ชุมชนจุลินทรีย์และการแสดงออกของยีน *nifH* ของแบคทีเรียตรึงไนโตรเจนเอนโดไฟท์ในข้าว

นางสาวจันทร์เพ็ญ ประกำแหง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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MICROBIAL COMMUNITIES AND THEIR *nifH* GENE EXPRESSION IN RICE ENDOPHYTIC DIAZOTROPH BACTERIA

Janpen Prakamhang

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

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MICROBIAL COMMUNITIES AND THEIR *nifH* GENE EXPRESSION IN RICE ENDOPHYTIC DIAZOTROPH BACTERIA

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

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จันทร์เพ็ญ ประกำแหง : ชุมชนจุลินทรีย์และการแสดงออกของยีน *nifH* ของแบคทีเรียตรึงไนโตรเจนเอนโดไฟท์ในข้าว (MICROBIAL COMMUNITIES AND THEIR *nifH* GENE EXPRESSION IN RICE ENDOPHYTIC DIAZOTROPH BACTERIA) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.หนึ่ง เตียอำรุง, 112 หน้า

การศึกษาโครงสร้างของชุมชนแบคทีเรียตรึงไนโตรเจนเอนโดไฟท์ในแต่ละส่วนของต้นข้าว และช่วงการเจริญเติบโตของข้าวขาวดอกมะลิ 105 ในดินแบบต่างๆ พบว่ามีจำนวนประชากรระหว่าง 10^3 - 10^6 CFU ต่อกรัมน้ำหนักสดเนื้อเยื่อข้าว จากเชื้อที่แยกได้คิดเป็นกลุ่มที่ตรึงไนโตรเจนได้เป็น 56% ของประชากรทั้งหมด เมื่อแยกเป็นไอโซเลทเดี่ยวจากแต่ละชุมชนของเชื้อ พบว่าแต่ละไอโซเลทมีคุณสมบัติทั้งยับยั้งและส่งเสริมการตรึงไนโตรเจนซึ่งกันและกัน เมื่อวิเคราะห์ถึงระดับสายพันธุ์ด้วยวิธีอ่านลำดับเบสดีเอ็นเอของยีน 16S rRNA พบว่าแบคทีเรียตรึงไนโตรเจนเอนโดไฟท์กลุ่มดังกล่าวได้แก่ *Enterobacter dissolvens, Brevundimonas aurantiaca, Pantoea agglomerans, Pseudomonas* spp. และ *Enterobacteriaceae bacterium* ศึกษาความสามารถในการใช้แหล่งคาร์บอน, การผลิต indole-3-acetic acid หรือ IAA การผลิตเอนไซม์ pectinase และ cellulase โดยแบคทีเรียที่สามารถผลิต cellulase, pectinase และ IAA สูง ได้แก่ *Rheinheimera* sp., *Brevundimonas* sp., *Citrobacter freundii*, และ *Pseudomonas mendocina* ตำแหน่งการอยู่อาศัยของแบคทีเรียในเนื้อเยื่อข้าวพบที่บริเวณราก ต้น และใบ โดยพบมากที่รากขนอ่อน ด้วยวิธียีนรายงาน GUS จากนั้นวิเคราะห์ชุมชนแบคทีเรียในเนื้อเยื่อข้าวด้วยวิธี PCR-DGGE โดยตรงจากต้นข้าวด้วย 16S rRNA primer สามารถวิเคราะห์ชุมชนจุลินทรีย์เอนโดไฟท์ได้ โดยแบคทีเรียสายพันธุ์หลักในชุมชนจุลินทรีย์นี้ได้แก่ *E. dissolvense, B. aurantiaca, P. agglomerans,* และ *Pseudomonas* spp. ในขณะที่ชุมชนของแบคทีเรียเอนโดไฟท์ตรึงไนโตรเจนสามารถแสดงให้เห็นโดยเทคนิค nested PCR-DGGE ร่วมกับ *nifH* primer สำหรับการแสดงออกของยีนที่เกี่ยวข้องกับการตรึงไนโตรเจนของแบคทีเรียตรึงไนโตรเจนเอนโดไฟท์สามารถวิเคราะห์ได้โดยวิธี RT-PCR ด้วย *nifH* primer สามารถพบความแตกต่างได้ในส่วนต่างๆ ของต้นข้าว ในแต่ละช่วงการเจริญเติบโต

สาขาวิชาเทคโนโลยีชีวภาพ
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ลายมือชื่อนักศึกษา_____
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JANPEN PRAKAMHANG : MICROBIAL COMMUNITIES AND THEIR *nifH* GENE EXPRESSION IN RICE ENDOPHYTIC DIAZOTROPH BACTERIA. THESIS ADVISOR : ASSOC. PROF. NEUNG TEAUMROONG, Dr.rer.nat. 112 PP.

ENDOPHYTIC DIAZOTROPH BACTERIA/MICROBIAL COMMUNITY/RICE/ *nifH* GENE

The community structure of endophytic diazotroph bacteria within each part, and growing stages of rice (*Oryza sativa* L. cultivar KDML-105) for each soil condition was determined. The population of the endophyte was in the range between 10^3 to 10^6 CFU g^{-1} fresh weight of rice tissue. The fifty-six percent from the total isolates was most likely diazotroph. The interaction between a single isolate from each diazotrophic consortium was determined. Both the inhibition and promotion of N_2-fixation from each individual strain were found. Some interested isolates were identified at the species level based on the full sequence analysis of the 16S rRNA gene. The results showed that they are closely related to *Enterobacter dissolvens*, *Brevundimonas aurantiaca*, *Pantoea agglomerans*, *Pseudomonas* spp., and *Enterobacteriaceae bacterium*. The carbon sources utilization, the production of indole-3-acetic acid, pectinase and cellulase were determined. Some of the highly-produced cellulase, pectinase, and IAA strains were identified as *Rheinheimera* sp., *Brevundimonas* sp., *Citrobacter freundii*, and *Pseudomonas mendocina*. The bacterial localization in the rice tissue was detected in roots, stems and leaves and most intensely on some of the younger lateral roots on the basis of GUS reporter gene. The

PCR-DGGE analysis directly from the rice tissue using 16S rRNA primer could elucidate the endophytic bacterial communities. The major bacterial strains in this community belong to *E. dissolvense, B. aurantiaca, P. agglomerans,* and *Pseudomonas* spp. The community of diazotrophic bacteria inside the rice tissue was exhibited using nested PCR-DGGE analysis with *nifH* primer. In order to detect the bacterial nitrogen fixing activity in the rice tissue, RT-PCR approach was carried out. The results demonstrated that the *nifH* gene expression could be detected in different parts and growing stages of rice plants.

School of Biotechnology Student's Signature_____

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Janpen Prakamhang

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LIST OF ABBREVIATIONS

°C	degree celcius
µg	microgram
µl	microlitre
bp	base pair
DEPC	Diethylpyrrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide 5’ triphosphate
et al.	Et alia (and other)
g	gram
h	hour
l	litre
M	molarity
M	molarity
mg	milligram
min	minute
ml	milliliter
mM	millimolar
N	normality
ng	nanogram
PCR	polymerase chain reaction

LIST OF ABBREVIATIONS (Continued)

pmol	picomol
RNA	ribonucleic acid
rpm	revolution per minute
rRNA	ribosomal ribonucleic acid
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume

CHAPTER I

INTRODUCTION

Symbiotic N_2 fixation by legumes is generally expected to be the dominant source of biological nitrogen input in the earth (Mark and Eric, 1992). Bacteria are capable of invading and proliferating in plants, they are likely to interact more intimately with the plant than bacteria from rhizosphere or root surface (Balandreau, 1986). When physical barriers are overcome by invading bacteria, a selective enrichment of certain genotypes of bacteria inside the plant and thus a lower diversity than in the surrounding soil will be achieved. Therefore, microorganism living endophytically will face less competition for nutrient. Another advantage of living inside a plant might be a partial protection from changes in the environment, such as osmotic stress due to lowered water activities. A well-studied example is the legume-nodule symbiosis, in which the bacteria fix nitrogen as endosymbionts inside the plant, in nutrient-rich, oxygen controlled microenvironment (Mylona et al., 1995). Therefore, the emphasis was on endophytic bacteria in several groups searching for diazotrophs that able to contribute to the nitrogen requirements of gramineous plants (Reinhold-Hurek and Hurek, 1998a).

Gramineous plants differ in their capacity to support associated nitrogen fixation. For example, sugar cane which was shown to support biological nitrogen fixation (BNF) was traditionally carried out without large amounts of N-fertilizer (Lima et al., 1987). Moreover, nitrogen fixation may be variety-specific, depending

on the plant genome: only some of the Brazilian sugar cane cultivars tested gained high amounts of their nitrogen content from biological nitrogen fixation in uninoculated Brazilian soil (Lima et al., 1987). Plants that were likely to support BNF were among the first that were screened successfully for the presence of endophytic diazptroph bacteria. In non-legume agrosystems, agriculturally important grasses such as sugar cane (*Saccharum* sp.), wheat (*Triticum aestivum*) sorghum (*Sorghum bicolor*), maize (*Zea mays*), contain numerous diazotrophic bacteria, such as *Gluconacetobacter diazotrophicus, Herbaspirillum* spp., *Azospirillum* spp. Endophytic diazotroph bacteria do not usually cause disease symptoms in the plants with which they are associated and the more numerous of them. There are capable of invading inner tissues including xylem vessels and of systemic spreading (James and Olivares, 1998). However their functions for the plants are still disputed (Reinhold-Hurek and Hurek, 1998a). Wild rice species are likely to harbor unique populations of nitrogen-fixing bacteria that differ from those in extensively bred modern varieties of cultivated rice (Elbeltagy et al., 2000).

Cultivated Rice (*Oryza sativa*) is the most important staple crop in the developing world. It is well known that a remarkable diversity of N_2-fixing bacteria is naturally associated with field-grown rice (Balandreau, 1986). However, in the case of wetland rice, even when specific varieties have been shown to fix N_2 (Ladha et al., 1997), it will be extremely difficult to isolate the organisms responsible, because approximately 90% of the bacteria isolated from surface-sterilized rice plants (several species and varieties, plus some related genera) using N-defficient media are non-diazotrophs (Barraquio et al., 1997). And the culturable diazotrophic population is extremely varied, and so far virtually uncharacterized (Stoltzfus et al., 1997). Also the

rhizosphere of rice may also contain an enormous bacterial population that has yet to be cultured (Ueda et al., 1995). Therefore, cultivated rice fields are considered to be ideal niches for BNF, especially endophytic diazotroph bacteria.

CHAPTER II

REVIEW AND LITERATURE

2.1 Endophytes definition

The term endophyte is defined as an organism inhabiting plant organs that at some time in its life, can colonize internal plant tissue without causing apparent harm to the host (Petrini, 1991). Endophytes have been discovered in high numbers within different tissues of various plants. In principle, all the bacterial endosymbionts of plants fit well within the endophytic definition, they usually are considered as a group of their own. Differing from endosymbionts, endophytes are not necessarily symbiotic, and some of them may even become pathogenic under defined conditions (James et al., 1997). Various endophytic nitrogen fixing bacteria, named endophytic diazotrophs have most frequently been detected in the nonsymbiotic root and vascular tissues of several nonleguminous plants (Hallmann et al., 1997). Endophytic diazotrophs have been proposed to be responsible for the supply of biologically fixed nitrogen to their host plant (Boddey et al., 1995). These endophytes do not cause damage to the host organism but they promote plant growth by one or more of three factors; the production and secretion of plant growth regulators (Verma et al., 2001), antagonistic activity against phytopathogens (Downing and Thomson, 2000) and the supply of biologically nitrogen fixation (Ladha et al., 1997).

Diazotrophs are bacteria that fix atmospheric nitrogen gas into a more usable form such as ammonia. Diazotrophs are scattered across bacterial taxonomic groups (mostly in the Eubacteria but also a couple of Archaea). Even within a species that can fix nitrogen there may be strains that do not fix nitrogen. Fixation is shut off when other sources of nitrogen are available, and, for many species, when oxygen is at high partial pressure. Bacteria have different ways of dealing with the debilitating effects of oxygen on nitrogenases. For example, anaerobes, these are obligate anaerobes that cannot tolerate oxygen even if they are not fixing nitrogen. They live in habitats low in oxygen, such as soils and decaying vegetable matter. Facultative anaerobes, these species can grow either with or without oxygen, but they only fix nitrogen anaerobically. Often, they respire oxygen as rapidly as it is supplied, keeping the amount of free oxygen low. Examples include *Klebsiella pneumonae*, *Bacillus polymyxa, B. macerans* and *Escherichia intermedia* (Postgate, 1998). Aerobes, these species require oxygen to grow, yet their nitrogenase is still debilitated if exposed to oxygen. Phototrophs, photosynthetic bacteria generate oxygen as a by-product of photosynthesis, yet some are able to fix nitrogen as well (www.en.wikipedia.org). Baldani and colleagues (1997) proposed to divide the endophytic diazotrophs into two groups: facultative and obligate. Facultative endophytes are described as those bacteria that survive in the soil and or on plant surfaces as well as being able to colonize the interior of some plants. Most endophytic *Azospirillum* strains are regarded as being facultative endophytes. However, *Herbaspirillum* sp., *Gluconacetobacter diazotrophicus, Burkholderia* sp. and other endophytes are obligate endophytes, as they survive poorly in the soil and appear to have a requirement for living within a host plant. On the other hand, Olivares and team

(1997) had reported that *H. rubrisubalbicans* would clearly live for some time on leaf surface. The scope for nitrogen fixation in cereals by means of endophytic nitrogen fixation has been increased, the endophytic association of *Azorhizobium caulinodans* for nodulation and nitrogen fixation in cereal crops was reported (Kannaiyan and Kumar, 2003).

The search of natural association and endophytic interaction of diazotrophs with rice is considered very promising, especially in primitive rice varieties not bred to efficiently respond to N fertilizer (Barraquio et al., 1997; Stoltzfus et al., 1997). Therefore, endophytic bacteria-plant interaction has a potential role in developing sustainable systems of crop production (Sturz et al., 2000).

2.2 Plant-associated bacterial endophytes

Bacteria belonging to the genera *Azospirillum*, *Herbaspirillum*, *Gluconacetobacter*, and *Azoarcus* are found as endophytes of many graminaceous plants mostly from the tropical regions. The ability to colonize the root interior, to survive only poorly in the soil, and to fix nitrogen in association with these plants is a characteristic of all these bacteria.

Azospirillum has been found in association with many cereals and forage grasses grown both in temperate and tropical climates (Baldani et al., 1997). Although these bacteria are regarded more as being rhizospheric bacteria colonizing mainly the elongation and root hair zones of roots, some strains of both *A. lipoferum* and *A. brasilense* are either facultatively or obligately endophytic (Baldani et al., 1997; James et al., 1997). Strains of *A. brasilense* can colonize plant tissues differentially; some strains live only on root surfaces, whereas others colonize cortical intercellular

spaces or even the vascular tissue (James and Olivares, 1998). Besides the ability of fixing nitrogen, both *A. brasilense* and *A. lipoferum* can produce auxin (Costacurta and Vanderleyden, 1995).

G. diazotrophicus is another restricted bacterium found in high numbers mainly in the roots, stems, and leaves of sugar cane. *G.diazotrophicus* has also been detected in *Pennissetum purpureum*, sweet potato (*Ipomoeabatatas*), coffee (*Coffea arabica*), and pineapple (*Ananas comosus*) (Jimenez-Salgado et al., 1997; James and Olivares, 1998). *G. diazotrophicus* is a nitrogen-fixing bacterium which, as with the two *Azospirillum* species, produces the phytohormones auxins and gibberellins (Bastián et al., 1998). Because the bacterium survives poorly in soil, it is considered an obligate endophyte, colonizing the intercellular spaces of sugar cane stem parenchyma tissue (Baldani et al., 1997). It has also been detected within the xylem vessels (James et al., 1996).

Nitrogen-fixing bacteria belonging to the genus *Azoarcus* are found mainly in roots of Kallar grass (*Leptochloa fusca*) in the intercellular spaces, xylem vessels, and dead root cells. *Azoarcus* has been demonstrated to spread systemically within the plant via the xylem vessels (Hurek et al., 1994). In addition to the plant roots, this bacterium has been discovered in close interaction with a rhizosphere fungus (Hurek et al., 1997).

