; synthesize phytohormones such as auxins, cytokinins and gibberellins which can act to enhance or regulate various stages of plant growth; solubilize minerals such as phosphorus, making them more readily available for plant growth (Bashan and De-Bashan, 2010; Glick, 1995; Glick et al., 2007).

2.4.3 Rice - PGPR interaction

Agricultural systems have now changed to improve environmental quality and avoid environmental degradation (Roesch et al., 2007). One popular approach of integrated plant nutrient management systems is the addition of biological agents to standard chemical fertilization methods to improve crop yields. In this regard, PGPR may have a potential role in the development of sustainable systems for crop production (Shoebitz et al., 2009). Such plant–bacteria association was found to have great potential in improving production of non-leguminous crops, manifested in terms of increased seedling vigor, yield, growth and nutrient uptake, photosynthetic activity, stomatal conductance and N content (Bhattacharjee et al., 2008). By fixing atmospheric nitrogen (performed by diazotrophic organisms), solubilizing minerals such as phosphorus, producing siderophores (iron chelators), producing plant growth regulators (hormones) such as indole acetic acid (IAA) (Osorio Vega, 2007; Selosse et al., 2004) and producing the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase to reduce ethylene levels in the roots of developing plants, thereby increasing root length and plants growth (Forchetti et al., 2007).

2.4.4 Endophytic bradyrhizobia in rice

Endophytes promote plant growth and yield, suppress pathogens, may help to solubilize phosphate and also contribute assimilable nitrogen to plants. Some endophytes are seed borne, but others have mechanisms to colonize the plants (Rosenblueth and Martínez-Romero, 2006). Various kinds of endophytic bacteria, such as *Pantoea*, *Methylobacterium*, *Azospirillum*, *Herbaspirillum*, *Burkholderia* including bradyrhizobia have been found inside rice plants (Table 2.3) and having no visibly harmful effects on the plants (Mano and Morisaki, 2008).

Table 2.3 Various endophytic bacteria colonized in rice plants.

Rice species	Endophytic bacteria	Rice response to the endophyte	Reference
Oryza. sativa L. cv. Yuefu	*Pantoea agglomerans*	Promotes host rice plant growth and affects allocations of host photosynthates.	Feng et al., (2006)
Oryza sativa L. cv. CO-43	*Methylobacterium* sp.	Improved the plant growth and lateral root formation	Senthilkumar et al., (2009)
Oryza sativa L. cv. Nipponbare	*Azospirillum* sp.	Induce disease resistance in rice	Yasuda et al., (2009)
Oryza sativa L. cvs. IR42 and IR72.	*Herbaspirillum seropedicae*	Increases in N content in grains and fresh weight.	James et al., (2002) Divan Baldani et al., (2000)
Oryza sativa L.	*Burkholderia kururiensis*	Promoting both plant growth and rice grain yield.	Mattos et al., (2008)
	Burkholderia vietnamiensis	Increased rice grain yield	Govindarajan et al., (2008)
Oryza breviligulata	*Bradyrhizobium sp.*	Enhance cultivated rice production	Chaintreuil et al., (2000)

In the absence of leguminous plants, populations of rhizobia are commonly found in soils where they can survive saprophytically. In addition, *Bradyhizobium* are also found to colonize various non-legumes, acting as non-nodular symbiotic endophytes. A few studies reported that *Bradyhizobium* association with plants like rice, sweet potato and maize as non-nodular (Bewley et al., 2006). Chaintreuil et al., (2000) investigated the natural existence of endophytic photosynthetic *Bradyhizobium* (an endosymbiont of *Aeschynomene indica* and *A.sensitiva*) within the root of wetland wild rice *Oryza breviligulata*. The results revealed that endophytic photosynthetic *Bradyhizobium* colonized the root surface and intercellular regions of the wetland wild rice (*O. breviligulata*) and also could increase 20% in the shoot growth and grain yield.

2.4.5 Negative impacts of rice endophytic bacteria via nitrogen metabolism

N fertilization is one of the major sources of nitrate and generally increases the nitrate concentration in the soil solution. Rice production currently depends on the large-scale use of chemical N fertilizers, which contribute to nitrate contamination of soils and groundwater supplies, often leading to health hazards and environmental pollution for rice producing areas (Bhumbla, 2005). The results obtained from investigation of nitrate pollution in 14 cities and counties in North China demonstrated that nitrate contents in ground water and drinking water exceed 50 mg/L, the allowable limit for nitrate content in drinking water (Zhang et al., 1996). N fertilizer applied to rice crops partially lost through different mechanisms including ammonia volatilization, leaching and denitrification. (Choudhury and Kennedy, 2005). Moreover, nitrate-fertilized plants emitted nitric oxide (NO) into soil or air (Rockel et al., 2002). The NO emission can become very toxic under certain

conditions determined by its rate of production and diffusion. NO can stimulate both beneficial and harmful effects, which depend on the concentration and location of NO in plant cells (Qiao and Fan, 2008). High levels of NO can be toxic for the plant cells (Beligni and Lamattina, 1999). In addition, Beligni and Lamattina, (2000) also revealed that NO inhibits hypocotyl and internode elongation in plants. Millar and Day, (1996) reported that cytochrome oxidase within higher plant mitochondria is also inhibited by NO. Several studies have clearly shown the production of NO in early stages of symbiosis and in mature nodules. Rhizobial denitrification in the symbiosomes is a likely source of NO in nodules (Meakin et al., 2007; Sánchez et al., 2010). It has been reported in *Lotus japonicas* roots inoculated with *Mesorhizobium loti* (Nagata et al., 2008). Several authors observed that a decrease in NO production in root nodules results in an increase in N_2-fixation activity in *Lotus japonicus* (Shimoda et al., 2009; Tominaga et al., 2009). NO has been reported as a potent inhibitor of nitrogenase activity, which can bind Lb (leghaemoglobin) to form LbNOs (nitrosyl–leghaemoglobin complexes) (Maskall et al., 1977). In addition, it has also been observed that NO formed by *Bradyrhizobium japonicum* in soybean nodules in response to nitrate has a negative effect on both nitrogenase activity and expression of the *nifH* and *nifD* genes, which encode the Fe protein and the *α*-chain of the FeMo protein from the nitrogenase complex (Sánchez et al., 2010).

However, one candidate for NO detoxification was found in *Bradyrhizobium***,** which reduces nitrate and nitric oxide during denitrification, *resulting* in NO detoxification and *decrease* in nitrate contamination from chemical N fertilizers in the rice producing areas. The schematic of denitrification in *Bradyrhizobium* is shown below:

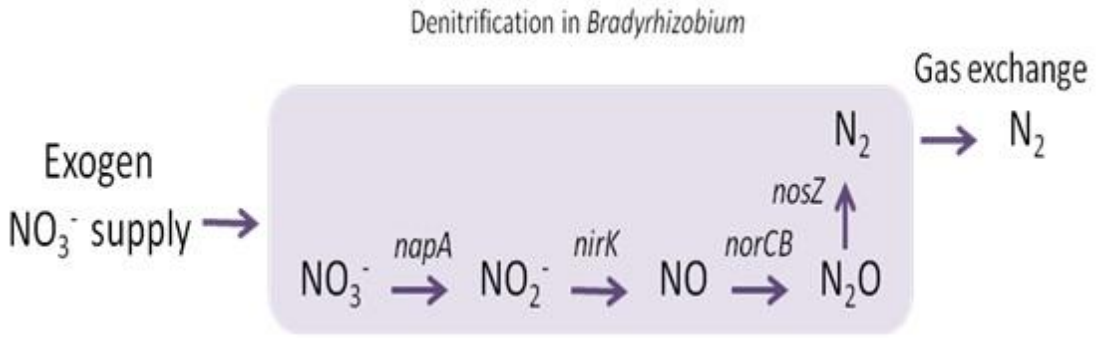

Figure 2.4 Schematic of denitrification in *Bradyrhizobium* (Hirayama et al., 2011).

Legume plants such as soybean can also form symbioses with bradyrhizobia that can reduce NO_3^- (nitrate) to N_2 through denitrification (Sánchez et al., 2014). *Bradyrhizobium* requires four enzymes for reduces nitrogen oxides during denitrification: *napA* (encoding periplasmic nitrate reductase), *nirK* (Cu-containing nitrite reductase), *norCB* (nitric oxide reductase), and *nosZ* (nitrous oxide reductase) (Bedmar et al., 2005). These observation suggested that NO_3^- reduction is the respiratory *napA* (nitrate reductase) and NO detoxification is the respiratory Nor (NO reductase) that catalyses the reduction of NO to nitrous oxide (Sánchez et al., 2011).

2.5 Rice – Legumes crops rotation

In rainfed lowland areas which are traditionally planted to only one crop of rice per year, land use can be optimized by using the pre- and/or post-rice wet period to grow-legume crops. Legumes are suitable rotational crops with rice because they: 1) can mature in 55-90 days, 2) can be grown as pre-rice crop when rainfall accumulation reaches 100 mm/month or as post rice crop using the receding rain and residual soil moisture, 3) are acceptable crops because they are easy to prepare for consumption or to sell at the market, 4) are drought-tolerant and 5) are capable of

using atmospheric nitrogen and contribute nitrogen to the soil (International institute of rural reconstruction, 1990**)**.

Organic rice production has played an important role in recent years in boosting the income of farmers in Northeast Thailand, due to expanding market demand in European countries since 2003 (Polthanee et al., 2008). One of its conditions at the production stage is that organic rice must be cultivated without chemical fertilizer and pesticides. With regard to soil fertility, compost, green manure and animal manure play an important role in improving the crop yield from organic rice farming. Legumes such as groundnut, mung bean (*Vigna radiate* (L.) Wilczek), soybean (*Glycine max* L.) and cowpea (*Vigna unguiculata* (L.)Walp) were well suited for rice based cropping system in peninsular India (Gowda et al., 2001). Groundnut, because of its high oil and protein content was a major crop grow in the post-rainy or summer season crop in rice-fallows in India (Pratibha et al., 2013).

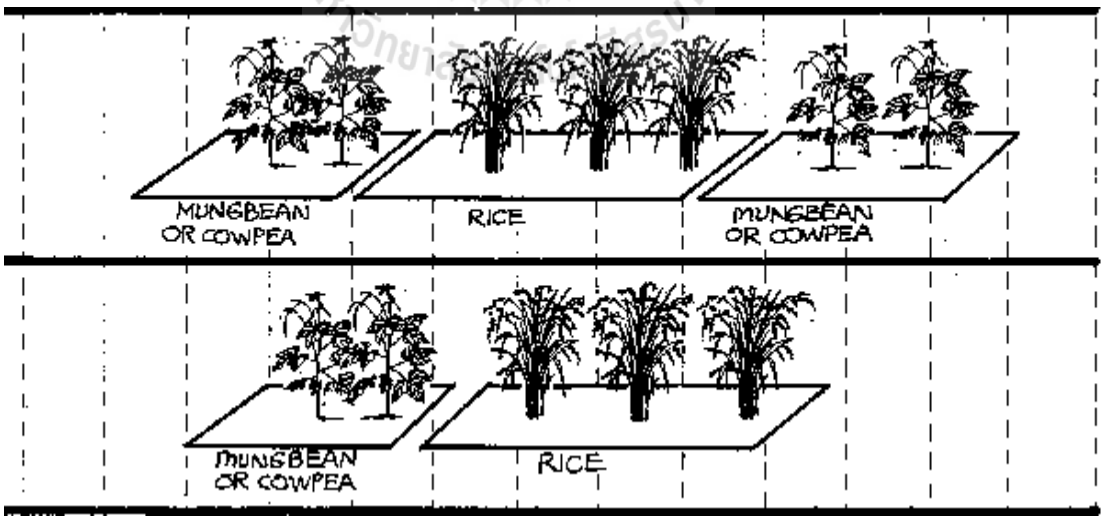

Figure 2.5 Legume crop rotation with rice (International institute of rural reconstruction, 1990**)**

In Thailand, mung bean residues incorporated into the soil significantly increased plant height and tiller number per hill but had no significant effect on top dry weight per hill of the succeeding rice crop at panicle initiation stage (PI). At harvest, pre-rice mung bean significantly increased top dry weight per hill of rice. In addition, growing mung bean before rice provides the advantage of marketable grain of mung bean to 1.6 t/ha. The net economic return was found highest in growing mung bean alone with transplanted rice later (2,855US $/ha) (Polthanee et al., 2012).