The genus *Herbaspirillum* contains an unusual group of endophytes in the respect that these bacteria may become pathogenic to their host under certain conditions. *H. rubrisubalbicans* has been identified in association with sugar cane, sorghum, rice, palm trees, and a C4 grass. *H. seropedicae* has been demonstrated within roots of 13 members of the *Gramineae*, as well as in the stem of sugar cane

(James et al., 1997). *H. seropedicae* is another root-associated bacterium capable of producing auxins and gibberellins (Bastián et al., 1998). *H. seropedicae* strain Z67 colonized mainly subepidermal regions of rice roots (Roncato-Maccari et al., 2003). *H. seropedicae* B501 colonized mainly intercellularly in wild rice plants (*O. officialis*) and express the ability to fix nitrogen endophytically (Elbeltagy et al., 2001).

There are several bacterial species, in addition to the most well studied root endophytes, which have been isolated from gramineous plants. Whereas certain species are less studied, but connected by their ability to fix nitrogen (James and Olivares, 1998). Genera *Burkholderia* and *Klebsiella* are preferentially regarded as endophytes (Palus et al., 1996; Baldani et al., 2000). Recently, the isolation of presumptive endophytic diazotroph bacteria from rice have been reported, including *K. oxytoca* and *Enterobacter cloacae* have been isolated from the rhizosphere of wetland rice (Fujii et al., 1987). *Serratia marcessens* IRBG500 was observed within rice roots, stems, and leaves and could also increase the root length and root dry weight of the inoculated plants (Gyaneshwar et al., 2001). Teaumroong and colleges (2001) found five endophytic bacteria isolates from Thai rice have been shown a high N_2-fixation potential and three out of the strains were able to produce the plant growth promoting substance as IAA. *Pantoea agglomerans* (Remus et al., 2000), *Alcaligenes faecalis* (You and Zhou, 1989), and a few other bacteria belonging to the genera *Pseudomonas*, *Enterobacter* and *Bacillus* (Watanabe and Lin, 1984; Lindberg et al., 1985; Persello-Cartieaux et al., 2003) are considered endophytic bacteria.

For cultivated rice, a considerable amount of work has been published on the interaction with endophytic bacteria (Table 1). The most studied belong *Herbaspirillum* sp, *Azospirillum* sp., *Azoarcus* sp and *Serratia marcesens.*

Table 1. Endophytic diazotroph colonization site in rice**.**

Strain	Colonization site	Reference
*Azoarcus sp.*BH72	Intercellular in root	(Hurek and Reinhold-Hurek, 2003)
Azospirillum sp.	root hair / elongation zones	(Bashan et al., 1991)
Azospirillum brasilense	Emerging zone in 20 roots	(Bacilio-Jimenez et al., 2001)
Herbaspirillum seropedicae	rice roots	(Roncato-Maccari et al., 2003)
H. seropedicae Z67	Subepidermal region of roots	(Barraquio et al., 1997)
H. seropedicae Z67	Intercellular spaces, aerenchyma, and xylem of the roots and aerial parts	(James et al., 1997)
*Herbaspirillum sp.*B501	Intercellular in root	(Elbeltagy et al., 2001)
Serratia marcesens	Intercellular spaces and aerenchyma in root, stem, leaves	(Gyaneshwar et al., 2001)

In addition to the root endophytes of gramineous plant discussed above, endophytic bacteria have been detected in citrus trees and grapevine. Bacteria belonging to the genera *Pseudomonas*, *Enterobacter*, *Bacillus* and *Corynebacterium* were found in the xylem of lemon roots, and the xylem tissues of grapevine stem contained bacteria belonging to the genera *Pseudomonas*, *Enterobacter*, *Pantoea* and *Rhodococcus* (Gardner et al., 1982; Bell et al., 1995). Some of these bacteria exhibited antagonistic effects towards other bacteria. *E. agglomerans*, *Rahnella*

aquatilis and *P. corrugata* isolated from grapevine were able to control phytopathogenic strains of *Agrobacterium vitis* (Gardner et al., 1982; Bell et al., 1995).

2.3 The endophytic role

Microbial promotion of plant growth may be the outcome of several additional factors besides the nitrogen fixation. For example, an indirect plant growth promotion is the production of phytohormones, which has been considered to be the main function in the symbioses. Almost all root endophytes fix also nitrogen (Baldani et al., 1997). However, the benefit of their nitrogen fixing ability for the plant has not indisputably been demonstrated (James, 2000). *P. agglomerans* can infect and colonize in the rice roots and producing IAA and have been shown to be potent biological control agent against fungal disease (Verma et al., 2001). Production of auxins and gibberellins is also typical for many root associated endophytic bacteria such as *Azospirillum* sp., *G. diazotrophicus*, and *H. seropedicae* (Bastián et al., 1998). The flavonoids, quercetin and diadzein, significantly increased the endophytic colonization ability of *Serratia* sp. than growth hormones. The induced colonization of *Serratia* sp. due to quercetin proportionally increased the *in planta* nitrogenase activity which reflected in the increased plant height, protein and chlorophyll contents of rice seedlings (Sandhiya et al., 2005). However, apart from the roots, the importance of the microbial production of phytohormones has been evaluated to be low, and the significance of these products for the plant has remained ambiguous (Zinniel et al., 2002). Therefore, endophytic function which is considered distinctively beneficial for the plant appears to be the protection of the host against pathogens.

Because not all endophytes are responsible of producing antagonistic substances, their role is yet to be discovered. Nevertheless, it appears that the function of an endophyte may be composed of several diverse factors that may together have a positive influence on the plant.

2.4 Colonization sites and infection pathways

According to Dobereiner (1997), endophytic diazotrophs, by inhabiting the interior of the plants, can avoid the competition with rhizospheric bacteria and derive nutrients directly from the host plants. In return, as the plant interior may provide an environment conducive to N_2-fixation by being low in O_2 and relatively high in carbon, the bacteria can fix N_2 more efficiently to the host (James and Olivares, 1998). The stele of plants has been considered to be colonized by pathogens only (Campbell and Greaves, 1990) or by saprophytes (Gagné et al., 1987). Vessels of non-diseased plants were thought to be sterile. This is not true for endophytic diazotrophs, as first shown *Azoarcus* sp. BH72 in Kallar grass and rice. It was demonstrated that the bacteria were present in vessels of roots in gnotobiotioc cultures by immunogold-labeling using genus-specific antibodies (Hurek et al., 1991; Hurek et al., 1994). Microscopical studies using immunological approaches and reporter genes have clearly shown similar colonization patterns for several nitrogen-fixing grass endophytes, such as *Azoarcus* sp. BH72 (Hurek et al., 1994), *H. seropedicae* (James and Olivares, 1998), *G. diazotrophicus* (Olivares et al., 1996) and certain strains of *Azospirillum* spp. (Schloter and Hartmann, 1998) (Figure 1).

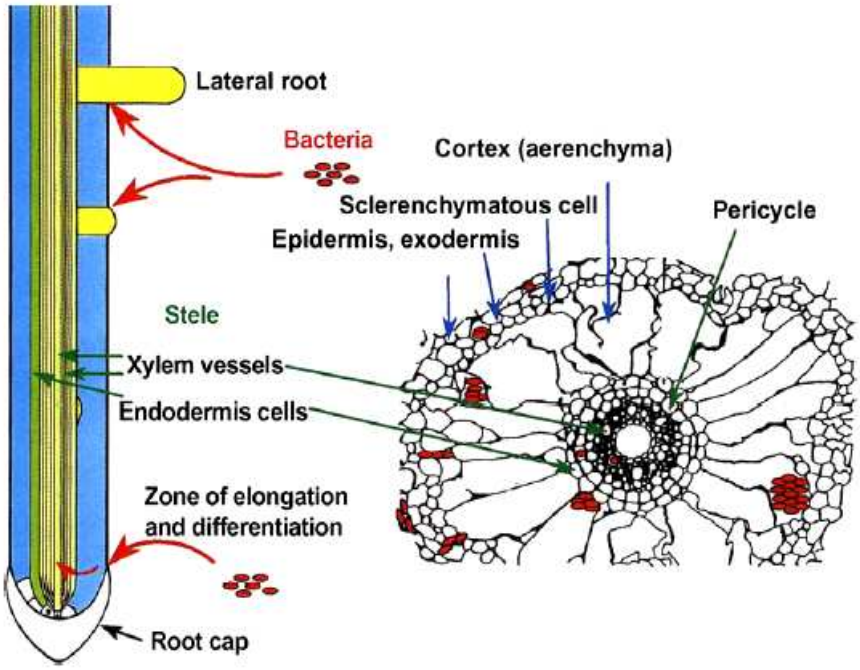


Figure 1. Possible sites of colonization and infection of diazotrophic endophytes in roots, shown in a sketch of a longitudinal (left) and a transversal (right) section of rice roots (Reinhold-Hurek and Hurek, 1998b).

In plants showing no symptoms of disease, *Azoarcus* sp. BH72 colonizes the original host Kallar grass and also rice seedlings in a similar way. Outer cell layers (exodermis, sclerenchyma) and the root cortex are colonized inter- and intracellularly within 2–3 weeks, the aerenchyma which forms in waterlogged plants being the main site for large microcolonies (Hurek et al., 1994; Egener et al., 1999). The mainly intercellular colonization pattern raises questions on the delivery of nutrients, especially carbon sources for the bacteria (Hurek et al., 1994). Rarely, the bacteria penetrate deeply into plant roots into the stele, where they may occur in the parenchyma and in xylem vessels. The detection of *Azoarcus* sp. in stelar parenchymatic cells of the culm and in vessels of Kallar grass and rice (Hurek et al.,

1991; Hurek et al., 1994) suggested that systemic spreading into shoots may be mediated through the transport in vessels (Figure 1). However, shoot colonization of *Gramineae* appears to be more obvious in *G. diazotrophicus* (James and Olivares, 1998) and *H. seropedicae* (Gyaneshwar et al., 2002).

Endophyte microorganisms differ remarkably from highly developed root nodule symbioses, in which rhizobia enter the plants through root hairs via infection threads. The infection of grasses by endophytes is similar to the crack entry. One site of primary colonization is the points of emergence of lateral roots, where bacterial microcolonies can readily be detected, and bacterial cells have been found between the cell layers of the lateral root and the cortex of the main root. Another route of entry is the root tip at the zone of elongation and differentiation. The bacteria can invade intercellular and intracellular and may penetrate into the central tissues (Hurek et al., 1991; Hurek et al., 1994). With the exception of *Azospirillum* spp. which is mainly regarded as a rhizoplane colonizer (Steenhoudt and Vanderleyden, 2000). The entry of bacteria into the root is most likely an active process, which might be mediated by enzymes degrading plant cell wall polymers. Two types of cellulolytic enzymes, cellobiohydrolase and β-glucosidase have been detected in *Azoarcus* sp. BH72 (Reinhold-Hurek and Hurek, 1998a). Further insights into the cellular machinery for plant invasion, and a comparison with pathogens and symbionts, will be fostered by the genome analysis.

2.5 Microbial community study

The microbial community in rice is inherently complex, and assessments performed with such a complex population do not always reveal its specific

components. Moreover, cultivation biased methods a limited because they do not assess the unculturable and unidentified fraction (Salles et al., 2002). In a recent study published by Minamisawa and colleges (2004) reported the existence of anaerobic nitrogen-fixing consortia (ANFICOs) consisting of N_2-fixing clostridia and diverse non-diazotrophic bacteria in many gramineous plants. Their work indicated that clostridia are naturally occurring endophytes in gramineous plants and that N_2 fixation by the clostridia arises in association with nondiazotrophic endophytes in culture. The detection of ANFICOs in plants indicates that clostridia should be candidates for diazotrophic endophytes in grasses and also demonstrates a new principle in environmental microbiology, namely that consortium of bacteria, rather than monocultures, may be responsible for a particular activity within a very complex environment. Seghers and colleages (2003) found a wide diversity of bacteria associated with the roots of *Z. mays* L. It was suggested that the bacteria associated with maize roots were a subset of the larger soil microbial community. This viewpoint postulates that a certain subpopulation of the wider soil community prospers in the root endophytic zone, suggesting that the root endophytic population composition is due to interactions of plant-specific and soil-specific factors. In a recent study of Seghers and colleges (2004) by accessed the influence of long-term applications (20 years) of herbicides and different fertilizer types on the endophytic community of maize plants grown in different field experiments. They results indicate that the effect of agrochemicals is not only limited to the bulk microbial community but also includes the root endophytic community. It is not clear if this effect is due to a direct effect on the root endophytic community or is due to changes in the bulk community, which are then reflected in the root endophytic community.

As well as Dalton and colleges (2004) suggested that respiratory activity by large population of non-diazotrophic bacteria within the cell wall of dune grasses (*Amophila arenaria* and *Elymus molis*) may create microaerobic condition favoring nitrogen fixation. However, they are not suitable for monitoring large numbers of sample e.g., for monitoring shifts in microbial communities during different stage of plant growth. Although such studies provided new insights into the genetic diversity of nitrogen-fixing bacteria, the cloning and sequencing strategies used were rather cumbersome and time-consuming. PCR has been adapted to the analysis of environmental DNA samples using denaturing gradient gel electrophoresis (DGGE). PCR-amplified fragment of *nifH* genes are separated by electrophoresis on the basis of nucleotide distribution, adapting DGGE to investigate DNA from samples, were able to observe different band patterns for DNA from different sites and DGGE can detect a 1% segment of the total community, including culturable and unculturable stage (Bowman and Sayler, 1996). This technique has been extensively used to examine the complexity and stability of the diazotroph assemblage found in the rhizosphere of smooth cordgrass *Spartina alterniflora* (Piceno et al., 1999). And sequence analysis of the DGGE bands has been used to determine phylogenetic relationships of the diazotrophic organisms represented (Lovell et al., 2000), and recovery and phylogenetic analysis of *nifH* sequences from diazotrophic bacteria associated with dead aboveground biomass of *Spatina alterniflora* (Lovell et al., 2001). PCR-DGGE has also been used to study the diversity of *nifH* gene sequences in *Paenibacillus azotofixans* and soil samples (Rosado et al., 1998).

2.6 *nifH* gene study

Nitrogen fixation is catalyzed by the enzyme nitrogenase complex. More than 20 genes have been identified as controlling the structure and function of the nitrogenase system, and much functional detail has been defined (Figure 2). The phylogenetic analysis of molecular sequences of *nifH*, which encodes the Fe protein component of nitrogenase are useful for identifying unknown diazotrophs. Dinitrogenase reductase gene sequences (*nifH*) have been amplified and sequenced from a number of environments, including rice roots, soil, oceans, and invertebrates such as zooplankton and termites.

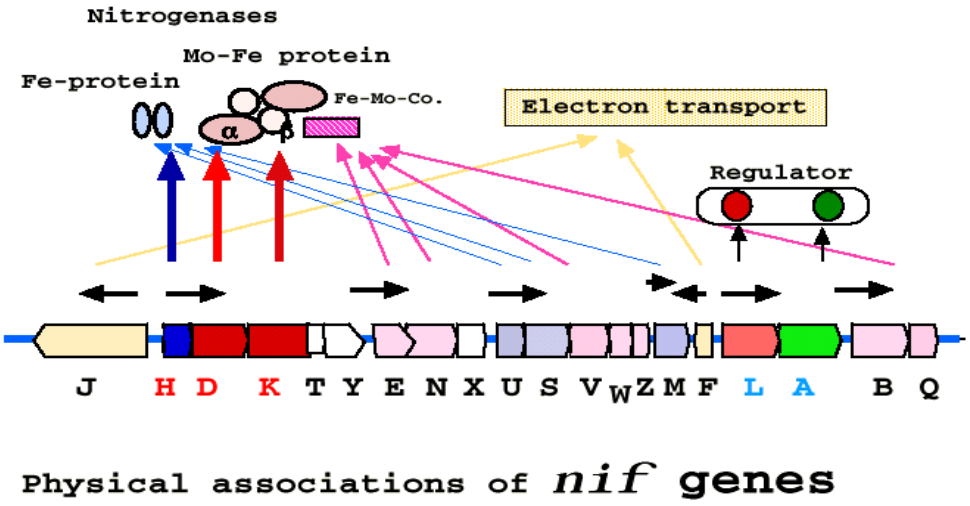


Figure 2. *nif* genes map in *Klebsiella pneumoneae* and their roles (www.asahi-net.or.jp).