2.6 Rice-endophyte-legume

Rice-legumes rotational cropping systems are useful for rice production in Thailand, since legumes can be planted after rice season and famer can earn some money from leguminous product. Moreover, legume-*Bradyrhizobium* can provide the nitrogen to soil fertility for zaanext rice cropping. Chaintreuil et al., (2000) reported that photosynthetic *Bradyrhizobium* strains, which are usually known to induce nitrogen-fixing nodules on the legume, are also natural true endophytes of the primitive rice *O. breviligulata* could significantly enhance cultivated rice production.

Therefore, if we can select endophytic bradyrhizobia that can nodulated legume as well as can live inside the rice plant tissue, this selected *Bradyrhizobium* still persist in the soil (facilitated on the basis of oligotrophic characteristics) and perform symbiosis with legume or rice along the rotational cropping system to support the growth of plant. Thus, it is possible that rice stubble can be used as inoculum in field grown legumes rendering not necessary to supply chemical nitrogen fertilizer or often inoculate *Bradyrhizobium* to legume.

CHAPTER III

MATERIALS AND METHODS

3.1 Plants and bacterial strains

Rice (*Oryza sativa* L.) cv. Pathum Thani 1 was obtained from organic farm and mung bean (*Vigna radiata* L.) cv. SUT4 was obtained from university farm, Suranaree University of Technology. *Bradyrhizobium* sp. PRC008 (commercial strain) was obtained from Department of Agriculture (DOA). This strain was recommended to use with mung bean (*Vigna radiate* L.) to reduce the use of chemical nitrogen fertilizer. Photosynthetic bradyrhizobial reference strain ORS285 was originally isolated from naturally occurring root or stem nodules of *Aeschynomene afraspera* collected in different regions of Senegal by (Molouba et al., 1999). Non-photosynthetic bradyrhizobial reference strain SUTN9-2 was isolated from nodules of *A. americana* , which grown in soil collected from rice field areas in Lampang province (17^{o}31’13.50”N, 99^{o}11’6.34”E) Thailand as described by (Noisangiam et al., 2012). Endophytic bradyrhizobia were isolated from rice (*Oryza sativa* L.) grown in 6 provinces of Northern and North-Eastern parts of Thailand as described by Pongdet Piromyou. BOX –PCR was carried out for screening the different strains of bacteria. The bacterial strains showing the different BOX-PCR patterns were selected for bacterial identification by 16s rRNA sequencing. The sequence of 16s rRNA gene of all isolates were identified and the results indicated that selected endophytic bacteria were closely related to genus *Bradyrhizobium* (Table 3.1).

Table 3.1 Identification of partial 16s rRNA gene of endophytic bradyrhizobial strains.

Provinces	Rice endophytic bradyrhizobial strains	Homology	% Homology	Accession number
Chiangmai: 19°13’11.3”N,98°50’51.7”E	*Bradyrhizobium* sp. SUT-R3	*Bradyrhizobium liaoningense*	98	AB973684
Uttaradit: 17°39’1.9”N, 100°8’34.2”E	*Bradyrhizobium* sp. SUT-R9	*Bradyrhizobium oligotrophicum* S58	97	AB973679
Surin: 14°39’41.3’N,103°17’09.5”E	*Bradyrhizobium* sp. SUT-PR48	*Bradyrhizobium* sp. ORS278	100	AB973680
Lampang: 18°49’42.5”N,99°56’37.2”E	*Bradyrhizobium* sp. SUT-R55	*Bradyrhizobium* sp. Ch25	99	AB973683
Khonkaen: 16°14’13.5”N,102°31’31.1”E	*Bradyrhizobium* sp. SUT-PR64	*Bradyrhizobium* sp. ORS278	100	AB973681
Chiangrai: 19°22’31.7”N,99°30’5.9”E	*Bradyrhizobium* sp. SUT-R74	*Bradyrhizobium liaoningense* 2281	100	AB973682

3.2 Physiological charecteristic of rice endophytic bradyrhizobia

The physiological characteristic of selected bradyrhizobial strains as nitrogenase activity, production of indole acetic acid (IAA) and 1-amino-cyclopropane-1-carboxylic acid (ACC) deaminase were assayed. Each isolate was grown in YEM medium at 28°C with agitation (125 rpm) for 5 days to used as inoculum.

3.2.1 Nitrogen fixation

The nitrogenase enzyme activities of rice endophytic bradyrhizobia in free living form were investigated on the basis of the acetylene reduction assay (ARA) (Chaintreuil et al., 2000). The selected bradyrhizobia were cultured in 5 ml of LG (N-free) broth in test tube and incubated for 7 days at 28±2^{o}C. Ten percentage (v/v) of gas phase in the headspace was replaced with acetylene and further incubated at 28±2^{o}C for 24 h. After incubation, the gas from vessel was injected into gas chromatograph (GC) with a flame ionization detector equipped with PE-alumina packed column (50 m x 0.32 mm x 0.25 µm). The standard curve of ethylene was constructed by varied concentration of pure ethylene following method described by Nuntagij et al., (1997). Then, total protein concentrations of cell suspension were determined using Lowry's method (Lowry et al., 1951).

3.2.2 IAA production

Extracellular indole compounds were colorimetrically determined using the xanthydrol assay of Dickman and Crockett (1956). The concentrations were calculated using L-tryptophan at different concentrations of 0, 10, 50, 100, 150 and

200 µM as the standard. While IAA gave one-third of absorption in the xanthydrol assay, as compared with L-tryptophan, the values for total indoles were collected for the contribution of IAA using the data of the Salkowski reaction (Gordon and Weber, 1951). After completion of ARA and IAA assays, total protein concentration of cell suspension were determined using Lowry's method (Lowry et al., 1951).

3.2.3 ACC deaminase activity

The selected bradyrhizobia were grown in YEM medium at 28°C with agitation (125 rpm) for 5 days until cell reached the early stationary phase. The cells were collected by centrifugation at 5000 rpm for 5 min and washed twice with minimal medium (Penrose and Glick, 2003). Cell pellets were suspended in 15 ml of minimal medium supplemented with 1 mM ACC (1-aminocyclopropane-1-carboxylate), and further incubated at 28°C for 40 h with shaking at 125 rpm to induce ACC-deaminase enzyme production. ACC-deaminase activity was measured following to a protocol of Tittabutr et al., (2008).

3.3 Investigation of rice growth promotion under endophytic bradyrhizobia inoculation

Leonard's jar was filled with 1:3 of sterilized sand and vermiculite. The N-free nutrient solution contained 7 mM $CaSO_4$. $2H_2O$, 17.8 mM Fe-EDTA, 1.0 mM K_2SO_4, 0.25 mM KH_2PO_4, 0.625 mM K_2HPO_4, 2.0 mM $MgSO_4.7H_2O$ and micronutrients adjust pH to 6.8 as described by Broughton and Dilworth, (1970) was added into the Leonard's jar. The different N sources of 0.1 and 1 mM KNO_3, Urea and NH_4NO_3 were added into the N-free solution and applied through a wick to provide nutrients to

plants. The whole apparatus were autoclaved (90 min at 121^{o}C) prior to apply the rice seedlings. Surface-disinfected rice seeds (*Oryza sativa* cultivar Pathum Thani 1) were germinated on 0.85% agar of YM medium. Three replicates of seeds were soaked in broths containing various bacterial isolates (5 ml of 10^8 CFUml^{-1}) for overnight and then transplanted into the Leonard's jar under aseptic conditions. This was conducted as three replicates per single bradyrhizobial isolate as well as the bradyrhizobial reference strains (SUTN9-2 and ORS285). Rices were grown under controlled environmental condition of 28 ± 2^{o}C on 16/8 h day/night cycle (full light, 639 μE m^{-2} S^{-1}). The rice plants were harvested after one month of planting and plant dry weight were determined.

3.4 Detection of nitric oxide (NO) production

The rice plants inoculated with bradyrhizobial strains were grown in the test tube containing N-free nutrient solution with different N sources of 1 mM KNO_3, Urea and NH_4NO_3. One and two week-old-plants were collected for DAF-FM DA detection. A stock solution of diaminofluorescein-FM diacetate (DAF-FM DA) 5 mM in dimethylsulfoxide was diluted 500-fold in water before used (Nagata et al., 2008). The rice roots were placed for 30 min on filter paper soaked with the DAF-FM DA solution and the fluorescence images of the roots were then observed under confocal laser scaning microscopy (excitation 488 nm, emission 515-530 nm; Nikon PCM2000).

3.5 Determination of rice endophytic bradyrhizobia on nodulation and growth promotion of mung bean

Mung bean (*Vigna radiata*) seeds were surface sterilized (Shaharoona et al., 2006). The seeds were put into sterilized Petri dishes containing wet sterilized tissue paper and kept at room temperature for 2 days. The germinated seeds were then transplanted into the Leonard's jar containing sterilized vermiculite and N-free solotion under aseptic conditions. One milliliter of $5x10^8$ CFU ml^{-1} rice endophytic bradyrhizobial inoculum was applied to each seedling at 2 days after transplanting. Plants were placed in a growth chamber receiving 639 μE m^{-2} S^{-1} of photosynthetically for 16 h daily. The plant dry weight and nitrogenase activity were determined after 4 weeks of inoculation.

3.6 Enumeration of endophytic bradyrhizobia

The plant most probable number (MPN) method was used to enumerate the bacterial endophytic population (Zumft, 1997). Rice plants were surface-sterilized and excised surface-sterilized samples of roots and shoots were macerated with a sterilized mortar and pestle, diluted in of saline solution (0.85% NaCl) and inoculated into plastic pouches using mung bean as plant host for counting the density of endophytic bradyrhizobia nodulating in mung bean.

3.7 Investigation of rice endophytic bradyrhizobia inside plant tissues using scanning electron microscopy (SEM)

The roots from rice after inoculated with *Bradyrhizobium* strains SUT-PR48 and SUTN9-2 at 3 and 7 days of culture were fixed with 2.5% (v/v) glutaraldehyde in

0.1 M sodium phosphate buffer pH 7.2, for 2 h, and postfixed in 1% (w/v) osmium tetroxide in the same buffer for 2 h. The fixed roots were dehydrated in a graded ethanol series. Then the samples were treated with CO_2 and mounted on an aluminum cylinder (stub), and finally covered with a steam of carbon and ionized gold (Bacilio-Jiménez et al., 2001; Nowell and Pawley, 1979). The samples were examined under a SEM (JSM 7800F) [magnification (the enlargement of an image) = 1 μm]. The SEM study was conducted at Electron Unit, Suranaree University of Technology, Thailand.

3.8 Gene expression analysis

3.8.1 Sample preparation

The fresh rice roots were harvested at 7 days after inoculated with endophytic bradyrhizobial strains SUT-PR48 and SUTN9-2. Rice samples were sterilized with 70% ethanol for 30 sec, 3% sodium hypochlorite and washed 5-6 times with sterilized water and then immediately frozen in liquid nitrogen and stored at –80°C for further total RNA extraction.

3.8.2 Total RNA extraction and RT-PCR analysis

Total RNA were directly isolated from plant samples using RNeasy Plant Mini Kit (QIAGEN, USA) according to the manufacturers protocol. RNAs were treated with the DNaseI to prevent contamination of genomic DNA, and then convert to cDNA by using iScript™ cDNA Synthesis (BIO-RAD). The transcription levels were determined by Reverse-Transcription Polymerase Chain Reaction (RT–PCR). Primers for amplification (*atpD*, *nirK*, *norB*, *nifH* and *nifV*) were listed in table 5. All RT-PCR were performed in Thermal cycler BIO-RAD T100^{TM} with PCR conditions

as follows: cycler for 35 cycles (2 min at 95°C, 30 sec at the annealing temperature of *atpD* (62°C), *nirK* (50°C), *norB* (50°C), *nifH* (48°C) and *nifV* (60°C) and 30 sec at 72°C), followed by a final 5 min extension at 72°C. The products were visualized using 1% agarose gel electrophoresis and stained in an ethidium bromide buffer, then documented on Gel Doc XR (BIO-RAD Laboratories, Inc.). The relative expression quantity was evaluated by the ratios of band intensity (Roth, 2002).