A substantial molecular diversity of N_2 fixing bacteria has been detected in field grown rice based on retrieval of *nifH* gene fragments from root DNA (Ueda et al., 1995). Palus et al., (1996) used PCR amplification for the identification of an

endophytic diazotroph bacterial, *Klebsiella*, isolated from the stems of *Z. mays* L. by amplifying portions of *nifH* and 16SrRNA genes from this organism. Cabellero-Mellado and Martinoz-Romero (1994) used multilocus enzymes profiles, plasmid and *nif*HDK restriction enzyme patterns of isolates in Mexico and Brazil to determine that populations of *G. diazotrophicus* are clonal. Based on genomic fingerprinting using BOX, ERIC, and REP-PCR provide more evidence for the existence of greater genetic diversity in *G. diazotrophicus* isolates (Sevilla et al., 1998). Nitrogen fixation by diazotrophic bacteria is a significant source of new nitrogen in salt marsh ecosystems. Five hundred and twenty-one isolates cultivated from the rhizoplanes of salt marsh grasses like *Juncus roemarianus*, *Spartina patens* and *S. alterniflora* were screened for the presence of plasmids. Analysis of the RAPD-PCR patterns using *nifH* primer indicated as many different plasmid genotypes occurring in diazotroph bacterial assemblages within and between the four different salt marsh grass rhizoplane habitats investigated (Beeson et al., 2002). The diazotrophic endophyte of rice, *Serratia* sp. was marked with enhance green fluorescence protein (egfp)-Km marker gene by biparental mating and used for colonization studies in rice. The conjugants established themselves endophytically in rice root, stem and leaves, of which the stem was preferentially colonized (Sandhiya et al., 2005). The interactions between maize, sorghum, wheat and rice plants and *H. seropedicae* were also examined microscopically following inoculation with the H. seropedicae LR15 strain, a Nif+ (Pnif::gusA) mutant strain. The expression of nif genes occurred in roots, stems and leaves as detected by the GUS reporter system. Moreover, the colonization of plant tissue by *H. seropedicae* did not depend on the nitrogen-fixing ability.(Roncato-Maccari et al., 2003). To detect N_2-fixing bacteria in a plant without

using culture methods, *nifH* gene segments were amplified with degenerate primers from DNA extracted from stems of and leave of pineapple. Sequences of the *nifH* clones were homologous to those of bacteria in the genera *Bradyrhizobium, Serratia,* and *Klebsiella* but no *nifH* sequence related to *G. diazotrophicus,* which is an endophytic diazotroph and had been isolated from sugarcane, was detected in sugarcane. This indicates the absence or the presence of few *G. diazotrophicus* in the stems of the sugarcane plants used in the current study (Ando et al., 2005). To demonstrate the extent of phylogenetic diversity of diazotrophic bacteria associated with rice roots by characterized phylogenetically 23 *nifH* gene sequences obtained by PCR amplification of mixed organism DNA extracted directly from rice roots without culturing the organisms. The results shown that eight novel NifH types, which appear to be a variety of significant components of the diazotrophic community, dominated mainly by proteobacteria (Ueda et al., 1995).

However, the only presence of nitrogenase gene does not indicate that bacteria are actively fixing nitrogen (James, 2000). Although ^{15}N or acetylene reduction techniques are available for detecting nitrogen fixation activity, they involve incubation of samples, can have limited sensitivity, and do not provide information on which microorganism are actively fixing nitrogen. Culturing techniques have been used to determine the type of individual species presents, but these techniques yield biased results and a misrepresentation of the types of bacterial species that are active in the environment. The reverse transcriptase PCR (RT-PCR) makes it possible to assay for cells that are actively expressing specific gene at the time of sampling, and it has been used recently to detect *nifH* expression in the freshwater plankton (Zani et al., 2000), *A. vinelandii* in soil (Burgmann et al., 2003), *Azoarcus sp* BH72 in Kallar

grass and rice (Hurek et al., 2002), and *Herbaspirillum* sp. B501 associated in shoot (leaf and stem) of wild rice (You et al., 2005). Thus *nifH* gene has provided a strategy to address the diversity of nitrogen fixation genes in bacteria of interest.

The hypothesis of this study is endophytic diazotroph bacteria community might present their establishment within rice tissue in a symbiotic relationship as well as *nif* genes expression. Therefore, prior to apply appropriate endophytic diazotroph as inoculum, the native bacterial community structure would be determined. The diversity of endophytic bacteria in rice as elucidated both of diazotrophic and non-diazotrophic bacteria on the basis of DGGE technique using *nifH* and 16S rRNA genes. The actively fixing nitrogen was also determined by RT-PCR using *nifH* gene in conjunction with culturable-based approach.

2.7 Research objectives

1. To determine the bacterial community structure of endophytic diazotroph bacteria within each part and growing stage of rice
2. To determine the expression of *nifH* gene within each part and growing stage of rice

CHAPTER III

MATERIALS AND METHODS

3.1 Apparatuses

Autoclave:	Hiclave HA-3000MIV, Hirayama, Japan
Balance:	Precisa 205A, Precisa Instruments, Switzerland
	Precisa 3000C, Precisa Instruments, Switzerland
Compound microscope:	Olympus SZ2-LGCL, Olympus, Japan
	Olympus CX 31-RBSF, Olympus, Japan
Deep freezer -70 °C:	Heto, Ultra Freeze, Denmark.
DGGE machine:	BIO RAD DCode ^{TM} Universal Mutation Detection System
Freezer -20 °C:	Heto, HLLF 370, Denmark.
	MyBio LFT420, DAIREI, Denmark
Gas Chromatography:	AutoSystem XL Gas Chromatography, Perkin Elmer, USA
Gel Document set:	White/Ultraviolet Transilluminator GDS7500, UVP, USA
	Digital Graphic Printer UP-D890, Sony, Japan.
Gel electrophoresis apparatus:	MyRun Mini Gel Migration Trough, Cosmo Bio, Japan

Heat Box:	HB1, Wealtee Corp., USA
Incubator shaker:	C24 Incubator shaker, New Brunswick Scientific, USA
Incubator:	Memmert, BE 500, WTB Binder BD115, Shel-Lab 2020 Low Temperature Incubator, Sheidon, USA
Laminar hood	Holten LaminAir HBB 2448, Denmark. BH2000 Series ClassII BiologicalSafety Cabinets, BHA120 & BHA180, Clyde-Apac,
Microcentrifuge:	Hereaus, Labofuge 400R Eppendorf 54154, Eppendorf, Germany
Mortar and pestle	-
pH meter:	Hanna instruments 8519, Italy
Shaker:	Innova 2300 platform shaker, New Brunswick Scientific, UK Certomat TC2, B. Braun Biotech International, Germany
Stirrer:	Variomag Electronicrührer Poly 15, Germany Magnetic stirrer MSH300,USA
Thermocycler:	Px2 Themal Cycler, Thermo Electron Corporation, USA
Vacuum dryer pump:	Glaswerk, Werthem, GL 32
Vortex:	Vortex-Genie2 G506, Scientific Industries, USA

Water bath: SWB 5050, National Labnet Company,
Comfort Heto Master Shake, Heto-Holten,

3.2 Methods

3.2.1 Rice resource

The cultivated rice (*Oryza sativa* cultivar KDML105) seeds were surface sterilized with 70% ethanol for 1 min and shaken in 10% (w/v) NaOCl solution for 30 min. Seeds were then washed three times with sterilized distilled water with shaking (15 min each). Surface sterilized seeds were gnotobiotically germinated on wet tissue paper. After 7 days, rice seedlings were transferred into cement pots (diameter 120 cm, high 60 cm) containing 3 soil types in three replicate pots; Paddy soil mixed, paddy soil mixed add with chemical fertilizer, and undisturbed soil or forest soil. Paddy soil mixture was obtained from 7 sites in Nakhorn Ratchasima rice fields. Soil samples were analyzed by Department of Soil Science, Faculty of Agriculture, Kasetsart University, Thailand (soil characteristics are presented in Table 2). In paddy soil mixture with chemical fertilizer experiment, plants was fertilized according to local custom; Seven days after transplantation, 100 g pot^{-1} [N:P:K (16-20-0)] were added into the pots and after 15 and 50 days, 50-60 g pot^{-1} urea [N:P:K (46-0-0)] were applied. Plants were grown under outdoor conditions in cement pot and all experiments were watered twice a week. Plants were studied in 3 growing stages (seedling stage, vegetative stage, and reproductive stage). Three rice plants were sampled in each pot as random and experimented by three different tissue parts: root, stem and leaf.

Table 2. Chemical and physical properties of soils used for rice cultivation.

Original	pH	Soil Texture				Organic matter		Phosphorus		Potassium		Calcium		Magnesium	
		%Sand	%Silt	%Clay	Texture	%	Rate	ppm	rate	ppm	Rate	ppm	Rate	ppm	Rate
Paddy soil	7.8	65	16	19	SL	0.9	VL	39	H	70	L	3520	H	420	H
Forrest soil	7.7	51	14	35	SC	2	M	11	M	330	VH	7600	H	1180	H

Texture; SL = sandy loam soil, SC = sandy clay soil

Rate; VH = Very high; H = high; M = Medium; L = Low; VL = Very low

3.2.2 Morphological and biochemical characteristies of culturable endophytic diazotroph bacteria

For isolation and maintenance of endophytic diazotroph bacteria, a modified version of Rennies medium supplemented with malate (RMR medium) was used (Elbeltagy et al., 2001). RMR medium was prepared from solutions A and B. Solution A consisted of 0.8 g of K_2HPO_4, 0.2 g of KH_2PO_4, 0.1 g of NaCl, 28 mg of $Na_2FeEDTA$, 25 mg of $Na_2MoO_4 \cdot 2H_2O$, 100 mg of yeast extract, 3.0 g of mannitol, 5.0 g of sucrose, 0.5 ml of 60% (v/v) sodium lactate, 2.0 g of sodium malate, 2.0 g (for semisolid medium in test tubes) or 15 g (for agar medium for plates) of agar, and 900 ml of distilled water (the final pH of solution A was adjusted to 7.0 before autoclaving). Solution B was consisted of 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.06 g of $CaCl_2 \cdot 2H_2O$, and 100 ml of distilled water. The solutions were autoclaved separately and mixed after cooling. Filter-sterilized biotin and *para*-aminobenzoic acid (100 µl each) were added at final concentrations of 5 and 10 µg/l, respectively.

3.2.2.1 Surface sterilization

Freshly collected plants were carefully washed with tap water and separated into leaf, stem, and root parts. They were cut into sections about 7 cm long. The samples were rinsed with 70% ethanol, flame sterilized and cut at the end of section. To ensure the complete surface sterilization of the plant materials, several sterilization conditions: 1% NaOCl for 0.5 min for leaves, and 2% NaOCl for 15 min for stems and roots were conducted. After surface sterilization, the plant materials were washed 3 times with sterilized 0.1% Tween 80 and 2 times with sterilized distilled water. CaOCl-treated 5-cm-long sections of leaves, stems, or roots samples were rolled on Nutrient agar (NA) (Difco, Detroit, Mich.) plates. To ensure the sterilization efficiency, NA plates were aerobically incubated at 30°C for one week and no bacterial growth was observed and used for further study (Miyamoto et al., 2004).

The surface-sterilized plant materials were mechanically macerated with 0.8% saline solution and quartz sand and then decimally diluted in 0.8% saline solution. The dilutions were used to seed N-free RMR semisolid medium for determinations of the N_2-fixing bacteria. The tubes were monitored for growth in the form of subsurface pellicles 5 days after inoculation. Growth-positive tubes were tested for nitrogen fixation ability on the basis of acetylene reduction assay (ARA).

3.2.2.3 Acetylene Reduction Assay (ARA)

The nitrogen-fixing activity of the bacterial culture was examined by acetylene reduction assay in a 21-ml test tube containing 7 ml of RMR semisolid culture. Acetylene gas was injected into the head atmosphere of the test tubes at a final concentration of 5% (v/v) and incubated for 24 h at 30°C. Ethylene concentration was

assayed on a gas chromatograph machine with a flame ionization detector and PE-Alumina column equipped, 50m x 0.32mm x 0.25μm (Perkin Elmer, USA).

3.2.2.3 Bacterial morphology and biochemistry properties

The pellicles from acetylene reduction-positive tubes were streaked on RMR agar plates. The Gram reaction was performed according to Hucker's method (Doetsch, 1981).

The ability of the bacteria to grow on various carbon substrates was assayed in M70 minimal medium supplemented with 1% (wt/vol) of the appropriate carbon substrate (lactose, xylose, rhamnose, manitol, glycerol, glucose, sorbitol, myo-inositol, arabinose, tartaric acid and fumaric acid).

Antibiotic resistance profile was tested individually on RMR agar plates containing antibiotics at the following concentrations: kanamycin, chloramphenicol, tetracycline, streptomycin, ampicillin, spectinomycin, and erythromycin 25 and 50 μg ml^{-1}. Bacterial isolates were plated onto RMR agar with or without antibiotic supplementation. All of antibiotics were purchased from Sigma- Aldrich (USA). Bacteria were considered sensitive to an antibiotic at the concentration tested if no visible growth was observed on plates containing the antibiotic when there was visible growth on control plates after 3-5 d of incubation at 30°C.

3.2.2.4 IAA production Assay

Production of indole acetic acid was colorimetrically determined as described by Minamisawa et al., (1992). The isolates were grown in RMR medium supplemented with 10 mM NH_4Cl and 100 ug tryptophan ml^{-1} at 30°C with shaking

for 48 h in dark. IAA produced ml^{-1} culture was estimated by mixing 5 ml Salkowsky reagent (0.01M $FeCl_2$ in $HClO_4$) with 1 ml culture supernatant followed by visualize the color changes (Costacurta et al., 1998). Pure indole-3-acetic acid (Sigma, USA) was used as standard.

3.2.2.5 Plant polymer hydrolyzing activities

Cellulase and pectinase activities were assayed on the indicator plates. For the cellulase assay, NFb plates supplemented with 0.25% carboxymethyl cellulose (CMC) were spotted with bacterial cells. After incubating for 48 h at 30 °C the plates were overlayed with congo red (1 mg ml^{-1}) solution for 30 min. Congo red solution was then poured off followed by washing the surface of the plate with 1 M NaCl solution (Andro et al., 1984). For the assay of pectinase, bacterial isolates were spotted on nutrient agar supplemented with 0.5% pectin. After incubating the plates at 30 °C for 5 days the surface of the medium was overlayed with 2% hexadecyl trimethyl ammonium bromide (CTAB) solution for 30 min. CTAB solution was then poured off and the surface of the plate was washed with 1 M NaCl to visualize the halo zone around the bacterial growth (Mateos et al., 1992).

3.2.3 Mornitoring of localization endophytic diazotroph bacteria in rice tissue

3.2.3.1 Recombinant plasmid

Plasmid pBBR_*nif*HGUS was obtained from Prof. Y. Murooka (Department of Engineering, Osaka University, Japan) and the map was depicted in Fig. 3. This

7,600 bp plasmid encodes kanamycin resistance and a *gus* translational fusion of the *nif*H gene derived from *Mesorhizobium huakii*.

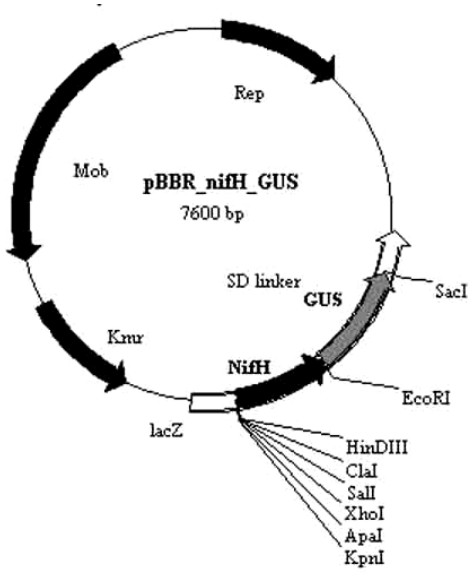


Figure 3. Diagram of recombinant plasmid pBBR_*nif*HGUS containing GUS gene, *nif*H promoter, Mob gene and Km^r gene.

3.2.3.2 Bacterial growth condition and plasmid

Escherichia coli DH5α donor strains (harboring plasmid pBBR_*nif*HGUS) and HB101 helper strains (haboring plasmid pRK2013 containing *tra* gene) which resistant to kanamycin were grown in Luria-Bertani (LB) broth contained the following components dissolved in 1 liter of water (pH 7.0): Tryptone, 10 g; yeast extract (Difco), 5 g; NaCl, 10 g containing kanamycin (50 μg ml^{-1}) at 37°C for overnight.

3.2.3.3 Triparental mating

Each of 1 ml of donor, helper and recipient mixture strain was pelleted by centrifugation at 3,000 rpm for 2 min. Cells mixtures were washed twice with RMR broth and resuspened in 0.5 ml in the same medium. Cell suspension from donor,

helper and recipient were mixed in 3:2:1 ratio, respectively and 100 µl of cell suspension were applied onto a membrane filter placed on RMR plate. The filters were then incubated at 28°C for 3 days. The cell slurry was scraped and transferred into a microcentrifuge tube before 1 ml RMR broth was added. The cell slurry was mixed with RMR and then 100 µl of cell suspension was plated on RMR agar containing 50 µg/ml of kanamycin, 50 µg ml^{-1} X-gluc and 50 µg ml^{-1} amplicillin. After incubation at 28°C for 5-7 days, blue forming colonies were selected as transconjugants.

3.2.3.4 Rice cultivation and inoculation with endophytic diazotroph bacteria

Seeds of cultivated rice were surface sterilized with 70% ethanol for 1 min and shaken in 10% (w/v) CaOCl solution for 30 min. Seeds were then washed three times with sterilized distilled water with shaking (15 min each). Surface sterilized seeds were gnotobiotically germinated on wet tissue paper. After 7 days, rice seedlings were transferred into the culture tubes containing 20 ml nitrogen-free medium. Nitrogen-free medium (Elbeltagy et al., 2001) was used for colonization and nitrogen fixation studies. The medium contained the following: 0.6 mM NaH_2PO_4. $2H_2O$, 0.3 mM K_2SO_4, 0.3 mM $CaCl_2.2H_2O$, 0.6 mM $MgCl_2.2H_2O$, and 0.045 mM FeEDTA as macroelements; 50 µM H_3BO_3, 9 µM $MnSO_4.5H_2O$, 0.3 µM $CuSO_4.5H_2O$, 0.7 µM $ZnSO_4.7H_2O$, and 0.1 µM $Na_2MoO_4.2H_2O$ as microelements; and 0.325% (w/v) agar. The pH was adjusted to 5.5. The macroelement and microelement solutions were sterilized separately and then poured into tubes before solidification. Three days after the transfer of seedling into the growth medium, Gus-tagged bacteria were inoculated

into the growth medium to a density of 10^5- 10^6 cell ml^{-1}. Roots, stems and leave of 5, 15 day old seedling were examined for bacterial colonization by GUS staining based histochemical staining.

3.2.3.5 Gus staining

For the detection of β-glucuronidase (GUS) expression of endophyte in plant tissue, the tissues were immersed in a microtiter plate containing the GUS assay solution (40 µl X-Gluc 20mg/ml in N, N-Dimethylformamide, 20 mg SDS, 2 ml methanol, 0.2 ml 1M sodium phosphate buffer and 7.76 ml distilled water), in vacuum for 120 min before incubated for overnight at 28°C. After the development of color, plant tissues were rinsed with phosphate buffer and immersed in commercial bleach for 30 sec to improve the contrast, followed by rinsing with water three to four times for 10 min each. The plant sample was then directly observed under a sterio microscope.

3.2.4 Analysis of community structure of rice endophytic bacteria

3.2.4.1 Extraction of endophytic bacterial DNA from rice tissue

Total DNA was extracted using a modified potassium acetate method (Dellaporta et al., 1983). The sterilized rice samples were homogenized in liquid N_2 and transferred into sterilized 1.5-ml tube containing 720 µl pre-heated extraction buffer (100 mM Tris-HCl, 50 mM EDTA, 500 mM NaCl and 1.25 % (w/v) SDS). The samples were mixed prior to incubated at 65 ºC for 15 min. Proteins were precipitated by adding 225 µl 5M potassium acetate and were incubated on ice for 20 min before decanting supernatant into new tube. The DNA precipitated by adding 2/3 volume

cold isopropanol and any remaining inhibitors were removed by reprecipitating the DNA with 300 µl 70% cold ethanol before resuspending it in 50 µl TE buffer and stored at -20ºC.

3.2.4.2 PCR amplification of 16S rRNA gene and *nifH* gene fragment

Amplification of 16S rRNA gene was performed using universal primers PBA338F (forward primer) and PRUN518R (reverse primer) and GC-clamps were added to the 5'end of PBA338F as summarized in table 3. Each PCR reaction contained 50 ng of DNA template, 0.5 µmol of each primer, 0.2 mM of dNTP, 10X PCR buffer, 0.1 mg/ml BSA, 3 mM $MgCl_2.2H_2O$ and 0.05 U Taq DNA polymerase (Promega, USA). The PCR reaction condition was used as follows; 94°C for 5 min 1 cycle, 94°C for 30 sec, 55°C for 45 sec, 72°C for 45 sec 35 cycle and final 73°C for 10 min 1 cycle. The PCR products were analyzed by electrophoresis in a 1 % (w/v) agarose gel in 1X TAE buffer. When necessary, products were stored at -20°C before DGGE analysis.

The *nifH* DNA sequences from divergent nitrogen-fixing microorganisms were amplified using a nested PCR to increase the sensitivity. The primers used were originally developed by Poly *et al*. (2001) and Roesch *et al*. (2006) (Table 3). The first PCR was performed with the forward primer PolF and the reverse primer PolR. The amplification product was ~317 bp. The second PCR was performed with the forward primer nifHFor containing the GC clamp and the reverse primer nifHRev. The amplification product was ~320 bp including the GC clamp sequence. The final PCR cocktails contained 5 µl of 10X PCR buffer (100 mM Tris-HCl, pH 9; 500 mM KCl),

1.5 mM $MgCl_2$, 0.5 mM each degenerated oligonucleotide primer, 200 mM each deoxynucleoside triphosphate, and 2.5 U of Taq DNA polymerase (Promega, USA). For the first PCR, 5 µl volume of the DNA was amplified. For the second PCR, 3 µl of the first PCR product was used as a template. Each mixture was adjusted to a final volume of 50 µl with sterilized water. The cycling conditions used are: 30 cycles consisting of denaturation at 94^{o}C for 45 sec, annealing for 45 sec at 55 ^{o}C for the first and at 48 ^{o}C for the second PCR, primer extension at 72 ^{o}C for 2 min, with a final extension at 72 ^{o}C for 5 min. The presence of PCR products as determined on 1% agarose gels, staining with ethidium bromide and comparison with a molecular weight marker. PCR products were stored at -20°C before DGGE analysis

Table 3. Summary of primers used in this study**.**

Primers		Sequences (5' to 3')	References
16S rRNA			
	PBA338F:	ACTCCTACGGGAGGCAGCAG	(Olivares et al.,
	PRUN518R	ATTACCGCGGCTGCTGG	1997)
	GC-clamp	GCGCCGCCGCGCGCGGCGGGCGGGGCGGGGG	
		CACGGGGGG	
nifH			
set 1	PolF	TGCGAYCCSAARGCBGACTC	(Poly et al.,
	PolR	ATSGCCATCATYNTCRCCGGA	2001)
Set 2	nifHFor	ACCCGCCTGATCCTGCACGCCAAGG	(Roesch et al.,
	nifHRev	ACGATGTAGATTTCCTGGGCCTTGTT	2006)
	GC-Clamp	CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCC	
		CCGCCCGACCCGCCTGATCCTGCACGCCAAGG	

R = A/G; N = A/G/C/T; H = T/C/A; Y = C/T; S = G/C.

3.2.4.3 Analysis of PCR Product by Denaturing Gradient Gel Electrophoresis (DGGE) analysis

All reagents were prepared as described in the Bio-Rad D Gene Instruction Manual and Applications Guide (Bio-Rad, Hercules, CA). DGGE gels were run using a BIO RAD DCode ^{TM} Universal Mutation Detection System (Bio-Rad, Hercules, CA). Three hundred µl of the PCR product were pooled, precipitated and resuspended in 30 µl of TE buffer. Before loading to the DGGE gel, the PCR products were incubated at 95°C for 5 min and gradually cooled to 4°C to avoid non-complementary annealing of DNA. Gels for DGGE were 10% polyacrylamide gel (40%-acrylamide and N, N-methylenebisacrylamide solution (37.5:1, v/v), 40% (v/v) formamide, 7 M urea and 1X TAE) containing a linear gradient of the denaturant concentration ranging from 40% to 60% with 1 mm thick,. The denaturing gradient gel was run for 300 min at 60°C and 120V. After completion of electrophoresis, the gels were stained in an ethidium bromide solution (0.5 µg/ml) and documented on Gel documentation and analysis (Ultra Violet Product, USA).

3.2.4.4 Sequencing from DGGE products

The interesting band of DNA was excised from DGGE gels and placed into sterilized vials. Twenty µl of sterilized distilled water were added to each of the vials, which were then kept at 4^{o}C overnight to allow the DNA to passively diffuse out from the gel strips. Two µl of eluted DNA were used as a DNA template along with each primer and PCR conditions as previously mentioned. The resulting DNA fragments were sequenced by Macrogen Corp., South Korea. In this study, to search for

homologous sequence in the data bank, Gene Bank (American), BlastN 2.0.13 was employed.

3.2.5 Detection of *nifH* gene expression by RT-PCR

3.2.5.1 RNA extraction

Total RNA were directly isolated from rice sample using RNeasy Plant Mini Kit (QIAGEN, USA) according to the manufacturers protocol. RNAs were treated with DNase to prevent contamination of genomic DNA and finally resuspended in diethylpyrocarbonate-treated water.

3.2.5.2 Reverse Transcription PCR (RT-PCR)

Reverse-transcription polymerase chain reaction (RT-PCR) was performed on RNA sample, using primers designed to target only *nifH* sequences. The total RNA was treated with 1 U of RNase free DNase (Promega, USA) and incubated at 37ºC for 30 min. One microliter of stop solution (20 mM EGTA [pH8.0] at 25ºC) was added and incubated at 70ºC for 15 min. Two step RT-PCR was chosen for this study. The target RNA was combined with either 25 ng/µl of Oligo$(dT)_{15}$ primer or 2.5 ng/µl of random hexamers. The combined RNA was preheated at 70ºC for 5 min and kept on ice until the reverse transcription reaction was added. Reverse transcription reaction mixture containing 4 µl of ImProm-II™ 5X reaction buffer, 3 mM $MgCl_2$, 0.67 mM dNTP mix, 20 U of ribonuclease inhibitor, 1 µl of ImProm-II™ reverse transcriptase (Promega, USA) was adjusted with nuclease-free water to 15 µl. Five microliters of RNA and primer mix were added into reverse transcription reaction mixture, giving a

final reaction volume of 20 µl. The solution was annealed at 25ºC for 5 min, extended at 42ºC for 60 min and inactivated reverse transcriptase at 70ºC for 15 min. Two microliters of the cDNA were amplified by PCR in same condition as DNA amplification. The 16S rRNA gene was used as a standard to calibrate the amount of RNA. All RT-PCR were performed in Thermal cycler either GeneAmp®PCR System 9700 (Perkin Elmer, USA) and GeneAmp® PCR System 2400 (Perkin Elmer, USA) and products were visualized using 1% agarose gel electrophoresis and stained with 0.5 µg/ml of ethidium bromide, then documented on Gel documentation and analysis (Ultra Violet Product, USA).

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Isolation and characterization of endophytic bacteria from rice

The culturable endophytic diazotroph bacteria were isolated from rice using Rennie Modified Rice (RMR) medium. The different growth patterns of various single isolates in RMR semi-solid medium was shown in Figure 4, culture tubes showed evidence of bacterial growth as turbid growth throughout the tube, a pellicle or subpellicle floats on the top of the tubes of semi-solid medium.

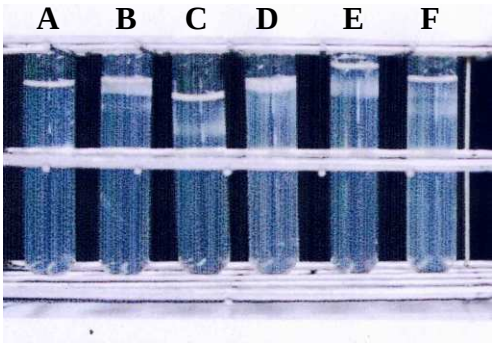


Figure 4. Growth Patterns in RMR semi-solid Medium: A and B, pellicle float; C, E and F, subpellicle float; D, turbidity.

Many researches have isolated diazotrophic bacteria from gramineous plants using nitrogen-free media such as Burk or Nitrogen-free (NFb) media (Baldani et al., 2000; Dalton et al., 2004). Rennie medium was proposed as a more efficient nitrogen-

free medium for isolation of nitrogen-fixing bacteria (Rennie, 1981). Dobereiner (1995) reported that the use of semi-solid N-free media was useful for isolating nitrogen-fixing endophytes because bacteria will form a subsurface pellicle depending on their oxygen requirements. Thus, use of a modified Rennie medium in this study probably enabled us to isolate appropriately endophytic diazotroph bacteria.

Table 4. Quantification of endophytic bacteria isolated from fresh rice tissue.

	CFU g^{-1}		**CFU g^{-1}**		**CFU g^{-1}**
Sample	**fresh weight**	**Sample**	**fresh weight**	**Sample**	**fresh weight**
Root					
SFR	1.09×10^6	VFR	5.10×10^5	RFR	2.65×10^5
SNR	2.25×10^6	VNR	3.51×10^6	RNR	1.81×10^5
SUR	1.11×10^6	VUR	1.42×10^6	RUR	1.27×10^6
Stem					
SFS	1.02×10^5	VFS	1.01×10^4	RFS	2.11×10^4
SNS	3.03×10^5	VNS	1.39×10^4	RNS	1.51×10^5
SUS	2.28×10^5	VUS	3.71×10^3	RUS	2.43×10^4
Leaf					
SFL	2.40×10^3	VFL	2.10×10^3	RFL	1.66×10^3
SNL	2.72×10^4	VNL	3.78×10^3	RNL	2.58×10^3
SUL	6.06×10^3	VUL	1.28×10^3	RUL	2.62×10^3

Abbreviations, First letter is growing stage of rice (S; seedling stage, V; vegetative stage, R; reproductive stage), second letter is soil type (F; fertilizer amendment, N; no-fertilizer experiment, U; undisturbed experiment), and third letter is rice part (R; root, S; stem, L; leaf)

The population size of the endophyte was detected in the range of 10^3 to 10^6 CFU g^{-1} of rice tissue (fresh weight) in surface sterilized leaves, stems, and roots of rice (Table 4). Irrespective of growing stage of rice, and soil type, the majority of endophytic bacteria were recovered from root (2×10^5 - 3×10^6 CFU g^{-1} fresh weigh) as compared to other part of rice plant. While endophytic bacteriapopulation number in stem was 4×10^3 - 3×10^5 CFU g^{-1} fresh weigh. The lowest population density occurred in the leaf with 1×10^3 - 2.72×10^4 fresh weigh. This suggests the most densely colonized of endophytic bacteria were recovered from a sterilized sample of root.

Sterilization by treating with ethanol, NaOCl and mild flaming was effective in removing most of the microbes from the plant surfaces. Even the conditions used for the surface sterilization by NaOCl were effective enough to completely avoid contamination by epiphytic bacteria. Furthermore, they may have underestimated the population levels of endophytic because NaOCl is able to penetrate the plant tissues and cause partial sterilization of endophytic (Miyamoto et al., 2004). Significant variations in the populations of both native and introduced endophytes have been reported. These variations are attributed to plant source, plant age, tissue type, time of sampling, and environment.

Similar results were obtained by Zinniel and team (2002) generally, bacterial populations are larger in roots and decrease in the stems and leaves. The number of colonies of both *G. diazotrophicus* and *Acetobacter peroxydans*, isolated from the surface sterilized roots and stems of rice varieties cultivated in flooded fields in South India, was in the range of 10^2–10^3 CFU g^{-1} fresh weight of rice (Muthukumarasamy et al., 2005). A similar study in South Korea indicates that *G. diazotrophicus* is naturally

associated with South Korean rice variety Hwsanbyeo in low numbers (10^4 CFU g^{-1} fresh tissue) (Muthukumarasamy et al., 2007). Natural endophyte concentrations can vary between 10^2 to 10^6 CFU g^{-1} for alfalfa, sweet corn, sugar beet, squash, cotton, and potato (Kobayashi and Palumbo, 2000). Similar results were obtained for endophytic bacteria inoculated by root or seed drenching, with the population levels reaching between 10^3 to 10^5 CFU g^{-1} of plant tissue for tomato and potato (Kobayashi and Palumbo, 2000). The levels of colonization by nonpathogenic endophytes tend to be far less than the levels of colonization by pathogenic bacteria; the concentrations of the latter organisms range from 10^7 to 10^{10} CFU g^{-1} fresh weight of tissue in susceptible infected plants (Zinniel et al., 2002).

4.2 Nitrogen fixing ability of rice endophytic strains

The nitrogen fixing ability of bacterial isolates grown in tubes containing RMR semisolid medium was assessed on the basis acetylene reduction assay (ARA). They consistently showed ARA in the medium during isolation steps (Table 5).

Though the ability to reduce acetylene is an indirect measure of N_2-fixation, it is specific for monitoring functional nitrogenase activity, and is indicative of N_2-fixing potential (Andrade et al., 1997). Hence, for further identification, screening and selection of prospective strains, ARA was used as a test for diazotrophy. Out of the 135 isolates from the primary selection process, only 75 isolates with an ARA activity of above 10 nmol C_2H_4 $h^{-1}tube^{-1}$ were qualified further (Table 5). Sixthy isolates were showed negative ARA, so in culturable isolates have both of diazotrophic and nondiazotrophic strain.