3.9 Preparation of rice stubble as bradyrhizobial inoculum for mung bean

The experiment was conducted under pot trial conditions. The experimental units consisted of pots (25.5×22.5 cm) sterilized with 3% sodium hypochlorite solution for overnight and then washed by added boiled water into pots before filled 5 kg soil of low-organic matter soil (pH: 6.8, EC: 408 µS/cm, %O.M.: 0.63, phosphorus (P): 49.9 ppm, potassium (K): 141 ppm and calcium (Ca): 689 ppm) and high-organic matter soil (paddy soil) (pH: 7.65, EC: 1066 µS/cm, %O.M.: 3.57, phosphorus (P): 86.1 ppm, potassium (K): 932 ppm and calcium (Ca): 3001 ppm). Soils were partially sterilized by added boiled water into pots before seedling in order to eliminate native (indigenous) bradyrhizobia.

Rice seeds were surface disinfected by washing them in 95% ethanol for 30 sec, followed by hydrogen peroxide (10%, v/v) for 10 min, then washed 3 min with sodium hypochlorite solution (3 %, v/v) and 5-6 times with sterilized water. Seeds were germinated in the dark at 30°C for 2 days on plates containing 0.85% agar YEM medium. Germinated seeds were soaked for overnight with the culture of *Bradyrhizobium* sp. SUTN9-2 containing GUS-reporter gene (10^8 CFU ml^{-1}). The

experiment was conducted as five replicates of the following treatments: (1) control (without inoculation), (2) low-organic matter soil inoculated with SUTN9-2 and (3) high-organic matter soil collected form paddy field inoculated with SUTN9-2. After seed maturation stage of rice, the rice biomass, number of plant per hill and number of panicles per hill were evaluated.

3.9.1 Enumeration of rice endophytic bradyrhizobia from rice tissues

To enumerate the endophytic bradyrhizobia in rice tissues, 1-4 weeks old rice tissues were surface-sterilized as previously described and excised surface-sterilized samples seeds, leaves, stems, roots and stubbles were macerated separately with a sterilized mortar and pestle, then, diluted in saline solution prior to spread on YM plates containing streptomycin (200 µg ml^{-1}) and X-gluc (10 mg ml^{-1}). After 5 days of incubation at 28^{o}C, the numbers of the blue colonies were counted to display the SUTN9-2 population densities in different rice tissues.

3.9.2 Enumeration of rice endophytic bradyrhizobia from soil

Plant Most-Probable-Number (MPN) counts was carried out for soil from rice cultures where the rices were harvested and after harvested 1 week. The soils were mixed with sterilized water (1:1, wt/wt). All visible roots were removed from each suspension. The plant-MPN using mung bean as plant host was calculated from the dry weight of the soil. The dilution factor and tables for three parallel dilution series based on a statistical treatment of the counting methods were conducted for enumeration analyses (Beliaeff and Mary, 1993).

3.9.3 Investigation of the nodulation in mung bean of endophytic bradyrhizobia from rice stubbles nodulation in mung bean

After harvesting the rice inoculated with SUTN9-2, the remained stubble was immediately incorporated into the soil in each pot. After 1 week, 3 seeds of mung bean were planted in each pot for 3 weeks. The nodules from mung bean roots were collected and stained by GUS-assay.

3.10 The statistical analysis

Statistical analysis was performed with the SPSS software (SPSS 16.0 for Windows; SPSS Inc., Chicago, IL).

Table 3.2 Primers used in this study.

Target gene	Primer name	Gene description	Primer sequence	Description of design and references
Housekeeping				
atpD	atpD F95-ORS278 atpD R526-ORS278	ATP synthase subunit beta	5'-CCGACCTACACCGACCAGT-3' 5'-GTTGTCGACGAAGAACAGCA-3'	Designed from *atpD* of *Bradyrhizobium* sp. strain ORS278 (CU234118.1)
Nitric oxide production				
norB	norB F33-ORS278 norB R587-ORS278	Nitric oxide reductase	5'- CTGCCTGATGTTCCTGTTCA-3' 5'- GGTGCCGTGGGTGTAGTAGT-3'	Designed from *norB* of *Bradyrhizobium* sp. strain ORS278 (CU234118.1)
nirK	nirK F198-ORS278 nirK R772-ORS278	Cu-containing nitrite reductase	5'- CGGCGTSTTCGTSTATCACT-3' 5'- ACTTGCCTTCGACCTTGAAA-3'	Designed from *nirK* of *Bradyrhizobium* sp. strain ORS278 (CU234118.1)
	nirK F199-SUTN9-2 nirK R744-SUTN9-2	Cu-containing nitrite reductase	5'- GGCGTGTTCGTGTATCACTG-3' 5'- CTCGATCAGGTTATGCGTGA-3'	Designed from *nirK* of *Bradyrhizobium* sp. strain SUTN9-2
Nitrogen fixation				
nifH	nifH F nifH R	Dinitrogenase reductase	5'- TACGGNAARGGSGGNATCGGCAA-3' 5'- AGCATGTCYTCSAGYTCNTCCA-3'	Noisangiam et al., (2012)
nifV	nifV9-2/102F nifV9-2/344R	Homocitrate synthase	5'- ACCTGGGTCGACGTTCACCACAGA GGAA-3' 5'- CGGAGGTTCTAGACGACATATTGA TCATGGAACC-3'	Designed from *nifV* of *Bradyrhizobium* sp. strain SUTN9-2

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Plant growth promotion characteristics of rice endophytic bradyrhizobia

All of selected endophytic bacteria showed the ability to produce IAA, ACC deaminase and nitrogenase enzymes. Among bradyrhizobial strains, SUT-PR9 produced the highest amount of IAA followed by SUT-R74 and SUT-PR48 (29.44, 10.04 and 5.41 mg/mg protein, respectively). However, the lowest production of IAA was found in SUT-R55 (0.44 mg^{-1} mg $protein^{-1}$). In case of ACC deaminase activity, SUT-PR9 also produced the highest ACC deaminase activity (5.34 µmole hr^{-1} mg $protein^{-1}$) and the lowest was detected in SUT-PR48 (2.18 µmole hr^{-1} mg $protein^{-1}$). All the strains in free-living form were also assessed for their nitrogenase activity. The highest nitrogenase activity was observed in SUT-R74 (0.93 nmol h^{-1} mg $protein^{-1}$). However, nitrogenase activity was neither detected in SUT-R3 nor ORS285 (Table 4.1).

Indole-3-acetic acid (IAA) is a quantitatively important phytohormone produced by several plant growth promoting rhizobacteria (PGPR) and treatment with such auxin-producing rhizobacteria increases plant growth (Spaepen and Vanderleyden, 2011). IAA levels produced by *Bradyrhizobium* sp. in our experiment were similar to *B. japonicum* E109 that performed nodulation in soybean with low levels of IAA (6.6 µg ml^{-1}) when compared to the *B. amyloliquefaciens* LL2012

(18.8 µg ml^{-1}) (Masciarelli et al., 2014). In contrast, IAA production was actually higher in *B. elkanii* (Boddey and Hungria, 1997). In case of, ACC-deaminase which can cleave the plant ethylene precursor ACC, thereby lower the level of ethylene in a developing or stressed plant (Glick et al., 1998). The detection of the ACC deaminase activities from bradyrhizobial strains in this study were varied from 2.18-5.34 µmole/h/mg protein. For the nitrogenase activity, bradyrhizobial strains are known to fix nitrogen under free living condition (Alazard, 1991). This feature may provide bradyrhizobia an advantage to survive under oligotrophic condition. However, this experiment aimed to explore the possibility derived from those PGPR characteristics which may facilitate rice (as non legume plant) growth promotion. Furthermore, it could not be concluded that the low nitrogen fixation activity detected from rice endophytic bradyrhizobial strains would support their efficiency on plant growth promotion.

Table 4.1 Plant growth promotion characteristics of the bradyrhizobial strains.

Bacterial strain	**Indole-3-acetic acid (IAA) mg mg protein^{-1}**	**µmole of alpha ketobutyrate h^{-1} mg of protein^{-1}**	**ARA nmol h^{-1} mg protein^{-1}**
SUT-R3	1.89±1.25^{b}	4.29±0.57^{ab}	0±0.00^{d}
SUT-PR9	29.44±15.29^{a}	5.34±0.26^{a}	0.01±0.00^{d}
SUT-PR48	5.41±2.20^{b}	2.18±0.10^{d}	0.11±0.07^{cd}
SUT-R55	0.44±0.00^{b}	3.29±0.29^{bcd}	0.08±0.06^{cd}
SUT-PR64	2.76±1.25^{b}	3.73±0.52^{bc}	0.43±0.12^{b}
SUT-R74	10.04±1.38^{b}	3.56±0.46^{bc}	0.93±0.24^{a}
ORS285	4.79±2.60^{b}	2.63±0.87^{cd}	0±0.00^{d}
SUTN9-2	0.75±0.36^{b}	3.48±0.10^{bc}	0.23±0.11^{c}

Different letter in the same column indicate significant different among treatment ($P \leq 0.05$)

4.2 Effect of different nitrogen sources on rice growth promotion when inoculated with rice endophytic bradryhizobia

Rice (*Oryza sativa* L. cv. Pathum thani 1) were inoculated with *Bradyrhizobium* strains and cultured for 4 weeks both in the presence and absence of various nitrogen sources. The nitrogen-free treatment was used as control. Rice dry weight was different when different amounts of N fertilizer and forms were applied (Figure 4.1A). In nitrogen-free treatment, almost all of bradyrhizobial strains (except SUT-PR48) showed no effect on rice growth promotion (Figure 4.1B-I) when compared with and without N-supplementation. However, rices grown on KNO_3 or NH_4NO_3 and inoculated with photosynthetic *Bradyrhizobium* sp. strains SUT-PR48, SUT-PR64 and ORS285 showed inhibition on rice growth and also reduced rice dry weight (Figure 4.1D, F and H). In contrast, in the same nitrogen source but inoculated with non photosynthetic *Bradyrhizobium* sp., especially, strain SUTN9-2 did not only inhibit rice growth but also promoted the highest rice dry weight (Figure 4.1I). The inhibition of rice growth with all strains inoculation was not observed under urea treatment.

Urea, NH_4NO_3 and KNO_3 are important available N sources for plant nutrition. Rice growth showed an increase in N applied treatments and their magnitudes of increment were different depending on different nitrogen fertilizer forms. Our results demonstrated that rice growth was differentially affected by KNO_3, NH_4NO_3 and Urea. The rice dry weights in the treatments of KNO_3 and NH_4NO_3 after inoculated with non photosynthetic bradyrhizobia were higher than in the treatments of urea. On the other hand, rice dry weights from inoculation with photosynthetic bradyrhizobia were lower in the treatments of KNO_3 and NH_4NO_3 than that of urea.

Jang et al., (2008) reported that rice (*Oryza sativa* L.) growth as also higher in ammonium based N fertilizer than that of nitrate based. In rice, the application of NH_4^+ is preferred to NO_3^- as nitrogen source because NH_4^+ metabolism requires less energy than that of NO_3^-. Since the absorption of NH_4^+ occurs faster than absorption of NO_3^- (Gaudin and Dupuy, 1999). While NH_4^+ can be assimilated directly into amino acid, NO_3^- must firstly be reduced into NO_2^- and then NH_4^+ via nitrate reductase and nitrite reductase (Hopkins and Hüner, 1995). In addition, assimilation of NO_3^- required energy equivalent up to 20 ATP/mol NO_3^-, whereas NH_4^+ assimilation required only 5 ATP/mol NH_4^+ (Salsac et al., 1987). Thus, NH_4^+ is the main form of N available to rice. In contrast, the previously study reported that in aerobic soil, NO_3^- is the dominant form of nitrogen for plant uptake. Fageria et al., (2006) reported that plant supplied with equal proportions of NH_4^+ and NO_3^- grew as well as supplied with any single amount of N form. In addition, they also revealed that plants can absorb both forms of N equally and that the N form absorbed is mainly determined by what form is abundant and assessable at any given times. The uptake rate of NH_4^+ and NO_3^- also depends on the availability of these ion in the nutrient medium (Mengel et al., 2001).

Bradyrhizobium is a facultative anaerobic soil bacterium with the capability to reduce NO_3^- simultaneously to NH_4^+ and N_2 when cultured anaerobically with nitrate via denitrification (Vairinhos et al., 1989). Denitrification has been defined as the dissimilatory reduction of nitrate (NO_3-) to N_2 via the gaseous intermediates nitric oxide (NO) and nitrous oxide (N_2O) (Zumft, 1997). NO is an inorganic free radical that can become very toxic under certain conditions and also toxic for the plant cells (Maxwell et al., 1989). Because NO inhibits hypocotyl and internode elongation in plants and also inhibit cytochrome oxidase within higher plant mitochondria. (Beligni

and Lamattina, 2000; Millar and Day, 1996). The results from this study suggested that some endophytic bradyrhizobial strains may produce nitric oxide and resulting in rice growth suppression (Figure 4.1D, F and H). Nitric oxide has also been reported as a potent inhibitor of nitrogenase activity (Sánchez et al., 2011). However, nitrogen fixation took place simultaneously with the assimilation of ammonium or nitrate was less inhibited by nitrate than by ammonium (Ito and Watanabe, 1983). These may be the reason why nitrogen accumulation in rice plants from different nitrogen sources and endophytic bradyrhizobia was varied. From these observations have led to the experiment of the detection of NO production and expression of genes involve in NO production (*nirK* and *norB*) and nitrogen fixation (*nifH* and *nifV)*.

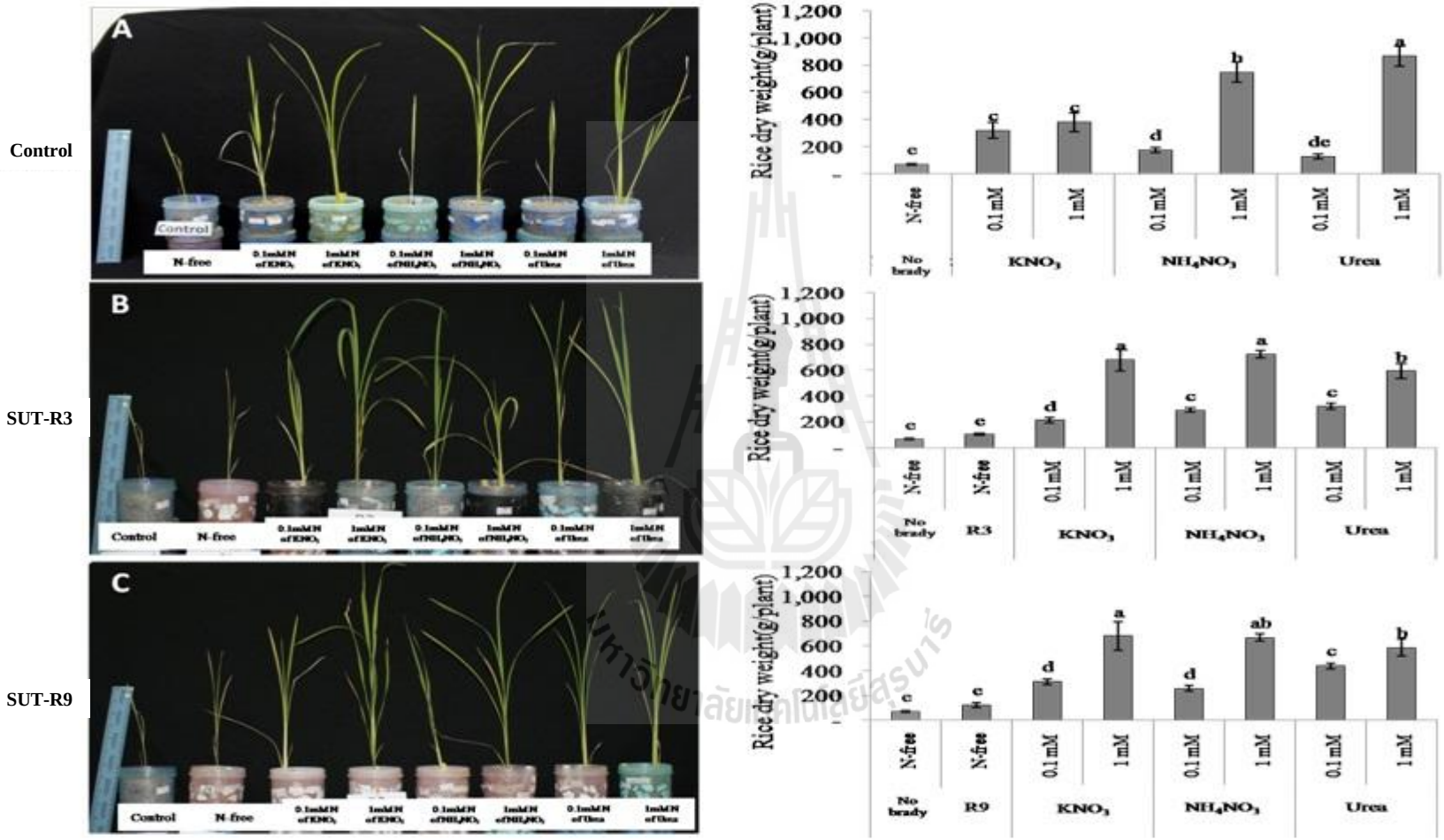

Figure 4.1 Effect of different nitrogen sources and bradyrhizobial strains on rice growth; un-inoculation control (A), inoculation of SUT-R3 (B), SUT-PR9 (C), SUT-PR48 (D), SUT-R55 (E), SUT-PR64 (F), SUT-R74 (G), ORS285 (H) and SUTN9-2 (I).

SUT-PR48

SUT-R55

SUT-PR64

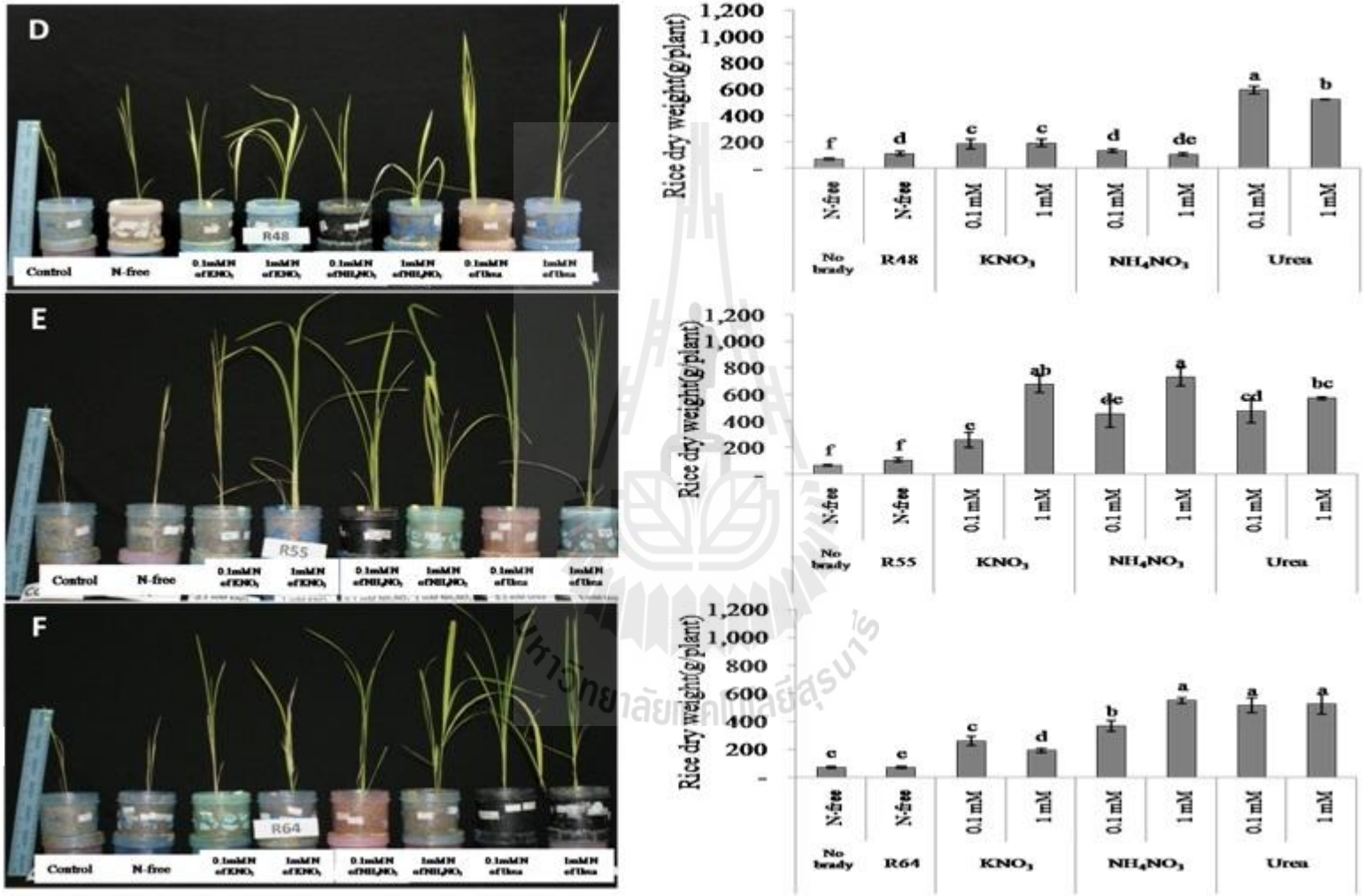

Figure 4.1 (Continued).

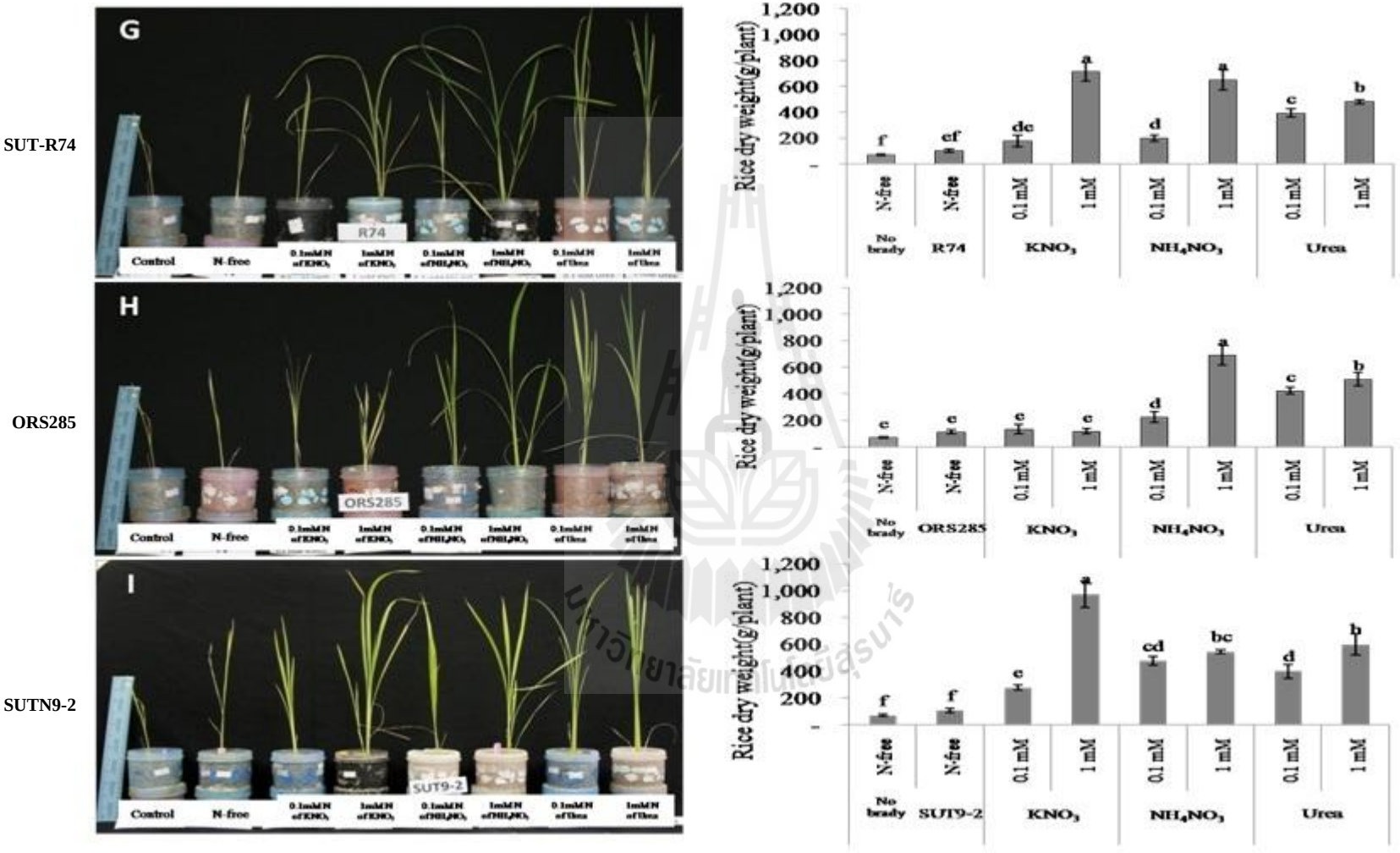

Figure 4.1 (Continued).