Table 5. Example of N_2-fixing activity of endophytic cultured (Consortium culture) in RMR semi-solid medium.

Seedling stage	N_2-fixing Activity	Vegetative stage	N_2-fixing Activity	Reproductive stage	N_2-fixing Activity
Root					
SFR5-1	527.44	VFR6-3	4,270.64	RFR5-2	1,276.70
SFR4-2	290.37	VFR5-3	3,859.50	RNR3-1	529.31
SFR2-3	254.78	VFR2-2	2,657.38	RNR4-2	43.4
SFR3-2	162.8	VFR3-3	1,842.81	RUR4-2	557.97
SNR2-1	936.84	VFR3-1	565.7	RUR2-2	328.93
SNR2-2	585.34	VNR3-3	355.84		
SNR4-3	287.9	VNR3-2	316.52		
SNR5-2	245.16	VNR6-1	132.13		
SNR5-3	142.85	VNR5-1	56.89		
SUR5-3	2,136.40	VNR4-1	53.45		
SUR6-2	1,662.14	VUR6-3	1,436.29		
SUR2-3	644.32	VUR4-1	1,069.52		
SUR3-2	487.24	VUR4-3	937.09		
SUR2-1	481.58	VUR3-3	635.04		
SUR5-1	149.2	VUR3-1	458.86		
Stem					
SFS3-2	3,817.33	VFS2-3	3,583.45	RFS3-3	991.75
SFS2-2	2,201.67	VFS4-1	295.24	RNS2-3	911.3
SNS3-1	5,840.07	VFS2-1	181.24		
SNS2-3	3,735.68	VNS3-3	926.84		
SUS2-1	2,905.17	VNS3-2	759.22		
SUS2-2	217.32				
Leave					
		VFL2-1	315.12	RUL2-2	949.24
		VUL3-1	817.25		

N_2-fixing activity = Ethylene concentration (nmol C_2H_4 $h^{-1}tube^{-1}$)

Abbreviations, First letter is growing stage of rice (S; seedling stage, V; vegetative stage, R; reproductive stage), second letter is soil type (F; fertilizer amendment, N; no-fertilizer experiment, U; undisturbed experiment), and third letter is rice part (R; root, S; stem, L; leaf)

N_2-fixing activity during isolation steps showing the capability of consortia, they showed N_2-fixing activity in coculture from each part of rice. Root consortium were showed highest number of samples and showed nitrogenase activity in the range between 40 to 4,000 nmol C_2H_4 $h^{-1}tube^{-1}$, following was the stem consortium (180–5,800 nmol C_2H_4 $h^{-1}tube^{-1}$) and leaf consortium (300– 900 nmol C_2H_4 $h^{-1}tube^{-1}$), respectly. Among the 75 isolates, the isolate SNS3-1 which was isolated from seedling stage growing in no-fertilizer soil on stem part was exhibited highest nitrogenase activity (5,840.07 nmol C_2H_4 $h^{-1}tube^{-1}$). However, the lowest number of ARA activity occurred in the leaf consortium which also showed weak activities of N_2-fixation, suggesting that rice leaf are not as much of N_2-fixing potential. This result correlated with the population size of the rice endophytic bacteria (Table 4). It is not surprising that endophytic bacteria reside in the leaf or aerial parts of rice tissues, which are exposed to the air and to O_2 produced by photosynthesis. The plant-inhabiting endophytic bacteria probably sometimes proliferate in anoxic microzones produced by consortium or plant respiration, while they under higher O_2 concentrations (Minamisawa et al., 2004).

N_2-fixing ability was re-measured using subculture from the two different colonies of the first positive ARA tubes that showed either positive or negative interaction (Table 6). The mixed or consortium cultures always exhibited the capability for N_2-fixation. The single isolate in consortium culture of the SUR2-3 expressed no N_2- fixing activity. This suggested the presence of nondiazotroph culture facilitated N_2–fixation activity in consortium culture. Similar results were obtained by Minamisawa (2004), the existence of anaerobic nitrogen-fixing consortia (ANFICOs) consisting of N_2-fixing clostridia and diverse nondiazotrophic bacteria in

nonleguminous plants. They found that a few ANFICOs, nondiazotrophic bacteria specifically induced nitrogen fixation of the clostridia in mixed culture.

Table 6. N_2-fixing activity of endophytic cultured (Consortium culture and Single culture) in RMR semi-solid medium.

Sample	**N_2-fixing Activity (nmol C_2H_4 $h^{-1}tube^{-1}$)**		
	Consortium culture	**Single isolate (1)**	**Single isolate (2)**
Root			
SUR2-3	644.32	-	72.05
VFR3-1	565.70	180.04	7,966.40
Stem			
SFS2-2	2,201.67	1,738.50	7,395.70
SNS3-1	5,840.70	18.34	23,319.17
SNS2-3	3,735.68	33,184.27	19,735.12
Leave			
RUL2-2	949.24	6,123.88	6,355.32

N_2-fixing activity = Ethylene concentration (nmol C_2H_4 $h^{-1}tube^{-1}$)

Abbreviations, First letter is growing stage of rice (S; seedling stage, V; vegetative stage, R; reproductive stage), second letter is soil type (F; fertilizer amendment, N; no-fertilizer experiment, U; undisturbed experiment), and third letter is rice part (R; root, S; stem, L; leaf)

The N_2-fixing activities of other single cultures occurred in higher than those of the original combinations (VFR3-1, SFS2-2, SNA3-1, SNS2-3, and RUL2-2). This suggests the presence of the accompanying bacteria produced specific metabolites of consortium that induced/reduced the N_2-fixation as well as association of

nondiazotrophic endophytes in culture. One of the most widely used methods to determine the susceptibility of microorganisms (single isolate (1)) to antimicrobial agents of other strain (single isolate (2)) is the culture filtrate. The inhibition of cell growth was shown by formation of a clear zone around the spotted culture filtrate (Figure 5).

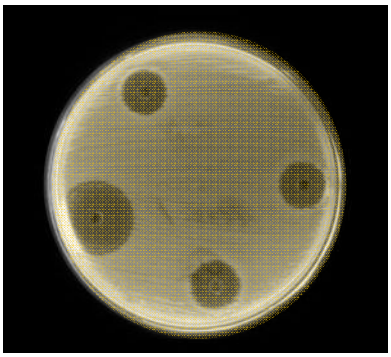


Figure 5. Antimicrobial activities of culture filtrate of strain VFR3-1 corresponding to bacterial growth as show as the inhibition zone in bacterial layer.

Single culture (2) of VFR3-1 consortium exhibited clear zone on the surface of single culture (1). This result suggests that one single isolate affected to other single isolate in the same consortium by producing agent that can kill the bacteria (bactericidal effect). However, SFS2-2, SNA3-1, SNS2-3, and RUL2-2 consortia do not show any clear zone around the spotted culture filtrate of each single isolate. Perhaps one single isolate of this consortium produced agents that can inhibit only N_2-fixing activity (bacteriostatic effect).

Similarly, a major feature of ANFICOs is that N_2- fixation by the anaerobic clostridia is supported by the consumption of oxygen by the accompanying bacteria in the culture (Minamisawa et al., 2004). The reason for this is unclear but it is possible that the N_2-fixing ability of endophyte is localized on extrachromosomal elements

present. Such extrachromosomal nitrogenase has been detected in other enteric bacteria such as *Enterobacter agglomerans* (Singh et al., 1983) and *Rahnella aquatilis* (Berge et al., 1990).

Some interested isolates were identified at species level based on the full sequence analysis of 16S rRNA gene (Table 7).

Table 7. 16S rRNA gene full length sequence analysis of some culturable N_2-fixing endophytic bacteria in rice.

Isolate Sample	**N_2-fixing activity (nmol C_2H_4 $h^{-1}tube^{-1}$)**	**% Similarity**
SFS2-2(1)	1,738.50	99% *Enterobacter dissolvens* LMG 2683
SFS2-2(2)	7,395.70	98% *Brevundimonas aurantiaca*
VNS3-1(1)	18.34	99% *Pantoea agglomerans* WAB1927
VNS3-1(2)	23,319.17	99% *Enterobacter dissolvens* LMG 2683
RUL2-2(1)	6,123.88	99% *Pseudomonas sp.* BWDY-42
RUL2-2(2)	6,355.38	99% *Enterobacteriaceae bacterium* ZHS050721

The results showed that the diazotrophic isolate SFS2-2(1) had highest similarity of 99% to a type strain of *Enterobacter dissolvens* LMG 2683. The name *E. cloacae* subsp. *dissolvens* comb. nov. is proposed for the group of bacteria formerly referred to as *E. dissolvens* which has high similarity to SFS2-2(1) (Hoffmann et al., 2005). This bacterial strain was characterized as endophytic strain from citrus plant (Araujo et al., 2002) and rice (Fujii et al., 1987). SFS2-2(2) has high similarity to *Brevundimonas aurantiaca* which was isolated and characterized as nitrogen-fixing

root nodule bacteria which collected from endemic tree species of *Dalbergia* in Madagascar (Rasolomampianina et al., 2005). VNS3-1(1) has high similarity *Pantoea agglomerans* which is a member of Enterobacteriaceae family that has been isolated endophytically and epiphytically from a wide variety of crops. Ruppel et al., (1992) observed that *P. agglomerans* colonized the intercellular spaces in root cortex of corn and stem mesophyll of wheat. Asis and Adachi (2004) found the stem of sweet potato cv. Koganesengan was colonized by diazotrophic endophyte *P. agglomerans*. This strain was also isolated from sugarcane stem tissue growing in Cuba without chemical fertilizers application (Loiret et al., 2004). *Pseudomonas sp*, was isolated and used as biocontrol strains for Tomato foot and root rot (TFRR) in stonewool (Roper and Ladha, 1995a). Dalton et al., (2004) presented evidence that sand dune grasses (*Ammophila arenaria* and *Elymus mollis*) from Oregon also contain non-diazotrophic pseudomonads, with authentic diazotrophs probably comprising a minor proportion of the population. The evidence for N_2 fixing and *nif* gene diversity in *Pseudomonas sp* from rice fields along the Yangtze River Plain was also discovered (Xie et al., 2006).

4.3 Biochemical characterization

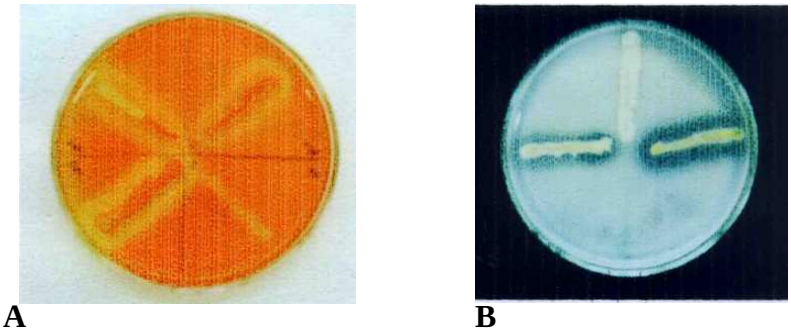
4.3.1 Carbon source utilization

Culturable endophytic diazotroph bacteria were determined on the basis of various carbon sources utilization (Appendix III). Out of 14 carbon substrates tested, all 51 endophytic diazotroph isolates could utilize arabinose and glucose as sole carbon sources. Suggesting that arabinose and glucose were prefered as carbon source for rice endophytic diazotroph bacteria while rhamnose, sorbitol, mannose, and tartaric acid were not favored (80.39, 74.51, 80.39, and 76.47% utilization,

respectively). Different results were found by Muthukumarasamy and colleages (2007), diazotrophic bacteria from Korean wetland rice were high in numbers when malate and azelaic acids were used as carbon source, but less when sucrose was used as a carbon substrate. Given such characteristics, some strains which could multiply both in sugars (sucrose, glucose, etc.) as energy sources, those are present abundantly in root environment as exudates, will aid the biofertilizer research (Muthukumarasamy et al., 2002). These nutritional traits indicated that it is seemingly suitable to bacterial life in plants (Minamisawa et al., 2004). However, arabinose, rhamnose, and tartaric acid were not favoured for their growth. Arabinose can be efficiently used as carbon sources by *G. diazotrophicus* but not by *G. johannae* and *G. azotocaptans* (Muthukumarasamy et al., 2002). *Methylobacterium populi*, a methane-utilizing bacterium isolated from poplar trees also could not utilize Arabinose (van Aken et al., 2004). But a diazotrophic bacterial endophyte isolated from stems of *Zea mays* L. and *Zea luxurians* were capable of utilizing arabinose and rhamnose (Palus et al., 1996). Diazotrophs from Korean rice varieties used arabinose as a carbon source while rhamnose and tartaric acid did not, but less when sucrose was used as a carbon substrate (Muthukumarasamy et al., 2007) like *Pseudomonas spp.* (Vlassak et al., 1992).

4.3.2 Plant polymer hydrolytic enzyme production

The production of pectinase and cellulase from cultirable endophytic diazotroph bacteria were determined (Figure 6).



A **B**

Figure 6. Production of cellulase (A) and pectinase (B) by rice endophytic diazotroph bacteria.

Out of 51 endophytic diazotroph isolates in this study, 60.8% showed pectinase and 96.08% showed cellulase activities (Appendix III). Figure 6 demonstrates the production of cellulase and pectinase of some endophytic strains. The plant polymer hydrolyzing enzymes were significantly presented in the most of endophytic strains. Pectinase is a key enzyme for colonization which degrades pectic substances present in the plant cell wall. Most endophytic strains also showed evident pectinase and cellulase activity, indicating the endophytic nature of these isolates. Plant cell walls consist mainly of cellulose, whereas middle lamella, which connects the cells, consists mainly of pectin. Pectinolytic activity has been proposed to be responsible for invasion of *Azospirillum* spp. in roots by the penetration of middle lamella and points of emergence of lateral roots (Bekri et al., 1999). Although pectinases might play an important role in plant–microbe interactions and intercellular colonization of roots they have not yet been studied in the endophytic diazotrophs (Reinhold-Hurek and Hurek, 1998a). Besides gaining entry into the plant through natural openings and wounds, endophytic bacteria actively go through plant tissues using hydrolytic enzymes like cellulase and pectinase. Since these enzymes are also

produced by pathogens, more knowledge on their regulation and expression is needed to distinguish endophytic bacteria from plant pathogens.

4.3.3 Indole acetic acid production from the rice endophytic bacteria

Out of 51 endophyic diazotroph isolates, 32 isolates (62.75%) in the minimal medium supplemented with tryptophan as precursor produced significant amounts of IAA (Figure 7 and Table 8) such as SUR5-3, SNR2-1, and VNS3-3.

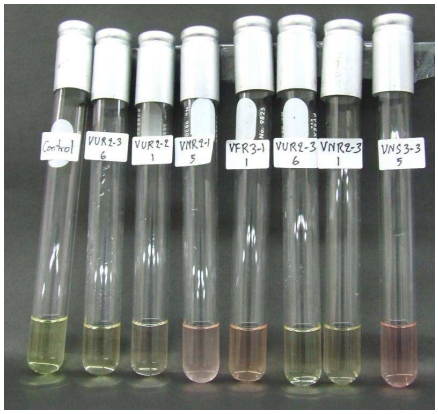


Figure 7. Indole Acetic Acid (IAA) production by rice endophytic bacteria.

Different strains of *E. cloacae*, *E. agglomerans*, and *P. agglomerans* have been shown to convert tryptophan to indole acetic acid (Costacurta et al., 1998). The capacity to synthesize IAA is widespread among soil and plant-associated bacteria. The ability to synthesize IAA helps in plant pathogenesis by *Pseudomonas syringae*, *Agrobacterium tumefaciens* and *A. rhizogenes* (Verma et al., 2001). It is also considered responsible for plant growth promotion by beneficial bacteria such as *Azospirillum* spp., *Alcaligenes faecalis, Klebsiella, E. cloacae, G. diazotrophicus, H. seropedicae,* symbiotic *Rhizobium* and *Bradyrhizobium* (Costacurta et al., 1998). The

plant growth promoting effect is brought about by causing root elongation and proliferation which leads to enhanced water and mineral uptake by the host plant. Some of strains highly produced cellulase, pectinase, and IAA were identified at species level based on the full sequence analysis of 16S rRNA gene as summerized in Table 8.

Table 8. Biochemical characteristics and 16S rRNA full length sequence analysis of isolates containing highest N_2-fixing activity.