4.3 NO production caused by different nitrogen sources and bradyrhizobial strains

To assess the NO production in rice tissues, different nitrogen sources and bradyrhizobial strains inoculation were performed using the dye diaminofluorescein-FM diacetate (DAF-FM DA) as a NO-specific detector. The distinct fluorescence indicating NO production was detected when the rice roots were inoculated with bradyrhizobial strains in treatments supplied with KNO_3 (Figure 4.2A) and NH_4NO_3 (Figure 4.2B). In contrast, the fluorescence was not observed when those rice plants were inoculated with all bradyrhizobial strains in treatment supplied with urea (Figure 4.2C). The strong fluorescence was observed at the 1st and the 2nd weeks after inoculated with photosynthetic bradyrhizobia especially strains SUT-PR48 and ORS285 in both treatments of KNO_3 and NH_4NO_3 amendments. However, some fluorescence was also observed in non photosynthetic bradyrhizobia strains R3.

The production of NO in the bradyrhizobia has been previously reported by *B. japonicum* (Bedmar et al., 2005; Meakin et al., 2006; Mesa et al., 2002). *B. japonicum* is the only *Bradyrhizobium* which is a true denitrifier. It has been shown that denitrification reduces nitrate (NO_3^-) to nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O) and N_2 when cultured microaerobically with nitrate as the terminal electron acceptor and the sole source of nitrogen (Bedmar et al., 2005). High NO levels can be toxic for the plant cell (Beligni and Lamattina, 1999) and also continuous production of NO may cause damage to roots of plants (Nagata et al., 2008). Our results revealed that NO in the rice root was produced from the treatments of NH_4NO_3 and KNO_3 and inoculated with some photosynthetic bradyrhizobia (SUT-PR48 and ORS285) (Figure 8A and B.). This result implied that some photosynthetic bradyrhizobia accumulated

NO using NO_3^- from NH_4NO_3 and KNO_3 via denitrification. On the other hand, an associated function of denitrification is the detoxification of cytotoxic compounds such as NO_2^- and NO produced as intermediates during denitrification reactions (Delgado et al., 2006). This function was found in some strains because NO was not observed from the treatment of NH_4NO_3 and KNO_3 and inoculated with non photosynthetic bradyrhizobia especially, strain SUTN9-2 (Figure 7A and B). Thus, it can be assumed that inoculation with bradyrhizobria producing NO in the rice roots may also inhibit rice growth. This experiment led to the confirmation of the expression of genes involve in NO production (*nirK* and *norB*) in denitrification of *Bradyrhizobium.*

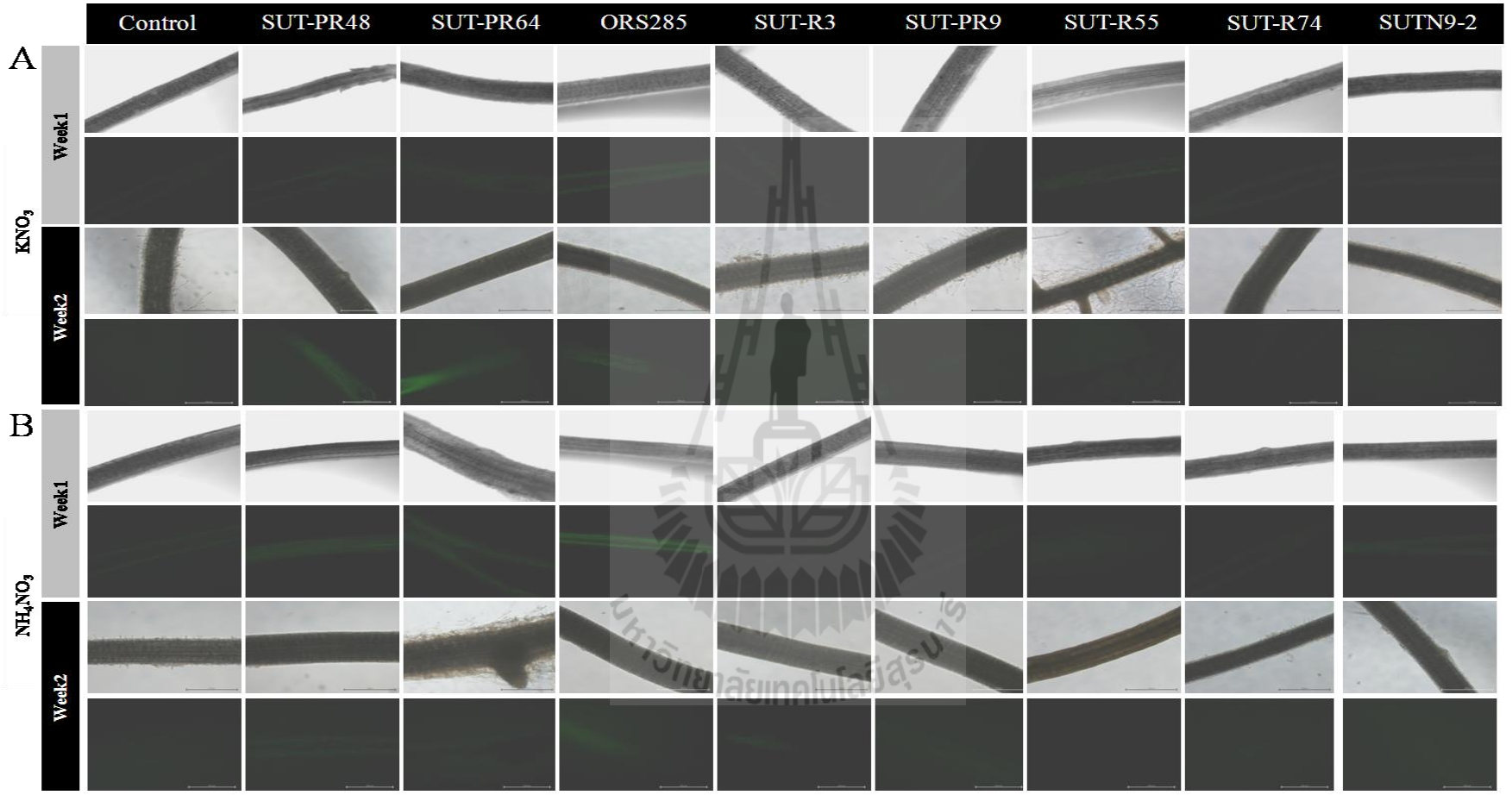

Figure 4.2 The production of NO (brightness) in rice roots fed with KNO_3 (A), NH_4NO_3 (B) and urea (C) and inoculated with bradyrhizobial strains. The NO production was detected by confocal fluorescence microscopy with DAF-FM DA (diaminofluorescein-FM diacetate). Scale bars = 1 mm.

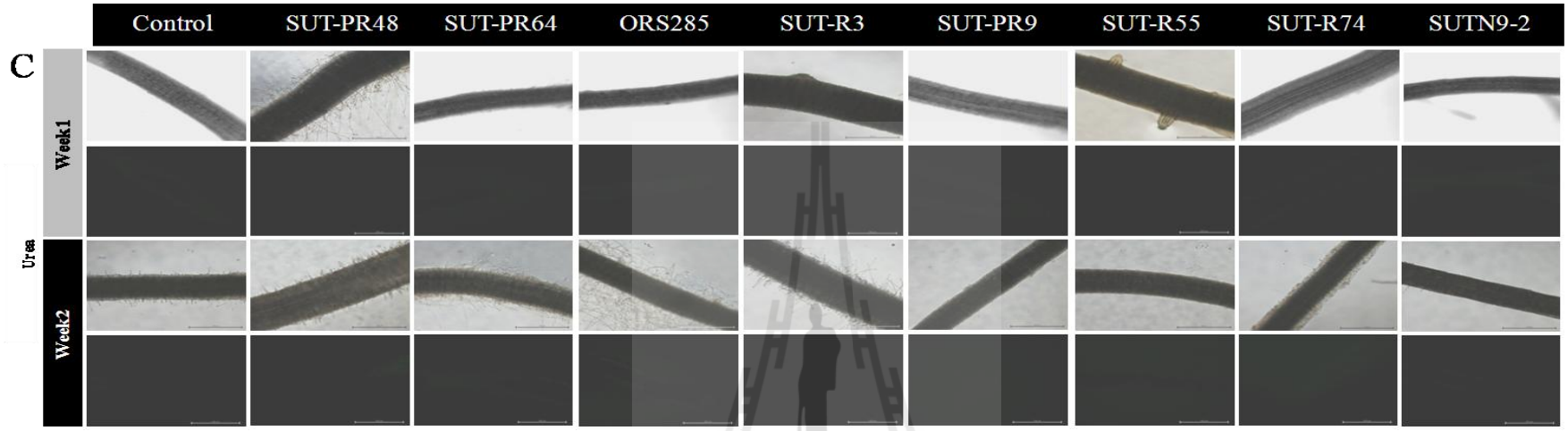

Figure 4.2 (Continued).

4.4 Some nitrogen metabolism related genes expression level of endophytic bradyrhizobia

4.4.1 The relative expression level of genes involve in nitric oxide production of *Bradyrhizobium* sp. SUT-PR48 and SUTN9-2 in rice roots

The relative expression levels of rice endophytic bradyrhizobia were identified in 7 DAI rice root using RT-PCR. The endophytic bradyrhizobia related genes were selected from their mode of nitric oxide production including *nirK* gene (Cu-containing nitrite reductase) and *norB* gene (nitric oxide reductase).

The expression levels of *nirK* and *norB* genes were presented in Figures 4.3A and B respectively. The inoculation of SUT-PR48 with KNO_3 demonstrated the highest *nirK* gene expression level (0.78 folds) followed by NH_4NO_3 (0.72 folds). However, the *nirK* gene expression level of strain SUT-PR48 was significantly lowest when inoculated with urea (0.45 folds). In contrast, for the highest relative expression level of *norB* gene (Figure 4.3B) was formed in the inoculation of SUT-PR48 with urea (0.82 folds) followed by NH_4NO_3 (0.65 folds) and KNO_3 (0.50 folds), respectively.

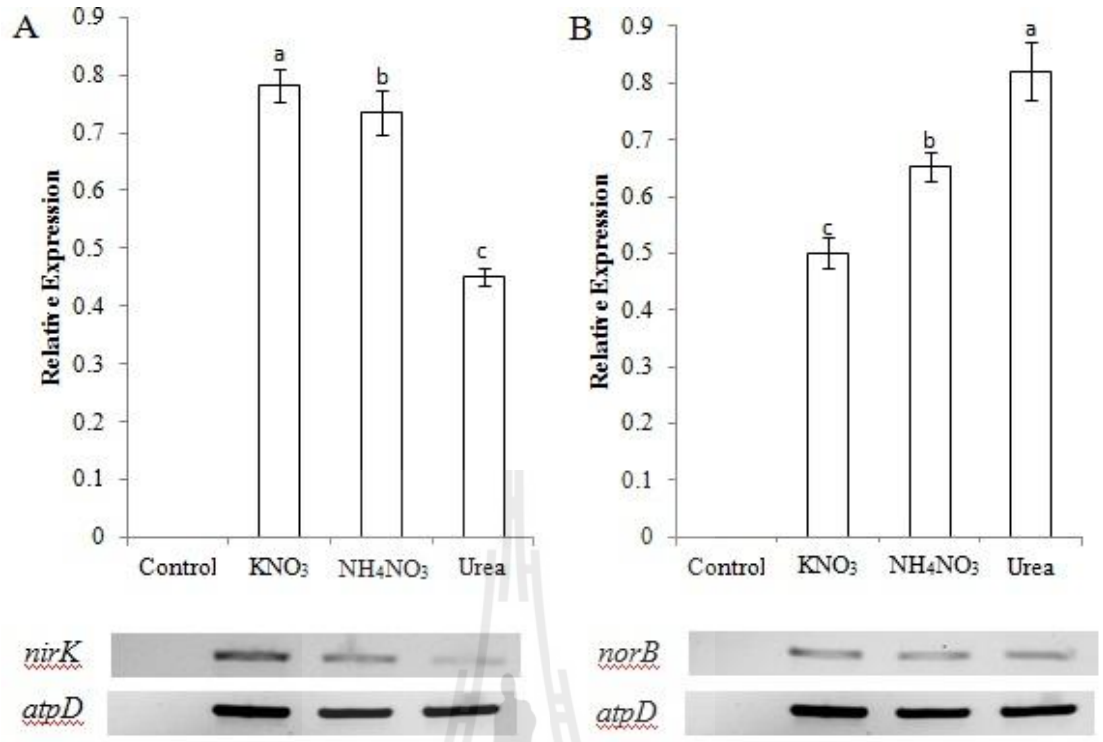

Figure 4.3 Relative expressions of *nirK* gene (A) and *norB* gene (B) of *Bradyrhizobium* sp. SUT-PR48 in rice roots at 7 days in response to different nitrogen source. The housekeeping gene, *atpD* was used as an internal control. Significant at $P \leq 0.05$ is indicated by mean standard error bar (n=3).