Sample	N_2-fixing activity (nmole/tube/h)	IAA	Cellulase	Pectinase	Antibiotic resistant	% Similarity
VFR5-3	3,859.5	+++	++++	+	Amp	99% *Rheinheimera* sp. J3-AN42
SUR5-3	2,136.4	++++	+	+	Amp	98% *Brevundimonas* sp. FWC30
SNR2-1	936.8	++++	++++	+	Amp	99% *Citrobacter freundii* strain 11
RNS2-3	911.3	++	+	++++	Amp	99% *Citrobacter freundii* SSCT56
VNS3-3	926.8	++++	+	+	Km	99% *Pseudomonas mendocina*

++++, very good growth; +++, good growth; ++, normal growth; +, slight growth

VFR5-3 has 99% similarity to *Rheinheimera* sp. J3-AN42, *Rheinheimera* is a recently named bacterial genus, and *R. baltica* is a novel, blue pigmented bacterium that was isolated from the Baltic Sea (Brettar et al., 2002) and the deep Pacific Ocean (Romanenko et al., 2003). The other strains of *Rheinheimera* have been reported as bacteria associated with spores of the arbuscular mycorrhizal fungi (Roesti et al., 2005) and root-associated of the tomato plant (Kim et al., 2006) but never been reported as diazotrophic strains. *Brevundimonas* sp., a potato-associated bacteria showed a production of indole-1,3-acetic acid (IAA) (Sessitsch et al., 2004). *Brevundimonas* sp. and *Pseudomonas* sp. have been reported as bacterial endophytes in processing carrots (*Daucus carota* L. var. sativus) and effected on plant growth

(Surette et al., 2003). *P. mendocina* strains produced enzymes, phytohormone auxin (IAA), and were antagonist against plant pathogenic fungi in in vitro experiments (Egamberdiyeva, 2005). Some of the *Pseudomonas* sp. have been found to increase the growth of cotton and sweet corn (McInroy and Kloepper, 1995). *Citrobacter freundii* was reported as a N_2-fixing bacteria have been isolated from the guts of several termites (Lilburn et al., 2001), fruit fly *Ceratitis capitata* (Behar et al., 2005) but never been reported as endophytic bacteria.

4.4 Monitoring of endophytic diazotroph bacteria

Since isolate VFR5-3 was the most dominant type among the isolates which showed the ability to fix nitrogen, produce indole acetic acid and moderate levels of cellulase and pectinase which might be involved in the spreading throughout rice tissues. Hence, we decided to use it for further studies on the colonization of rice. The localization of endophytic diazotroph bacteria in plant tissue could be detected by virtue of GUS activity which forming a blue colony and use as transcojugant for inoculated to rice seedling under gnotobiotic conditions. Thus, GUS-marked endophyte in plant tissue could be detected (Figure 8, and 9).

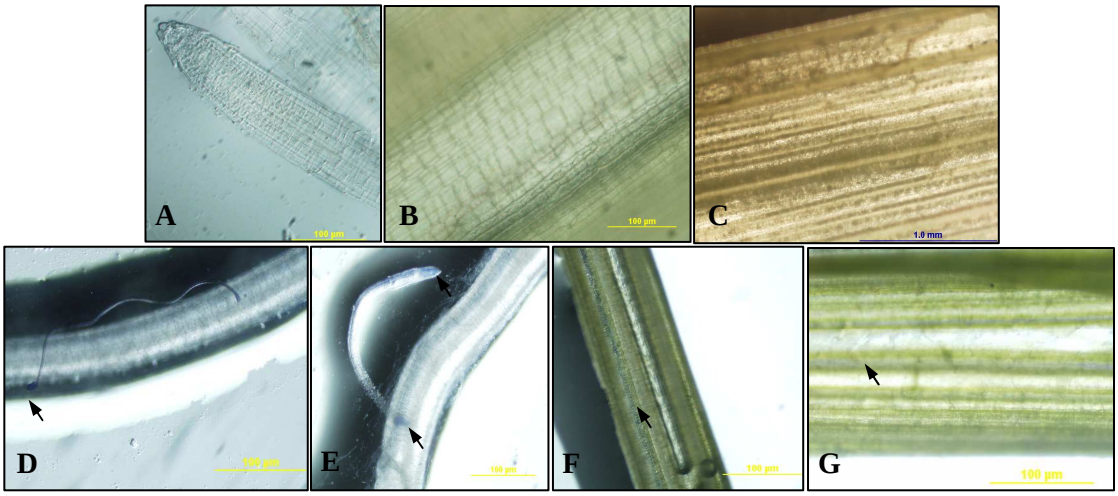


Figure 8. Light micrographs of GUS-stained roots, stems, and leaves of rice at 5 days after inoculation with strain VFR5-3 marked with GUS. Control uninoculated plants; root (A), stem (B) and leaf (C). GUS activity was observed on roots with the most intense color development on the lateral roots (D) and root junction (E). GUS staining was also observed on rice stems (F), and leaf (G).

At 5 days after inoculation (DAI), uninoculated control plants did not show any blue coloration with X-Gluc (Figure 8A, B, and C). The localization of *nifH* gene occurred in roots, stems and leaves as detected by the GUS reporter system (Figure 8D, E, F, and G). The results of 15 DAI were similar to that of 5 DAI (Figure 9).

Staining of the whole roots showed that the GUS activity was most intense on some of the younger lateral roots, and it is possible that the bacteria entered these at their junctions with the primary roots. This type of infection has also been observed with other diazotrophic endophytes, such as *G. diazotrophicus* in sugarcane (James and Olivares, 1998), *Azoarcus* spp. in rice and Kallar grass (Hurek et al., 1991), and *Herbaspirillum* spp. in sugarcane and rice (James and Olivares, 1998; James, 2000;

James et al., 2002). James and Olivares (1998) have suggested that stem could be a suitable niche for N_2-fixation because it can provide the low pO_2 required for the expression and function of nitrogenase.

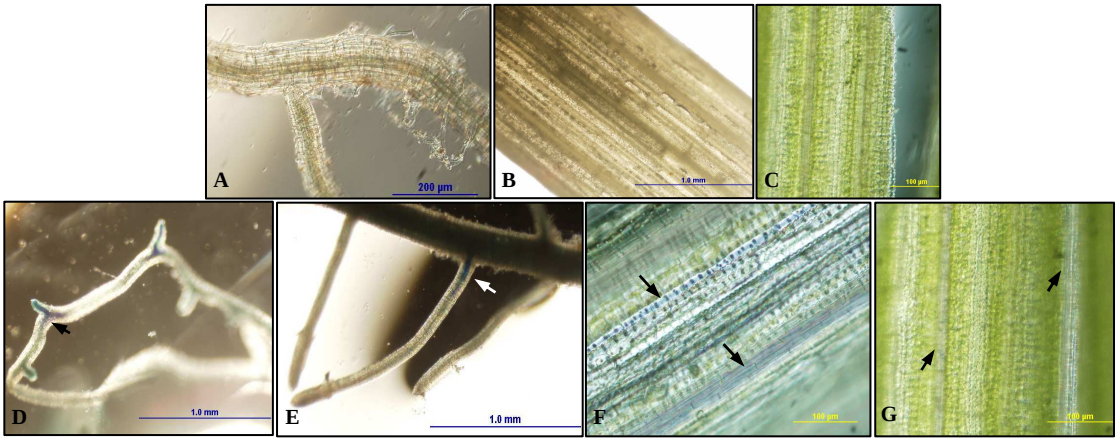


Figure 9. Light micrographs of rice roots, stems, and leaves at 15 days after inoculation with strain VFR5-3 marked with GUS. Control uninoculated plants; root (A), stem (B) and leaf (C). GUS activity was observed on roots with the most intense color development on the lateral roots (D) and root junction (E). GUS staining was also observed on rice stems (F), and leaf (G).

Strain VFR5-3 has 99% similarity to *Rheinheimera* sp., proteobacteria, gram negative and rod shape. Although this is the first report of endophytic diazotrophy by *Rheinheimera* sp. as detected by the GUS reporter system. The only other report of diazotrophy in the genus *Rheinheimera* is that of an *R. baltica* that was associated with spores of the arbuscular mycorrhizal fungi (Roesti et al., 2005) and root-associated of the tomato plant (Kim et al., 2006). A major drawback of in situ GUS

staining is that the presence of blue color does not unequivocally confirm the location, or even the presence, of the GUS-labeled bacteria because the color can diffuse into bacterium-free plant material (Hurek et al., 1994; Reinhold-Hurek and Hurek, 1998a). Therefore, to prove that bacteria is genuinely endophytic in rice, tissues stained for GUS activity were fixed with glutaraldehyde, embedded in resin, and sectioned for optical and microscopy. This also reduced the possibility that the bacteria observed within the plants had accidentally moved into them from the external population (Reinhold-Hurek and Hurek, 1998a; James, 2000). In contrast to the root, the stems and leaves showed little sign of degradation associated with bacterial colonization. A possible reason for the lack of an obvious defense response from the stems and leaves could be that the bacteria were primarily localized within intercellular spaces or within already dead cells, such as xylem and aerenchyma, and were not observed penetrating intact host cells (Gyaneshwar et al., 2001).

4.5 Analysis of community structure of rice endophytic diazotroph bacteria

4.5.1 Amplification of 16S rRNA gene fragments by a PCR-DGGE approach

In this study a PCR approach was characterized to facilitate the analysis of the 16S rRNA gene fragments of rice endophytic bacteria (Figure 10).

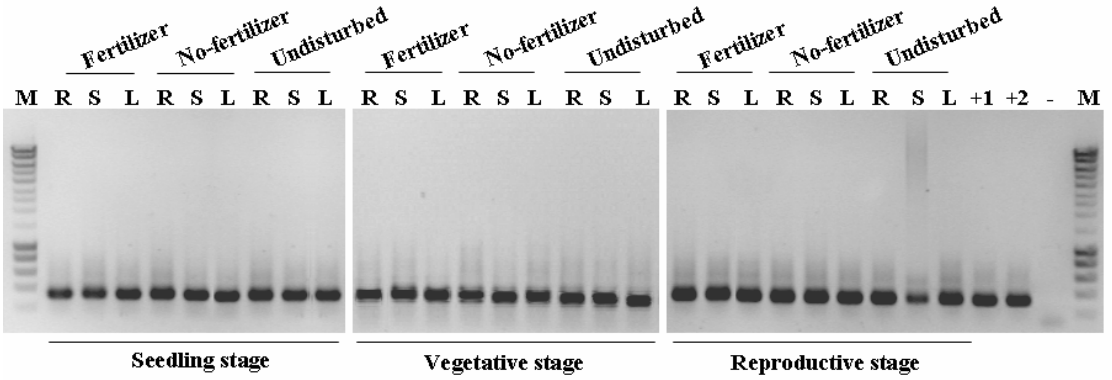


Figure 10. 16S rRNA primer PCR amplification of rice endophytic diazotroph bacteria. R, root; S, stem; L, leaf, +1, *Azotobacter* sp.; +2, *Azospirillum* spp.; -, negative control; M, 100 bp hyper ladder.

The PCR-DGGE analysis incorporating with 16S rRNA primer was elucidated endophytic bacterial communities. The results was demonstrated in Figure 11 indicate the simple community structure of rice endophytic bacteria.

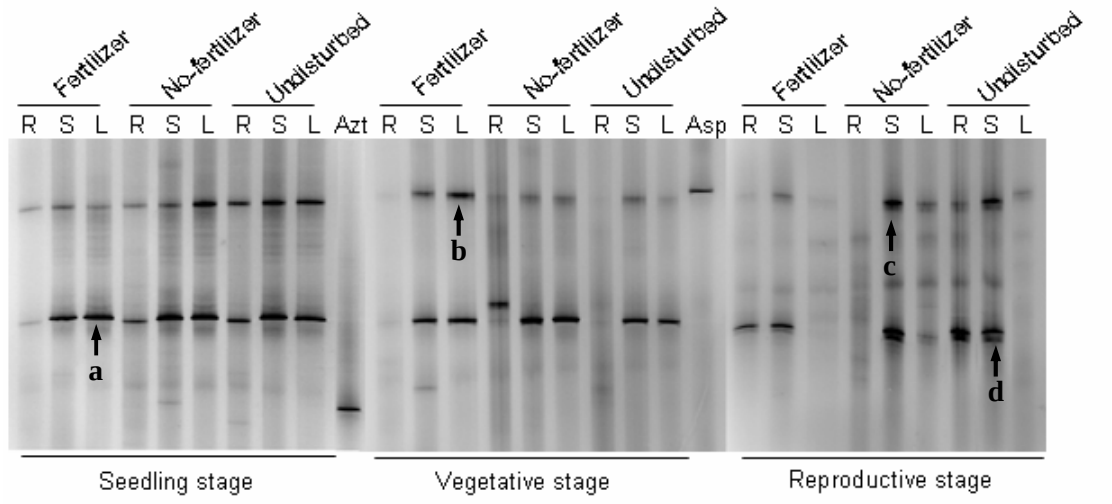


Figure 11. PCR-DGGE of 16s rRNA gene banding patterns from rice endophytic bacteria. Arrows show excised and sequenced bands (a-d). Azt., *Azotobacter* sp.; Asp., *Azospirillum* sp.; R, root; S, stem; L, leaf

Almost of the samples contained 2 major bands of DGGE-PCR products except from reproductive stage of rice. The results show that almost of samples have same major band. In seedling stage, the banding pattern show only two identical major bands in every soil sample. This could imply that no effect from environment to community structure of rice endophytic bacteria in this stage of growing. In vegetative stage of rice, the results were similar to seedling stage except in the root. The DGGE-PCR products generated from root sample could not be detected except in the sample from chemical fertilizer amendment. And in the reproductive stage of rice, this stage has more effected on microbial community structure especially in leaf of fertilizer soil was less detected. This can be concluded that soil condition have less effect than stage of growing and part of rice plant.

Sequences retrieved from the bands a–d were similarity to different strain, while a band (a) is high similarity to *Enterobacter dissolvens.* Band (b) is similarity to *Brevundimonas aurantiaca,* band (c) to *Pantoea agglomerans,* and band (d) to *Pseudomonas sp. E. dissolvens* is associated with a rhizome root of ediable ginger in Hawaii (Nishijima et al., 2004), in cereal straw as Biological N_2 fixation by heterotrophic and phototrophic bacteria (Roper and Ladha, 1995b). The *Enterobacteriaceae* strains were most commonly associated with maize roots and rhizosphere on different French soils (Berge et al., 1991), in addition *Pseudomonas* sp was also found. The stem of sweetpotato cv. Koganesengan was colonized by diazotrophic endophyte *P. agglomerans* and nondiazotrophic endophyte *E. asburiae* (Asis and Adachi, 2004).

The PCR-DGGE analysis using 16s rRNA primer was conducted for investigating endophytic bacterial communities. The result in several bands that

matched those from the bacterial isolates (culturable approach), indicating that DGGE profiles can be used to detect some endophytic bacteria of rice plants. However, some bands do not match with any isolates, suggesting the occurrence of other, nonculturable or as yet uncultured, endophytic bacteria. In the present study, the most isolates were detected both by culturable approach and by DGGE, suggesting that the molecular approach directly reported culturable endophyte bacteria. However, some isolates such as from leave with no-fertilizer soil of all stages of growing (SNL, VNL, RNL) and SFL, RFS, RFL, SUL, and RUS were not detected by ARA procedure, suggesting that these endophytic bacteria are nonculturable (or not yet cultured) endophytic bacteria from rice plants.

The plant-associated habitat is a dynamic environment in which many factors affect the structure and species composition of the microbial communities that colonize roots, stems, and leaves. It has previously been shown that endophytic communities vary spatially in the plant (Fisher et al., 1992) or may be dependent on the interaction with other endophytic or pathogenic bacteria (Araujo et al., 2002). Fingerprinting of endophytic bacterial communities by separation of amplified 16S rDNA fragments by DGGE provides the opportunity to compare the community structure features of multiple plant samples. In a study of Reiter and team (2002), the endophytic community of potato plants was evaluated by PCR-DGGE and the data obtained validated this approach to the analysis of culturable and nonculturable endophytic communities. The 16S rDNA pattern suggests that bacterial diversity was lower at the root than the leaf in fertilizer soil. This observation contrasts with the hypothesis that the roots are initially colonized more or less randomly by rapidly growing, opportunistic bacteria due to a temporary accumulation of organic acid,

sugars and other root exudates (Burgmann et al., 2005). The example band patterns showed that many bacteria groups appear to be ubiquitous but differ in abundance in the different environment. That can also be observed in the DGGE band patterns in other studies, e.g. (Yang et al., 2001; Araujo et al., 2002; Dar et al., 2005; James et al., 2006).