The expressions of *nirK* and *norB* genes of SUTN9-2 were depicted in figure 4.4A and B recpectively. The *nirK* gene expression level of SUTN9-2 was not detected when KNO_3 or urea was used as nitrogen sources. However, the inoculation of SUTN9-2 with NH_4NO_3 induced the *nirK* expression level by 0.34 folds. In case of *norB* gene, the expression level from NH_4NO_3 (1.05 folds) also significantly higher than other nitrogen sources of KNO_3 (0.72 folds) and urea (0.52 folds), respectively.

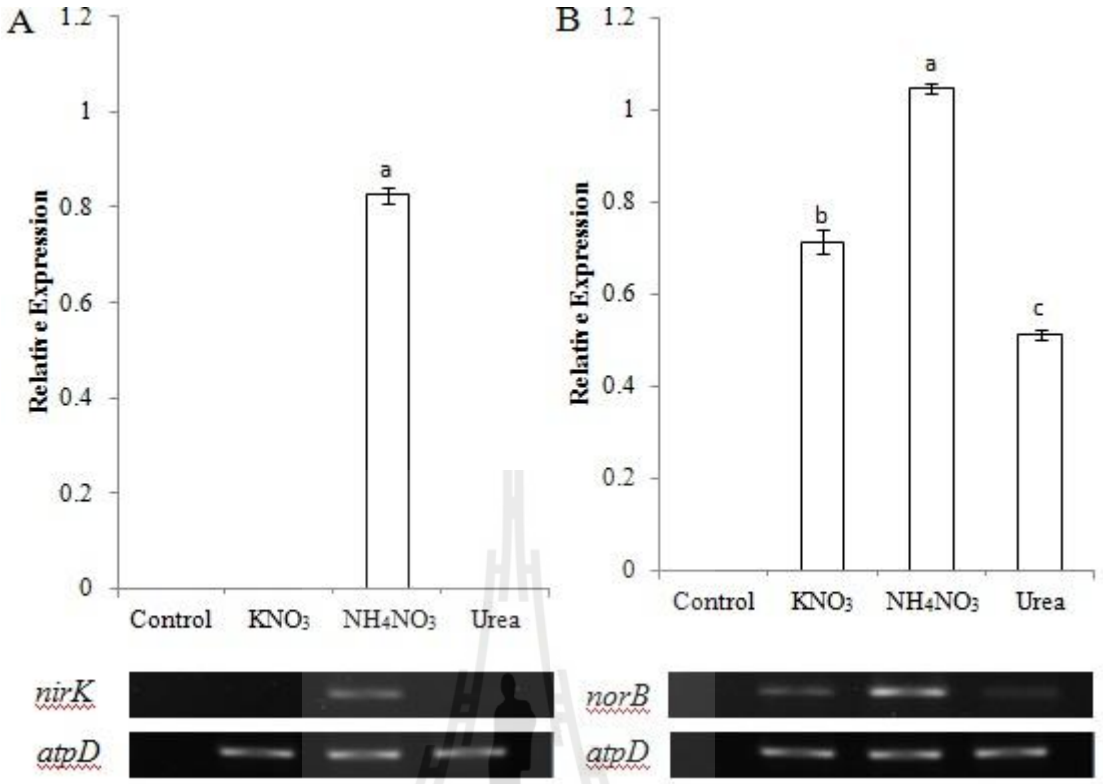

Figure 4.4 Relative expression of *nirK* gene (A) and *norB* gene (B) of *Bradyrhizobium* sp. SUTN9-2 in rice roots at 7 days in response to different nitrogen source. The housekeeping gene, *atpD* was used as an internal control. Significant at $P \leq 0.05$ is indicated by mean standard error bar (n=3).

Several studies have clearly shown that the production of NO occurred in early stages of rhizobia–legume symbiosis (Baudouin et al., 2006; Hérouart et al., 2002; Meilhoc et al., 2011; Sánchez et al., 2011). It was also demonstrated that *B. japonicum* reduces NO_3^- simultaneously to N_2 when cultured microaerobically with nitrate and also produced NO_2^- and NO as intermediates during denitrification reactions (Delgado et al., 2006). The *nirK* and *norB* genes of *Bradyrhizobium* were found to be essential for denitrification. In addition, *norB* gene was required for NO

detoxification. Nor catalyses the two-electron reduction of two molecules of NO to the greenhouse gas N_2O (nitrous oxide) through the denitrification process (Sánchez et al., 2011). The results of this study revealed that the *nirK* gene expression was activated by nitrogen source containing nitrate especially in *Bradyrhizobium* sp. SUT-PR48. In contrast, nitrate did not activate the expression of *norB* gene, which required for NO detoxification and led to the accumulation of NO in rice plants. This may be the reason why SUT-PR48 from pervious experiments showed the suppression of rice growth when nitrate was used as nitrogen source.

It has been reported that anaerobic ammonium oxidation (anammox) bacterium strain KSU-1 and denitrifying bacteria *Marinobacter maritimus* showed the expression of *nirK* gene (Hira et al., 2012; Oakley et al., 2007). NirK is likely to catalyze the proposed initial step of the anammox process, which is a reaction that oxidizes ammonium to dinitrogen gas using nitrite as the electron acceptor under anoxic conditions (Hu et al., 2011). These reports are in accordance with our results from *nirK* gene expression of *Bradyrhizobium* sp. SUTN9-2. The *nirK* gene expression was detected only in the treatment of NH_4NO_3. This suggested that ammonium may also involve in the expression of *nirK* gene. However, the *norB* gene of SUTN9-2 showed higher expression level than *nirK* gene. This result implied that SUTN9-2 may perform the NO detoxification which contributed to the promotion of rice growth.

4.4.2 The relative expression level of genes involve in nitrogen fixation of *Bradyrhizobium* sp. SUTN9-2 in rice roots

The expression level of *nifH* gene was detected only in the inoculation of SUTN9-2 with urea at 1 month after inoculation and induced the expression level by

0.095 folds. In contrast, *nifH* expression was not detected in the inoculation of SUTN9-2 with N-free condition (Figure 4.5). However, the expression level of *nifV* was detected neither urea nor N-free at 2 weeks and 1 month after inoculation (data not shown).

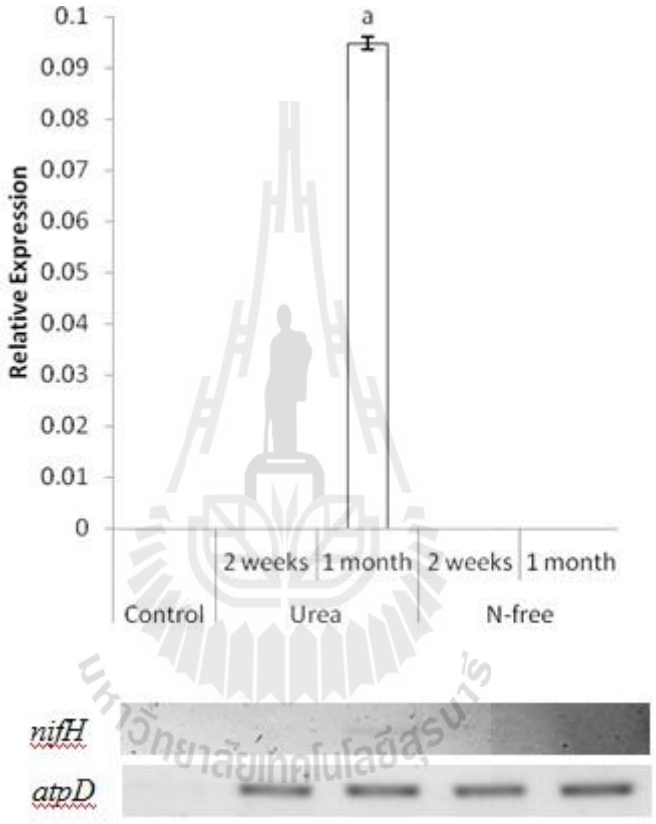

Figure 4.5 Relative expression of *nifH* gene of *Bradyrhizobium* sp. SUTN9-2 in rice roots at 7 days in response to urea as nitrogen source. The housekeeping gene, *atpD* was used as an internal control. Significant at $P \leq 0.05$ is indicated by mean standard error bar (n=3).

From the experiment of nitrogen accumulation, our results revealed that inoculation of endophytic byadyrhizobia with different nitrogen sources especially urea stimulated nitrogen accumulation in rice plant higher than in the absence of

nitrogen source (N-free). In this case, nitrogen accumulation in rice may be caused by biological nitrogen fixation of endophytic byadyrhizobia. This reaction is occurred by the activity of nitrogenase enzyme which is used to fix atmospheric nitrogen into ammonia and gene that encodes the nitrogenase structural component is *nifH*. However, another gene involved in nitrogen fixation is *nifV* gene, which is implicated in biosynthesis of the homocitrate synthase, a necessary component of the FeMo cofactor synthesis and activation of the nitrogenase Fe protein in free-living diazotrophs (Howard and Rees, 1994; Kalra). This gene is not present in most rhizobial species that perform efficient nitrogen fixation only in symbiotic association with legumes (Hakoyama et al., 2009). However, free-living nitrogen fixation by *Bradyrhizobium* was firstly shown by Pagan et al., (1975). In addition, *nifV* gene was also found in *Bradyrhizobium* sp. SUTN9-2.

During nitrogen fixation, the *nifH* gene was expressed in the treatment containing 1 mM of urea at 1 month but not expressed in the treatment without nitrogen source (N-free). This indicated that this gene was induced under nitrogen treatment. Similarly, Kitoh and Shiomi, (1991) reported that the addition of urea resulted in a substantial increase in the growth rate and nitrogen content of the *Azolla*. Even when *Azolla* is grown on a medium containing nitrogen, *Anabaena* in the leaf cavities still fixes atmospheric nitrogen and supplies it to the host plants (Ito and Watanabe, 1983). Moreover, Yang et al., (2014) reported that at under low-N treatment, fungal endophyte *Phomopsis liquidambari* symbiosis increased the rice yield and N used efficiency by 12% and by 11.59%, respectively. Because, low-N fertilization may induce a physiological state of rice favorable for *P. liquidambari* symbiosis and that the resulting higher density of the fungal endophyte is necessary for the beneficial effects on plant performance. In addition, they also showed that the

N metabolism-relevant genes (*OsNR1:* putative nitrate reducase, *OsGS1:* cytosolic glutamine synthetase, *OsGS2:* chloroplastic glutamine synthetase, *OsNADH-GOGAT*: NADH-dependent glutamine-2-oxoglutarate aminotransferase) of young rice seedlings were up-regulated in *P. liquidambari*-infected plants under low N treatment. The *possibilities of these results are* consistent with our case, since gene involve in nitrogen fixation (*nifH*) of SUTN9-2 was also induced under low N treatment. However, in this experiment *nifV* expression was not detected in both treatments of urea and N-free. This suggested that nitrogen accumulation in rice plants may partially supported by nitrogen fixation in endophytic association with rice.

4.5 Mung bean nodulation and growth promotion by rice endophytic bradyrhizobia

To examine whether rice endophytic bradyrhizobial strains can form nodules in mung bean and also promote mung bean growth, they were inoculated into mung bean for observed nitrogenase activity and plant dry weight (Table 4.2). The strain SUTN9-2 produced the highest nitrogenase activity, while SUT-R74 produced the lowest. The highest number and dry weight of nodules were produced by commercial strain PRC008 followed by SUT-R55. In the meantime, SUT-PR9, SUT-PR48, SUT-PR64 and SUT-R74 did not form nodules after one month of inoculation. Maximum plant dry weight was obtained from treatment inoculated with commercial strain PRC008. However, the strain SUTN9-2 did not produce plant dry weight significantly different from commercial strain PRC008 (non endophyte in rice). The results suggested that SUTN9-2 is more effective than other strains and were then selected for the next experiment for using rice stubble as inoculum for legume (Table 4.2).

The results are in accordance with several previous studies**,** *Rhizohium* inoculation in mung bean increases number of pods and seed yield (Bhuiyan, 2004). Solaiman, (1999) and Shukla and Dixit, (1996) also found that *Rhizobium* inoculation increased mung bean seed yield over un-inoculated control. In addition, Shaharoona et al., (2006) *found that Bradyrhizobium* had significant effect on root elongation, total biomass and nodulation of mung bean.

Table 4.2 Effect of rice endophytic bradyrhizobia on growth, nodulation and nitrogen fixation on mung bean (cv. SUT4).

Bacterial strain	**ARA (nmole of ethylene hr^{-1} nodule dry $weigth^{-1}$)**	**Nodule numbers $plant^{-1}$**	**Nodule dry weight (mg pl^{-1})**	**Plant dry weight (g pl^{-1})**
Control	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{e}	0.43 ± 0.02^{cd}
SUT-R3	7.07 ± 0.15^{c}	6.00 ± 1.53^{b}	2.63 ± 2.01^{d}	0.42 ± 0.07^{bcd}
SUT-PR9	0.78 ± 0.68^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{e}	0.58 ± 0.16^{bc}
SUT-PR48	0.41 ± 0.71^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{e}	0.32 ± 0.10^{cd}
SUT-R55	44.88 ± 9.99^{b}	16.00 ± 0.57^{b}	8.67 ± 0.50^{b}	0.62 ± 0.19^{bc}
SUT-PR64	0.68 ± 1.18^{c}	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}	0.45 ± 0.08^{bcd}
SUT-R74	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{e}	0.40 ± 0.07^{bcd}
SUTN9-2	88.99 ± 32.31^{a}	10.00 ± 2.00^{b}	5.37 ± 0.64^{c}	0.70 ± 0.04^{ab}
PRC008 (Non rice endophyte)	17.30 ± 10.91^{c}	157.00 ± 37.00^{a}	85.17 ± 4.01^{a}	0.97 ± 0.18^{a}

Different letter in the same column indicate significant different among treatment ($P \leq 0.05$)

4.6 Confirmation of endophytic bradyrhizobia in rice

Interaction between rice roots from 3 and 7 days plantlets and *Bradyrhizobium* sp. SUTN9-2 and SUT-PR48 were observed by SEM. Rod-shaped bacteria were observed and mostly located in groups or distributed covering the root surface (Figure 4.6A and B). A major presence of bacteria was observed mainly on the root surface, where bacteria were apparently adhered to the root epidermal cells. Such damage of the epidermal surface on heavily colonized areas suggests an active invasion mechanism, probably associated to a high density of bacterial population. Figure 4.6A and B showed that bradyrhizobia invade the inner tissues through the epidermal cells, eventually migrating to the cortex cells after 3 days of inoculation (Figure 4.6C and D). At 7 days, *Bradyrhizobium* sp. was observed entering the inner cortex along the vascular cylinder and inside xylem vessels (Figure 4.6E and F). Both strains SUTN9-2 and SUT-PR48 showed a similar invasion at 3 and 7 days (Figure 4.6A-F). In this experiment, we demonstrated that *Bradyrhizobium* sp. can invade rice roots, spreading rapidly and systematically through the plant tissues and finally leading to confirmation that bradyrhizobia are ehdophytes of rice. Moreover, previous studies also revealed that rhizobia and bradyrhizobia are natural endophytes of the African wild rice (*Oryza breviligulata*) (Chaintreuil et al., 2000) and rice (*Oryza sativa*) (Singh et al., 2006; Tan et al., 2001).

Interestingly, the results also revealed that cell elongation of SUTN9-2 and SUT-PR48 were observed at 7 days when compared with 3 days after inoculation. The cell length of SUTN9-2 after inoculation at 7 days (~ 3 µm) (Figure 4.6E) showed longer than that of at 3 days (~2 µm) (Figure 4.6C and D). Similarly, cell elongation of SUT-PR48 showed cell length at 7 days (~3-4 µm) (Figure 4.6B and F) longer than

that of at 3 days (~2-2.5 μm) (Figure 4.6A). The previous studies reported that differentiation of bacteroids nodules of *Medicago* and related legume from the galegoid clade is controlled by the host plant. During bacteroid maturation, repeated DNA replication without cytokinesis results in extensive amplification of the entire bacterial genome and elongation of bacteria (Mergaert et al., 2006). Therefore, further study should be conducted in order to understand the mechanisms and factors involved in cell elongation from there strains.

4.7 Enumeration of rice endophytic *Bradyrhizobium* SUTN9-2 in rice tissues

The population densities and the persistence of SUTN9-2 in rice tissues were determined by most probable number count (MPN). The population size of SUTN9-2 was found in both root and shoot tissues and the population densities varied from 10^4 to 10^6 (MPN/g of inoculant/g rice fresh weight). Consistent with previous study, the population densities of endophytic diazotrophic bacterial within rice roots and leaf sheaths varied from 10^4 to 10^6 and 10^3 to 10^5 (MPN/g of inoculant/g rice fresh weight), respectively (Rangjaroen et al., 2014). The population densities of shoot significantly decreased at 2nd week and root at 3rd week after inoculation. However, no significant difference of population densities in shoot at week 2-4 and root at week 1, 2 and 4 were observed. In addition, SUTN9-2 also persists in rice tissues from 1st to 4th week after inoculation (Figure 4.7). Chi et al., (2005) examined the persistence of viable populations of endophytic rhizobia within rice plants and found that rhizobia inoculated into the rhizosphere of rice were recovered from within surface-sterilized leaf sheaths, leaves and roots. These findings indicated that the natural endophytic

Bradyrhizobium-rice association is an alternative that can be used in sustainable agriculture to produce the rice crops.

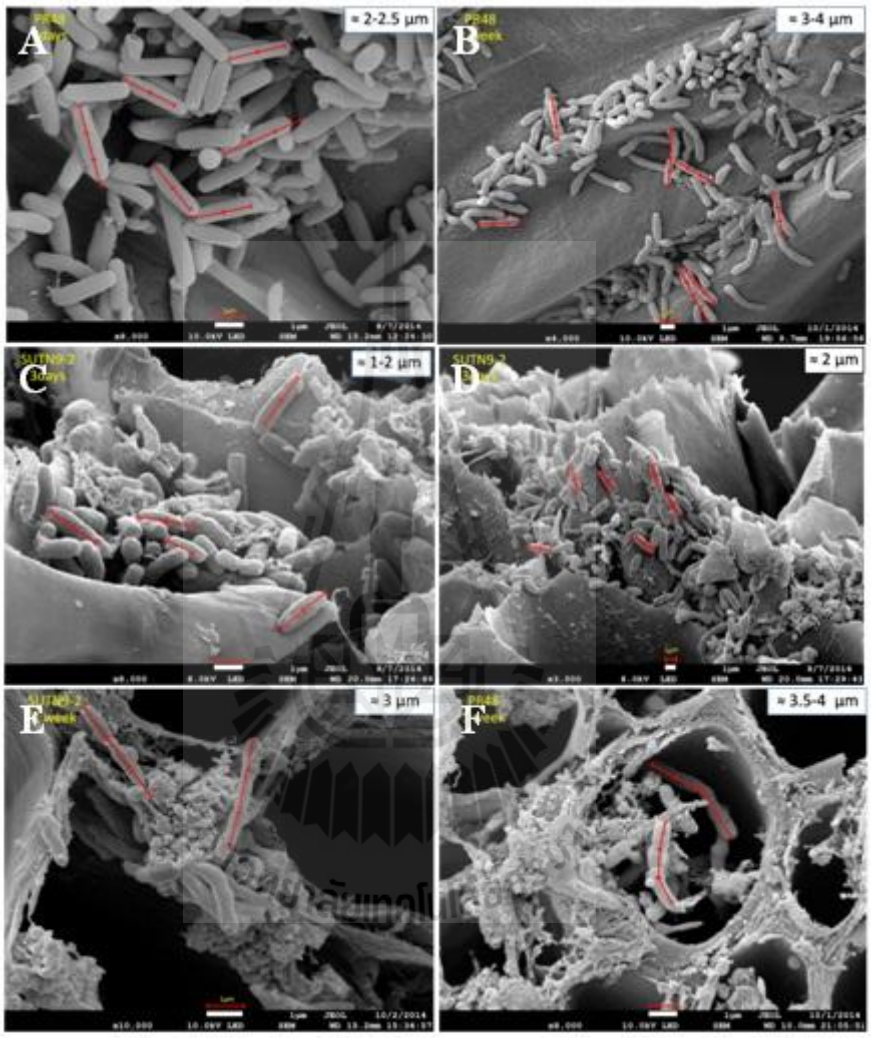

Figure 4.6 Scanning electron microscope (SEM) images of 3 days of *Bradyrhizobium* sp. SUT-PR48 on surface at 3 days (A) and 1 week (cell elongation) (B). SUTN9-2 inside rice roots at 3 days (C and D), at 1 week (E) and SUT-PR48 (F) (cell elongation) The magnification (the enlargement of an image) is the same in panels A, B, C, D, E and F (bar = 1 µm).

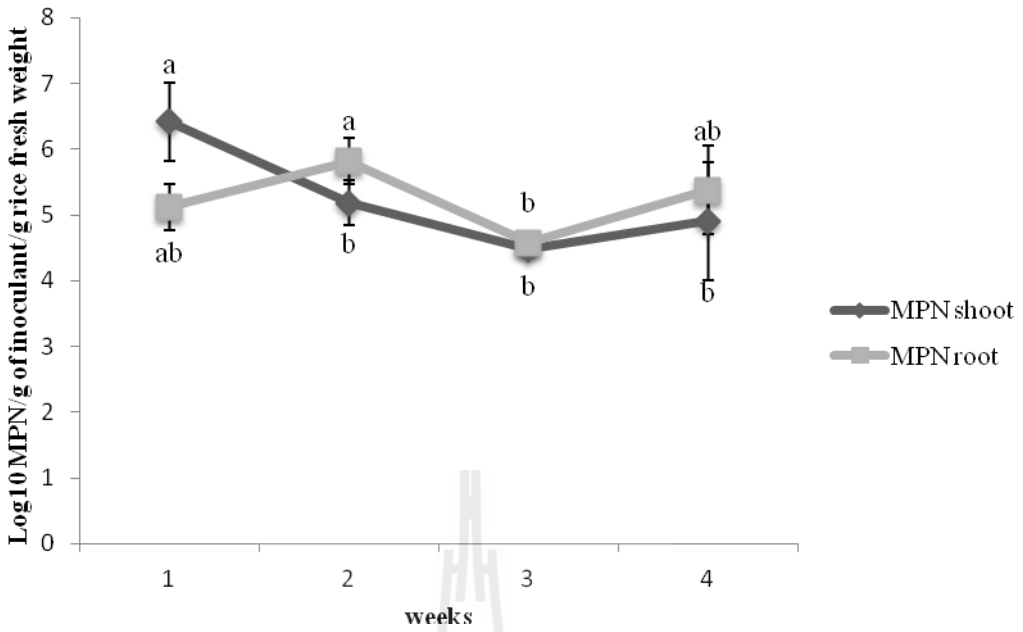

Figure 4.7 Enumeration of rice endophytic *Bradyrhizobium* sp. SUTN9-2 in rice tissues at different time using mung bean most probable number (MPN).

4.8 Investigation the persistence of the selected bradyrhizobia SUTN9-2 in the rice plant under pot trial condition

In order to evaluate the possibility to use SUTN9-2 in rice stubble as inoculum for growing mung bean in the system of rice-legume crops rotation, the experiments were carried out under pot trial condition.

4.8.1 Number of plants and panicles per hill

The inoculation of SUTN9-2 in both of low-nutrient and paddy soils has been recorded slightly higher number of plants per hill (18 plants/hill) than uninoculation (17 plants/hill). In contrast, the lowest number of panicles per hill (14 panicles/hill) was recorded in the inoculation of SUTN9-2 in low-nutrient soil followed by in paddy soil (15 panicles/hill) compared to uninoculation (Figure 4.8).