The number and intensity of bands in a DGGE gel do not necessarily give an accurate picture of the number and abundance of the corresponding species within the microbial community. One organism may produce more than one DGGE band because of multiple, heterogeneous rRNA operons (Nubel et al., 1996). On the other hand, partial 16S rDNA sequences do not always allow discrimination between species, such that one DGGE band may represent several species with identical partial 16S rDNA sequences (Vallaeys et al., 1997). In addition, in a mixture of target rDNAs present at very different concentrations, the less abundant sequences are not amplified sufficiently to be visualized as bands on a DGGE gel. Therefore, the banding pattern reflects only the most abundant rDNA types in the microbial community. Because of these shortcomings inherent in 16S rDNA DGGE, the diversity index calculated from the DGGE banding patterns of amplified 16S rDNA sequences must be interpreted as only an indication and not an absolute measure of the degree of diversity in a bacterial community (Muyzer et al., 1993). Muyzer et al., (1993) also showed that the presence of a few dominant species leads to a simple pattern, and that species of less than 1% of the analyzed community were not represented in the microbial community pattern. This will also be true for PCR-DGGE for certain very diverse bacterial groups: only few species that are dominant within this specific group will be visible. For subgroups that still contain a large

number of different 16S rRNA types, further subdivision using more specific primers could be useful if more detailed analysis is desired. In this study *nifH* gene primer, that specific for diazotrophic species was used on the basis of nested PCR-DGGE.

4.5.2 Amplification of *nifH* gene fragments by using a nested PCR-DGGE approach

A nested PCR approach performed with degenerated primers was used to amplify *nifH* gene fragments from the total DNA (Figure 12).

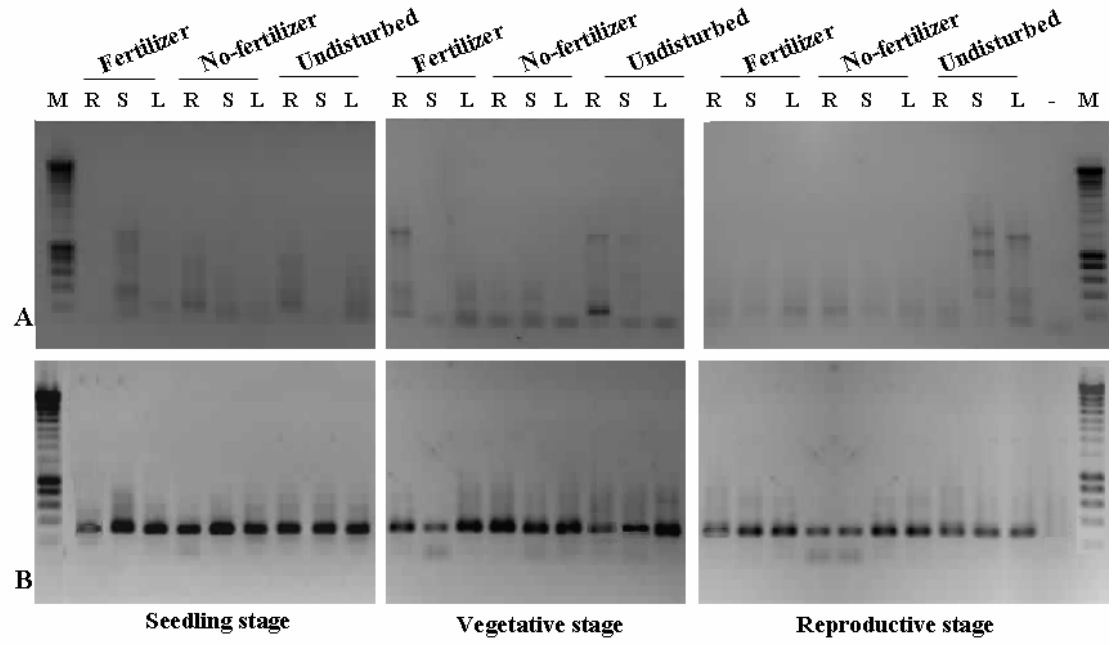


Figure 12. Agarose gel electrophoresis of nested-PCR products of *nifH* primer 1st set: polF and polR (A), and 2nd set: nifHFor and nifHRev (B). R, root; S, stem; L, leaf, -, H_2O; M = 100 bp hyper ladder.

The first amplification was performed with *nifH* PCR primers polF and polR and usually yielded lesswell- resolved background smearing (Fig. 12A). The nested

PCR amplification was performed with *nifH* PCR primers *nifH*For and *nifH*Rev; for all three parts of rice (root, stem and leaf) this amplification yielded single product bands at the expected *nifH* gene fragment size, about 370 bp (Fig. 12B). This specificity permitted us to perform DGGE analyses directly with the *nifH* PCR products and thus facilitated evaluation of the amplified gene pools.

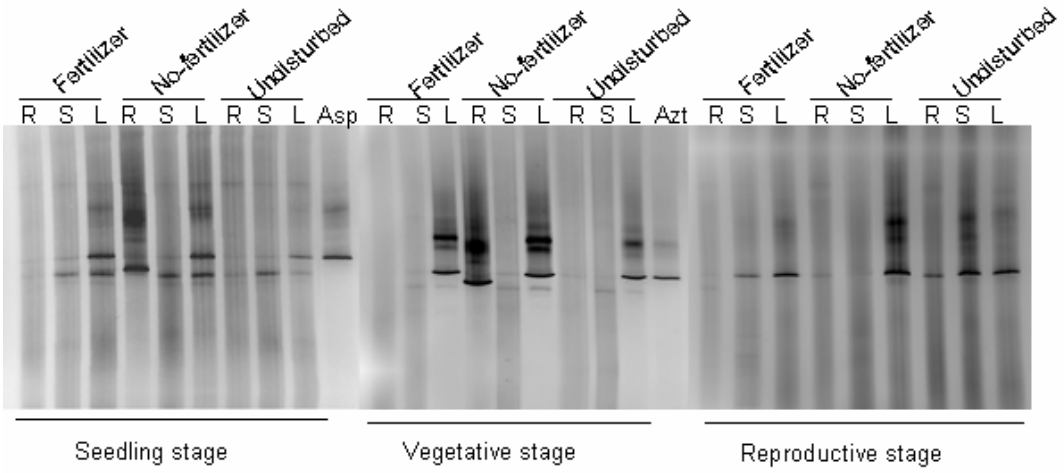


Figure 13. Nested PCR-DGGE of *nifH* banding patterns from rice endophytic bacteria. R, root; S, stem; L, leaf.

The fingerprinting of *nifH* primer are somewhat correlated to 16S rRNA gene fingerprinting which have 2-3 bands in each growing stage. Fertilizer has effect to root of every growing stage. Normally in wetland rice or flood field provides good condition for *nif* gene expression due to less amount of O_2. But in this study, it seems to be N from chemical fertilizer has more effect than that of O_2. Without chemical fertilization amendment, the diazotrophic bacteria population in the roots was changed (Figure 13). The endophytic diazotroph bacteria community in rice cultivars varied during plant development, increasing progressively during the seedling stage,

reaching a maximum during the vegetative stage, and decreasing during the reproductive stage. During the seedling stage, the diazotrophic bacterial population in the roots of fertilization and undisturbed soil was lower but higher in root of no-fertilizer soil. But in stem and leave of every soil conditions were not changed. In vegetative stage, DGGE bands of stem from all soil conditions could not be detected. Root of this stage was similarly to seedling stage, despite in leaf which higher than seedling stage in no-fertilizer and undisturbed soil. Interesingly, reproductive stage has different pattern from seedling and vegetative stage in leaf of all phase and soil condition.

However, the endophytic populations of diazotrophic bacteria in the part of rice plant have no significant changes in size during the different ontogenic plant stages of rice cultivars. There was no significant variation in the diazotrophic population in rice during the times of sampling. The endophytic population present in the stem presumably suffered from less competition with other microorganisms, and in plant tissues, carbon sources probably were more available (Olivares et al. 1997). The increase of endophytic bacterial population in reproductive stage are opposite of the study of Munoz-Rojas and Caballero-Mellado (2003) which found the decrease of bacterial population related to the age of the plant was a general characteristic of *G. diazotrophicus* in association with sugarcane. These authors suggested that physiological and metabolic changes during the sugarcane growth might modify the establishment and even the endophytic permanence of *G. diazotrophicus* or that the endophytic population decreases as a result of host plant defense. According to Reis et al. (2000), variations in diazotrophic bacterial numbers can also be explained by the changes in environmental factors like hydric stress and seasonal variation.

The *nifH* diversity pattern was less in fertilizer amendment and undisturbed soil than that of no-fertilizer amendment soil. In root of all stage of growing in fertilizer amendment soil were found no evidence of *nifH* diversity. Also in vegetative stage, stem of all condition were not found *nifH* diversity while leaf of every phase were found *nifH* diversity. Indeed, as many of the endophytic bacteria were presented in leaf. Perhaps they may actually have been a net drain on the plant's resources. These results show that rice plants that can provide additional carbon to diazotrophs may not necessarily result in a drain on their resources. Another possibility is that the growth increases shown by these varieties are due to a congruence between plant nitrogen demand and supply, resulting in an enhanced carbon sink and hence increased photosynthesis (Gyaneshwar et al., 2002). Symbiotic N_2-fixing bacteria often show a larger stimulation of growth and photosynthetic rate to elevated atmospheric carbon dioxide concentration ($[CO_2]$). One explanation for the greater stimulation in the growth of N_2-fixing species grown at elevated $[CO_2]$ is a smaller tendency toward photosynthetic acclimation, i.e. down-regulation of photosynthetic capacity in leaves developed in elevated $[CO_2]$ (Ainsworth et al., 2003). Moreover, the light and/or presumably photosynthesis in response to that light enhances rather than represses *nifH* transcription (You et al., 2005).

Interestingly, ARA-positive isolates were not corresponding to *nifH* community DGGE profiles. For example, the root of rice in seedling stage which treated with chemical fertilizer amendment (SFR5-1, SFR4-2, and SFR3-2) which have N_2-fixing activity as 527.44, 290.37, and 162.80 nmol C_2H_4 $h^{-1}tube^{-1}$, respectively) could not be detected by unculturable approach. Despite the high sensitivity of this method, some distinct species showed similar band migration

position, as previously reported in other PCR-DGGE studies (Salles et al., 2002) Indeed, DGGE has a theoretical resolution of 1 bp difference between two amplicons (Muyzer et al., 1993; Muyzer, 1999), but this separation power will largely depend on the length and sequence of the products. In fact, it has been shown that multiple nucleotide differences do not always produce differences in electrophoretic mobility (Hayes et al., 1999). Moreover, amplicons obtained from two different species can migrate at a similar position even if their sequences are largely dissimilar (Muyzer, 1999; Sekiguchi et al., 2001). To overcome these limitations, band migrating at positions that are known to be common to more than one species potentially present in a sample, should always be excised and sequenced. And some interested DGGE bands were selected for further analysis by sequencing, and this analysis showed that the strongest bacterial band. Large numbers of bands were common to all growing stage, part of rice plant and soil types, but varied in relative band intensity. The diversity of the endophytic bacterial community was significantly higher in the sandy soil and the clay, but not in the loamy sand (Javier and Klaus, 2006). It seems that the community structure was significantly affected by soil type, plant species and part of rice, but not by nitrogen fertilizer. Since chemical fertilizer had no significant effect on community structure, no differentiation was made between samples from with or without fertilizer in which the interaction between parts of rice plant, growing stage would examined in more detail. The finding that variation in nitrogen supply had no significant effect on the bacterial community composition in rice plant was somewhat surprising and it is contrast to the shift in community composition observed by Seghers and colleages (2004) indicate that the effect of agrochemicals is not limited to the bulk microbial community but also includes the root endophytic community.

Chi *et al.*,(2005) reported endophytic plant-bacterium association is far more inclusive, invasive, and dynamic including dissemination in both below-ground and above-ground tissues. But similarly, Liljeroth (1990) reported an overall increase in bacterial population densities with nitrogen fertilizer. Although simple difference is population densities would not be detected by DGGE, it is unlikely that nitrogen fertilizer would uniformly influence the growth of soil bacterial species. The endophytic community compositions examined in the present study were also shown to be affected by soil type, plant location, stage of growing, and by interactions of these variables. The relative importance of the different variables differed between the plant parts. The relative importance of soil type on microbial community structure is still difficult question, and there are no genera principles that have emerged yet. Comparisons of the different soils require consideration of many variables including difference in mineral nutrient, soil texture, pH, and organic matter, as well as physical structure and management history (Schmalenberger and Tebbe, 2003). All of these variables may affect the composition of the microbial community in plant. However, examination of this hypothesis will require further study in which single variables are adjusted in a controlled manner.

When interpreting DGGE patterns a few points should be noted. Part of the DNA extracted may derive from DNA released from dead cell and therefore the band pattern does not only reflect the current community composition. In PCR not all bacterial species may be amplified as primer sequences are based on known sequence and some not yet identified bacterial species may contain other sequences. Since the bands are separated out by their denaturation characteristics and GC content, the band may contain more than one species, thus underestimating species number. On the

other hand some species contain several copies of the amplified section and can generate several bands leading to an overestimation of the species numbers. Usually band intensity difference between species cannot be used as an indicator for species abundance (Muyzer, 1999). However if the intensity of a given band increase or decrease in different samples this does indicate a relative increase or decrease in abundance of this species. Despite these points, PCR-DGGE and nested PCR-DGGE are a powerful method foe assessing shift in microbial community composition since the ability to use small amounts of sample allows examination of rice microsite effects, and it is possible to quickly analyze large numbers of samples without the artifacts of culture based techniques. In accordance with previous research, this study showed that soil type (Tan et al., 2003) stage of growing (Feng et al., 2006; Green et al., 2006; Roesch et al., 2006) and plant parts effects (James et al., 1997) all contributed to the development of distinct microbial communities. By comparing these factors simultaneously, this research further shows that there is also a complex interaction between growing stage of rice, parts of rice plant and soil types that might not readily be discerned by studying each of these parameters as fully independent variables.

4.6 Detection of *nifH* gene expression by RT-PCR

The study of endophytic diazotroph bacteria in rice is hampered by the great diversity of endophyte communities and the difficulty of relating nitrogen-fixation activities to individual members of the diazotroph population. Total RNA extraction was performed with direct extraction (Figure 14).

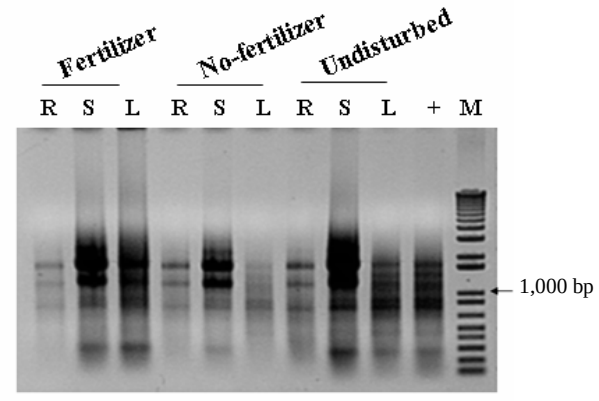


Figure 14. Total RNA extraction from seedling stage of rice, R; root, S; stem, L; leaf, +; rice inoculated with *Azotobacter* sp. as positive control, M; 1 kb ladder.

The RNA extracts were of high purity, free of DNA contamination and allowed highly sensitive and specific detection of *nifH* gene by use of RT-PCR. It must be noted here that the existence of *nifH* genes does not always mean the activity of nitrogenase, since this enzyme is regulated at both pre- and posttranslational levels (Dean and Jacobson, 1992). It must also be noted that the distribution of clones in final PCR products may not match the distribution of all *nifH* genes in the original rice DNA because of differences in amplification efficiency. The nitrogen fixation activity of endophytic diazotroph bacteria could be detected with the primers specific for *nifH* gene using RT-PCR procedure (Figure 15). In this study, reverse transcription (RT) and amplification of *nifH* cDNA by PCR from total RNA extracts of rice allowed an assessment as to whether nitrogenase genes were expressed at detectable levels in and on rice plant.

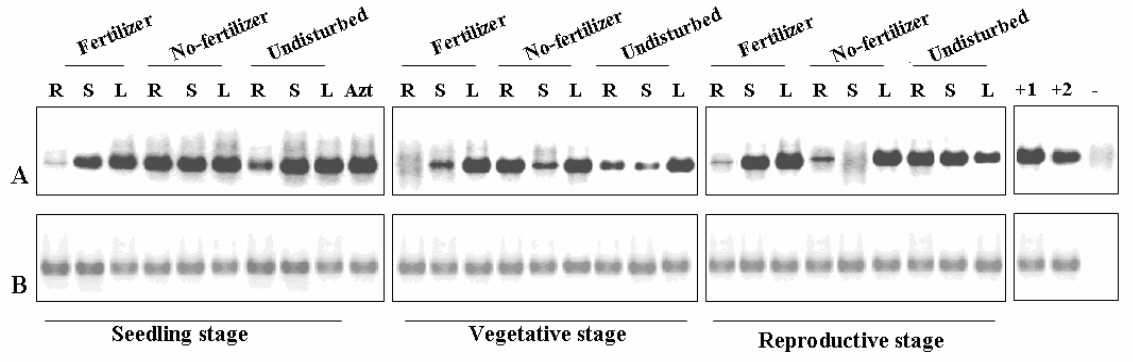


Figure 15. Gel electrophoresis analysis of RT-PCR for *nifH* gene expression. Reverse transcription and nested PCR amplification was performed using *nifH* primers (A). The 16S rRNA gene was used as a standard to calibrate the amount of RNA (B). R, root; S, stem; L, leaf; Azt., *Azotobacte*r sp.; +1, rice inoculated with *Azotobacter* sp. as positive control; +2, Positive Control; -, negative control (no-template).