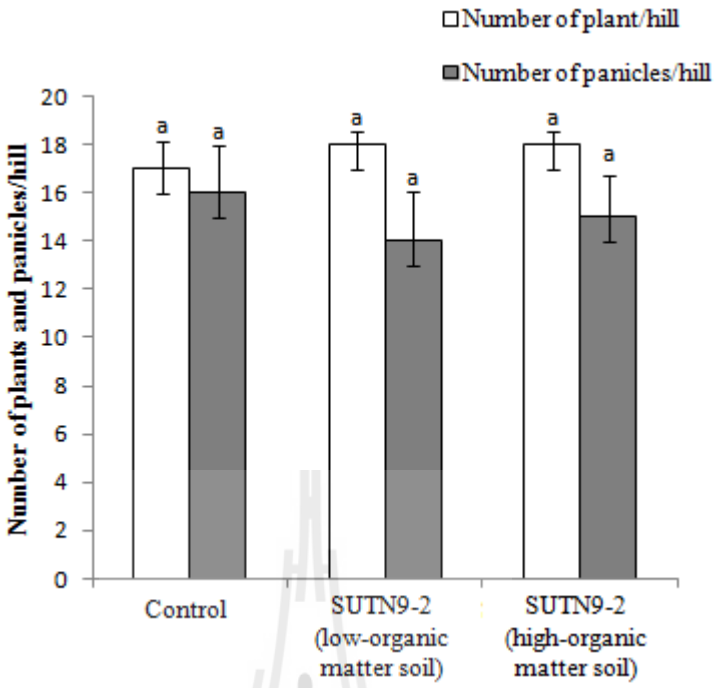

Figure 4.8 Effects of inoculation of endophytic bradyrhizobia SUTN9-2 on number of plants and panicles per hill. Data present the means of the experiment, each with three replicates. Significant at $P \leq 0.05$ is indicated by mean standard error bar (n=3).

4.8.2 Enumeration of the persistence of rice endophytic bradyrhizobia SUTN9-2

The persistence of SUTN9-2 in rice grown in pots under actual field condition was evaluated by plate count colony forming unit (CFU), the numbers of the blue colonies were counted to display the SUTN9-2 population densities in different rice tissues (Figure 14). The result revealed that the population density of SUTN9-2 was found in all tissues of rice and showed the similarity in both of soil containing low-organic matter soil and high-organic matter soil (paddy soil). The highest population density was observed in stems, stubbles and roots tissues, respectively (10^3 to 10^4 CFU/gram fresh weight). However, the population density

was not significantly different from each tissue. On the other hand, the lowest population density was observed in seed and leave tissues (10^1 to 10^3 CFU/gram fresh weights) (Figure 4.9). These results indicated that the population density declines from the under-ground tissues to the above-ground tissue. In addition, *Rhizobium leguminosarum* bv. trifol also utilize a dynamic infection process that permits them to migrate endophytically upward into the stem base, leaf base, leaf sheaths, and some leaves of rice (Yanni et al., 1997). However, our result also revealed that endophytic bradyrhizobia SUTN9-2 still persists in rice tissues until rice-harvesting season. The previous study also reported that endophytic rhizobia persist in rice throughout the vegetative and into the reproductive phases of development (Chi et al., 2005). These indicated that it is possible to use endophytic bradyrhizobia in the rice-legume cropping system.

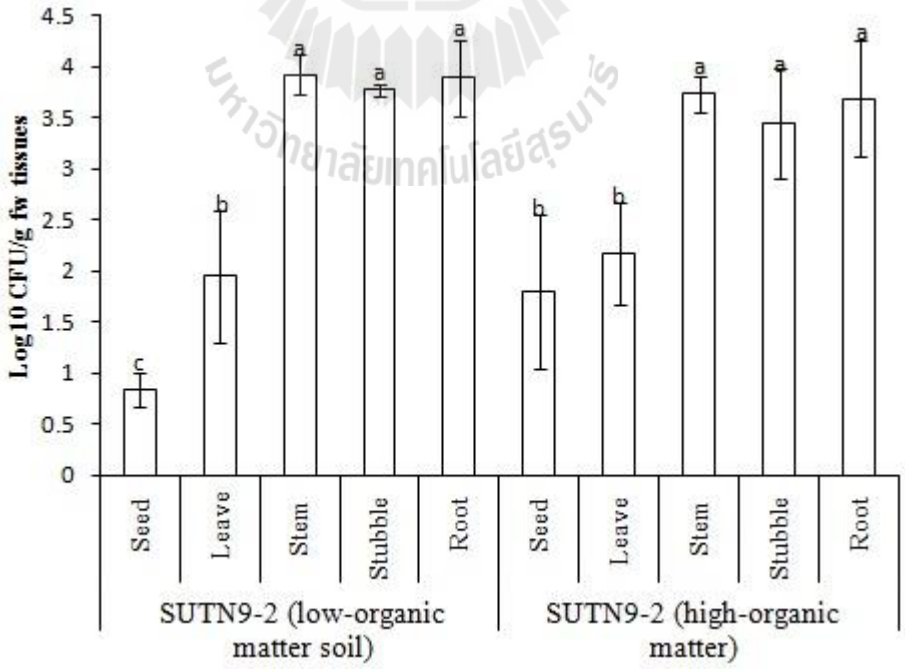

Figure 4.9 Population densities of GUS-tagging bradyrhizobia SUTN9-2 in different tissues after rice harvested.

4.8.3 Investigation of the nodulation of SUTN9-2 from rice stubbles in mung bean

To investigate the capability of SUTN9-2 from rice stubbles on nodulation in mung bean, mung beans were grown after incorporated rice stubbles into the soil for 1 week, 2 weeks and 5 weeks. Analyses were conducted for nodule number, acetylene reduction activity and dry matter of nodules and plants.

At 1 week after incorporated rice stubbles, the mung bean nodulation from rice stubbles was not observed. However, the nodulation was observed at 2 weeks after incorporated rice stubbles into the soil with nodule number around 3-4 nodules. Increasing in number of mung bean nodule obtained at 5 weeks after incorporated rice stubbles into the low-organic matter soil and high-organic matter soil with nodule number around 60 and 35 nodules, respectively. No significant differences in the nodule dry weight, plant dry weight and acetylene reduction activity were observed between low and high-organic matter soil but significantly higher than that of the un-inoculation control (Table 4.3). In addition, the population number of SUTN9-2 from soil at before and after incorporated rice stubbles into the soil at 1week were also determined by the pouch (MPN) method, nodules formed by SUTN9-2 were confirmed by GUS assay. The results revealed that after rice harvesting, low amount of SUTN9-2 still remains in the soil (8 MPN/g of inoculant/g soil dry weight). Furthermore, the population densities of SUTN9-2 increased after incorporated rice stubbles into the soil for 1week (50 MPN/ g of inoculant/g soil dry weight).

The influence of cell number on nodulation has been reported in several studies. It has been reported that the maximum viable number of rhizobia per seed of mung bean (*Vigna radiata*) was 10^7- 10^8 rhizobia/seed (Hafeez et al., 1989). Perkins, (1925) found that increasing the inoculum level above 100 per seed did not increase

the nodule number. However, lesser amounts of inoculum resulted in abundant nodulation on the lateral roots of soybeans grown in the growth chamber but small amounts of inoculum failed to produce good nodulation in the field (Weaver, 1970). Result of this study indicated that low and high amount of SUTN9-2 were released from rice stubbles after harvested 1 week and 1 month, respectively. These results implied that the nodulation of mung bean by using rice stubble as incolum may be affected by degradation of rice stubble which release endophytic bradyrhizobia into the soil.

Table 4.3 Nodulation of SUTN9-2 in mung bean after incorporated rice stubbles into the soil 5 weeks.

Soil	**ARA (nmole of ethylene h^{-1} nodule $^{-1}$ dry weight)**	**Nodule number $plant^{-1}$**	**Nodule dry weight (mg $plant^{-1}$)**	**Plant dry weight (g $plant^{-1}$)**
Control (without SUT9-2)	0.00±0.00[b]	0.00±0.00[c]	0.00±0.00[b]	0.13±0.006[b]
Low-organic matter soil	26.30±6.20[a]	60.00±5.00[a]	0.58±0.16[a]	0.26±0.07[a]
high-organic matter soil	25.90±5.17[a]	35.00±11.00[b]	0.70±0.35[a]	0.22±0.04[a]

Different letter in the same column indicate significant different among treatment ($P \leq 0.05$)

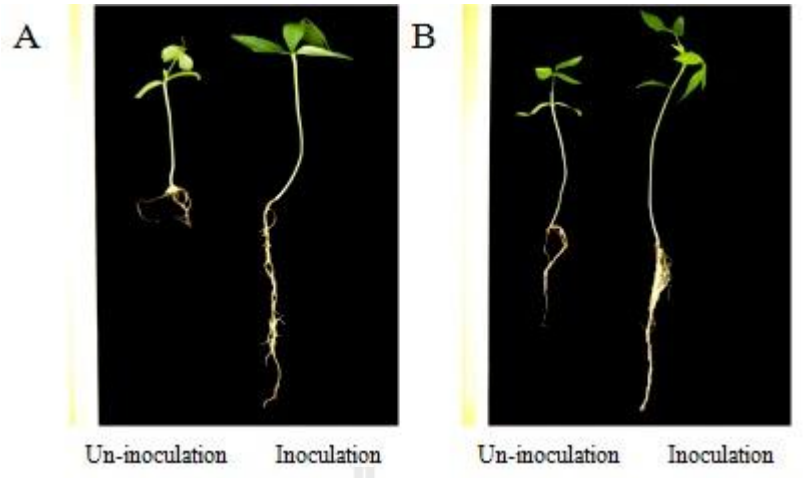

Figure 4.10 Mung bean growth at 14 days under pot trial condition with inoculation of SUTN9-2 in the low-organic matter soil (A) and inoculation of SUTN9-2 in the high-organic matter soil (B) compared with un-inoculation (control).

This is the first report to apply endophytic bradyrhizobia in rice stubbles and directly used as inoculum for leguminous plant (mung bean). Further, investigation of rice stubbles inoculum should be performed under field conditions compared with normal inoculum to assessed the ability of nitrogen fixation in legume plants as well as frequency of normal inoculum.

CHAPTER V

CONCLUSION

All the strains of rice endophytic bradyrhizobia in this study were performed capable of IAA production and ACC deaminase activity. However, high N_2-fixation was not observed in all the strains. This result suggested that bradyrhizobial strains in this study may use the capability of IAA and ACC deaminase production for plant growth promotion. For the N_2-fixation, may only provide bradyrhizobia an advantage to survive under oligotrophic condition.

The rice growth was affected by different nitrogen sources and bradyrhizobial strains. The application of NO_3^- and inoculated with non-photosynthetic bradyrhizobia may result in positive effect on rice growth promotion. In contrast the application of NO_3^- and photosynthetic bradyrhizobia may result in negative effect on rice growth and this may depend on the nitric oxide (NO) production of denitrification pathway by some endophytic bradyrhizobia.

The effect of different nitrogen sources and bradyrhizobial strains on rice growth was also identified by using RT-PCR. The relative expression level of NO production related genes including *nirK* and *norB* were induced in both strains SUT-PR48 and SUTN9-2. However, the *nirK* gene expression level of strain SUT-PR48 was higher than that of the expression level of *norB* gene. In contrast, the *nirK* gene expression level of strain SUTN9-2 was lower than that of the expression level of *norB* gene. These results were accordingly related with NO detection experiment

because SUT-PR48 (PB) may accumulate NO more than SUTN9-2 (non-PB) and resulting in negative effect on rice growth.

The SEM confirmation of endophytic bradyrhizobia in rice demonstrated that bradyrhizobial strains in this study invade the inner tissues through the epidermal cells and migrating to the cortex cells and inside xylem vessels after 3 days and 7 days of inoculation respectively. Moreover, cell elongation of SUTN9-2 and SUT-PR48 were observed at 7 days when compared with 3 days after inoculation. Therefore, further study should be conducted in order to understand the mechanisms of cell elongation from there strains.

To evaluate the possibility to use SUTN9-2 in rice stubbles as inoculum for growing mung beans, the experiment was carried out under pot trial condition. The results revealed that SUTN9-2 still persist in rice tissues until 4^{th} week. In addition, it also still persists until rice-harvesting season. The nodulation in mung bean of SUTN9-2 by using rice stubbles as incolum was observed at 2 weeks and 5 weeks after incorporated rice stubbles into the soil with nodule number around 3-4 nodules and 35-60 nodules respectively. The degradation of rice stubbles leads to an increased number of SUTN9-2 in the soil and may result in variable effect on nodulation in mung bean.

Therefore, the persistence of endophytic bradyrhizobia in rice tissue can be developed for using rice stubble as inoculum for mung bean in the system of rice – legume crops rotation.

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