The expression of this gene could be detected correlate in the different part and growing stage of rice plant. In root of fertilizer amenmented soil of all stages of growing were found no expression of *nifH* gene while leaf of all stages of growing even with or without fertilizer have an expression of these gene. Furthermore, stem of all stage of growing except without fertilizer amendment in reproductive stage was expressed of *nifH* gene. Nitrogen-fixing endophytes in rice were reported to be higher in stems than in roots, indicating that rice stems probably provide a suitable niche.

These results were related to *nifH* DGGE fingerprinting and culturable approach. Interestingly, ARA-positive isolates were not corresponding to *nifH* community DGGE profiles. For example, *nifH* gene expression in the root of rice in seedling stage which treated with chemical fertilizer could not be detected by

unculturable approach while diazotroph strains as SFR5-1, SFR4-2, and SFR3-2 were detected using culturable approach.

4.7 Effect of chemical-fertilization, growing stage and part of rice plant on endophytic diazotroph bacteria community

The results of community structure of endophytic diazotroph bacteria within each part, growing stages of rice and soil condition using culturable and unculturable approach were summarized in Table 9. In three experiment soils, the diazotrophic bacterial population was detected in the stem and leaf of rice plants during all stage of growth even chemical fertilizer was added while root of fertilizer amendment was not detected. Differ from report of Muthukumarasamy *et al* (2006) that the negative correlation between mineral nitrogen and the actively fixing nitrogen of endophytic diazotroph bacteria showed that during the first stage of growth, the diazotrophic bacterial population was decreased by increasing nitrogen fertilization. However, after the reproductive stage, there was a non-significant negative correlation detected between N-fertilization and the diazotrophic population. Also, there was an increase in the diazotrophic bacterial population in roots in all treatments, even when the amount of mineral nitrogen in the soil was high. These results demonstrated that the colonization of rice plants by diazotrophic bacteria was inhibited by high N-fertilization during the first stages of growth but not during the subsequent stages. Since the N-fertilization did not affect the diazotrophic bacterial population in all stages of growth, the observed effect does not seem to be a direct negative effect of the fertilizer on the bacteria.

Table 9. Summary results of culturable and non-culturable study of rice endophytic diazotroph bacteria.

	Seedling stage									**Vegetative stage**									**Reproductive stage**								
	Root			**Stem**			**Leaf**			**Root**			**Stem**			**Leaf**			**Root**			**Stem**			**Leaf**		
1. Culturable N_2- fixating	+++			++			-			+++			++			+			++			+			+		
2. Non-Culturable	**F**	**NF**	**U**	**F**	**NF**	**U**	**F**	**NF**	**U**	**F**	**NF**	**U**	**F**	**NF**	**U**	**F**	**NF**	**U**	**F**	**NF**	**U**	**F**	**NF**	**U**	**F**	**NF**	**U**
• 16S rRNA	++	++	++	++	++	++	++	++	++	-	+	-	++	++	++	++	++	++	+	++	++	++	+++	+++	-	+++	-
• N_2 - fixing	-	++	+	+	+	+	++	++	++	-	++	-	-	-	-	+++	+++	+++	-	+	+	+	+	++	++	++	++
• *nifH* gene expression	-	++	+	++	++	++	+++	+++	+++	-	+++	+	+	+	+	+++	+++	+++	-	++	+++	+++	-	++	+++	+++	++

Abbreviations, F; fertilizer amendment, NF; no-fertilizer experiment, U; undisturbed experiment;
+++, good growth; ++, normal growth; +, slight growth; -, no growth

16S rRNA is include positive ARA data and non-diazotroph bacteria data

N_2-fixing is include population of endophytic bacteria and *nifH* gene expression in each part of rice plant

The nitrogen alters the physiological state of the plant, and this subsequently affects its association with the diazotrophic bacterial population (Muthukumarasamy et al., 1999; Reis et al., 2000). *Herbaspirillum* spp. was found to occur both in N fertilised and unfertilized samples. The fact that N is not the limiting factor for *Herbaspirillum* spp. was evidenced in a similar study of its growth pattern in different N sources (Muthukumarasamy et al., 1999).

A poor relationship between *nifH* community structure and nitrogenase activity at this site, factors that control the distribution of genotypes may not be directly related to the expression of *nifH* genes. Similarly, in a *Spartina saltmarsh*, acetylene reduction rates increased in N and N & P amended plots 2 weeks post fertilization although no corresponding change in *nifH* community DGGE profiles were observed (Piceno and Lovell, 2000).

Each of the soils varied with respect to soil texture, pH, nutrient and organic matter concentration (Table 2), all of which may act as potential selection factors affecting rhizo-deposition, plant growth, and rhizosphere communities. Additionally, the physical structure of the soils varied, with the clay soil having a higher bulk density and poorer aeration. Even low concentrations of combined nitrogen as ammonium or nitrate lead to repression of nitrogenase genes (Egener et al., 1999) and to an inactivation of nitrogenase activity (Martin and Reinhold-Hurek, 2002) in most bacteria. Consequently, nitrogen fixation in soils and agricultural systems, especially in N_2 - fixing symbioses with legumes, often decreases in response to nitrogen fertilizer (Peoples et al., 1995; Limmer and Drake, 1997). In contrast, in a *nifH* DGGE-based study on *in situ* grown *Spartina alterniflora*, the population of diazotrophs in the rhizosphere soil appeared to be fairly stable after short-term

addition of combined N and P while long-term fertilizations treatment applied every 2 weeks for 8 weeks did elicit community change. The contrasting results might be explained by the application of less of nitrogen in the case of *Spartina*, which did not lead to inhibition of nitrogen fixation but even to an increase (Piceno and Lovell, 2000). In a 16S rRNA-based study of soil bacterial communities in response to grassland successions, the population was remarkably stable in fertilized and unfertilized plots (Felske et al., 2000). However, data on root-associated diazotrophs are consistent with results on the endophytic diazotroph *G. diazotrophicus*, which showed a severe decrease in numbers of culturable cells inside sugar cane fertilized with high levels of nitrogen (Fuentes-Ramirez et al., 1999). The diazotrophs abundant in rice plants may have been either rapidly decaying or overgrown by others after fertilizer application. This suggests that the original diazotrophic community consisted mainly of autochtonic bacteria for which N-depletion conferred a selective advantage. These results also demonstrate that rice represent a highly dynamic microenvironment for bacteria. In addition to fertilization with combined nitrogen, variation of the growing stage and part of rice plant and the environmental conditions caused large differences of the population structure of endophytic diazotrophs, as demonstrated in a culturable approach. Under controlled conditions in undisturbed soil, the DGGE patterns of prevalent *nifH* genes were dependent on the plant parts (root, stem and leave). An impact of the rice genotype on microbial diversity has also been demonstrated for root-associated communities of nitrifyers at the *O. sativa* cultivar level (Briones et al., 2002). Species- and environmentally-dependent shifts of *nifH* pools were also corroborated by the analysis of rice samples taken in Nepal and Philippines, the influence of environmental factors on the population structure of

diazotrophs appeared to be greater than the influence of the plant species (Tan et al., 2003). The observation that the biodiversity of diazotrophs may be higher in modern rice cultivars than in wild rice (Engelhard et al., 2000). In 16S rRNA-based studies on the microbial community of rhye, clover, bean and alfalfa roots, significant variations were found, however, the plant type had a stronger influence than the soil type (Miethling, 2000; Wieland et al., 2001). These contrasting results might be due to plant specie specific effects or to the experimental setup, as different plant genera and not species were compared, and similar soils from the same regions were used under otherwise identical environmental conditions.

Interestingly, *Azospirillum* sp. which had been isolated from rice roots (Engelhard *et al*., 2000) and can often be cultured from rice (Stoltzfus et al., 1997; Trân Vân et al., 1997) could not be detected in any rice root sample by this study. This might indicate that *Azospirillum* is not among the prevalent diazotrophs associated with rice roots when unculturable strains are also taken into account; it underlines that some diazotrophs, although able to grow rapidly on laboratory culture media, may not play an important role in the natural environment. We were unable to detect changes in diazotroph communities from fertilization treatments. Higher photosynthetic rates in plants from plots may also lead to allocation of excess carbohydrate to roots and rhizosphere organisms. Increased exudation of labile carbon compounds by plant roots may select for certain *nifH* genotypes, and this mechanism may have been important in causing structural shifts in the diazotroph community.

CHAPTER V

CONCLUSION

The culturable endoophytic diazotrophic bacteria could be isolated from rice and significantly demonstrated N_2-fixing activity. Fingerprinting of endophytic bacterial communities by separation of amplified 16S rRNA and *nifH* genes fragments by DGGE able to compare the community among the rice plants investigated differ present in the different part and growing stage of rice. With the 16S rRNA primer and PCR-DGGE analysis could be used to observe both of diazotroph and nondiazotroph endophytic bacterial communities.

Nested PCR-DGGE using *nifH* genes as primer could be explored to compare the community among the rice plants. The actively fixing nitrogen of endophytic bacteria was no relations to growing stages, part of rice plant and soil types. While it seems fertilizer has effected to *nifH* gene expression in root of rice. The results presented in this study have shown that monitoring of *nifH* mRNA in rice is a suitable and promising approach to link population structures and activities of diazotroph communities. This approach could be used to study the dynamics of microbial communities. Therefore, the combination data of *nifH* gene expression is fundamental to understanding how and when endophytic communities associate in rice plant. The knowledge of this interaction is an essential to investigate ability of endophytic diazotroph bacteria especially culturable strain to compete with other endophyte

strains and contribute nitrogen to host plant prior to apply as rice biofertilizer in Thailand.

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Table 10 Growth pattern in RMR semi-solid medium of endophytic bacteria consortium and colony morphology

Sample	Growth on RMR semi-solid medium			No. of Colony	Colony morphology			
	Surface	Sub-surface	Growth		Form	Elevation	Margin	Color
SNR5-2	-	turbid/clear	+	3	circular	convex	entire	white
					irregular	convex	lobate	clear
					irregular	convex	undulate	light blue
SNR5-3	-	turbid/clear	+	1	circular	convex	entire	white
SNS2-3	-	turbid	+++	2	circular	convex	entire	white
					circular	convex	entire	beige
SNS3-1	pellicle 1 cm	turbid	+++	2	circular	convex	entire	white
					irregular	convex	lobate	clear
SUR2-1	-	turbid	+++	2	irregular	convex	lobate	clear
					punotiform	convex	entire	orange
SUR2-3	-	turbid	+++	2	circular	convex	entire	beige
					punotiform	convex	entire	orange
SUR3-2	subpellicle	turbid	+++	2	circular	convex	entire	white
					irregular	convex	lobate	clear
SUR5-1	subpellicle	-	+	2	circular	convex	entire	White
					circular	convex	undulate	pink
SUR5-3	subpellicle	slime	++	1	circular	convex	entire	white
SUR6-2	pellicle/foam	turbid	+	1	circular	convex	entire	white
SUS2-1	foam	slime, green	+++	1	circular	convex	entire	white
SUS2-2	foam	slime, green	+++	2	circular	convex	entire	white
					irregular	convex	lobate	clear
VFL2-1	foam (small)	slime	++	1	irregular	convex	lobate	clear

Abbreviations, First letter is growing stage of rice (S; seedling stage, V; vegetative stage, R; reproductive stage), second letter is soil type (F; fertilizer amendment, N; no-fertilizer experiment, U; undisturbed experiment), and third letter is rice part (R; root, S; stem, L; leaf)

+, little growth; ++, normal growth; +++, Very good growth

Table 10 Growth pattern in RMR semi-solid medium of endophytic bacteria consortium and colony morphology (Continued)

Sample	Growth on RMR semi-solid medium			No. of Colony	Colony morphology			
	Surface	Sub-surface	Growth		Form	Elevation	Margin	Color
VFR2-2	-	turbid	+++	1	circular	convex	entire	beige
VFR3-1	foam (big)	turbid	+++	2	circular	convex	entire	white
					irregular	convex	lobate	clear
VFR3-3	-	turbid	+++	1	circular	convex	entire	white
VFR4-2	pellicle/foam	turbid	+	1	circular	convex	entire	white
VFR5-3	pellicle	turbid	++	2	circular	convex	entire	white
					irregular	umbonate	undulate	yellow
VFR6-3	pellicle	turbid	++	1	circular	convex	entire	white
VFS2-1	-	slime, green	+++	2	circular	convex	entire	white
					irregular	raised	lobate	yellow
VFS2-3	-	slime, green	+++	1	circular	convex	entire	white
VFS4-1	pellicle	turbid	+	1	irregular	convex	lobate	clear
VNR3-2	-	turbid	+++	2	circular	convex	entire	white
					irregular	umbonate	undulate	yellow
VNR3-3	foam	turbid	+++	1	circular	convex	entire	white
VNR4-1	foam	turbid	++	1	circular	convex	entire	white
VNR5-1	-	slime/clear	+	2	circular	convex	entire	white
					irregular	convex	lobate	clear
VNR6-1	-	slime/clear	+	2	circular	convex	entire	beige
					irregular	convex	lobate	clear
VNS3-2	pellicle	clear	+++	2	irregular	convex	lobate	clear
					circular	raised	entire	white

Abbreviations, First letter is growing stage of rice (S; seedling stage, V; vegetative stage, R; reproductive stage), second letter is soil type (F; fertilizer amendment, N; no-fertilizer experiment, U; undisturbed experiment), and third letter is rice part (R; root, S; stem, L; leaf)

+, little growth; ++, normal growth; +++, Very good growth

Table 10 Growth pattern in RMR semi-solid medium of endophytic bacteria consortium and colony morphology (Continued)

Sample	Growth on RMR semi-solid medium			No. of Colony	Colony morphology			
	Surface	Sub-surface	Growth		Form	Elevation	Margin	Color
VNS3-3	foam	turbid	+++	2	circular	convex	entire	white
					irregular	convex	lobate	clear
VUL3-1	pellicle	turbid	+++	1	circular	convex	entire	white
VUR3-1	-	turbid	+++	2	circular	convex	entire	white
					irregular	convex	lobate	clear
VUR3-3	subpellicle	turbid	+++	2	circular	convex	entire	white
					irregular	convex	lobate	clear
VUR4-1	pellicle	turbid	++	1	circular	convex	entire	white
VUR4-3	pellicle	turbid	++	2	circular	convex	entire	white
					punotiform	convex	entire	orange
VUR6-3	pellicle/foam	turbid	+	1	circular	convex	undulate	pink
RFR5-2	pellicle	turbid	++	1	circular	convex	entire	white
RFS3-3	subpellicle	turbid	+	2	circular	convex	entire	white
					circular	convex	entire	beige
RNR2-3	foam	turbid	+++	2	circular	convex	entire	white
					irregular	convex	lobate	clear
RNR3-1	-	turbid	+++	2	circular	convex	entire	white
					circular	convex	entire	beige
RNR4-2	foam	turbid	++	2	circular	convex	entire	white
					irregular	convex	lobate	clear

Abbreviations, First letter is growing stage of rice (S; seedling stage, V; vegetative stage, R; reproductive stage), second letter is soil type (F; fertilizer amendment, N; no-fertilizer experiment, U; undisturbed experiment), and third letter is rice part (R; root, S; stem, L; leaf)

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Table 10 Growth pattern in RMR semi-solid medium of endophytic bacteria consortium and colony morphology (Continued)

Sample	Growth on RMR semi-solid medium			No. of Colony	Colony morphology			
	Surface	Sub-surface	Growth		Form	Elevation	Margin	color
SFR2-3	-	turbid	+++	1	circular	convex	entire	white
SFR3-2	foam	turbid	+++	1	circular	convex	entire	white
SFR5-1	-	turbid	++	1	circular	convex	entire	white
SFS2-2	-	turbid, green	+++	2	circular	convex	entire	white
					circular	convex	entire	beige
SFS3-2	subpellicle	turbid	+	1	circular	convex	entire	white
SNR2-1	foam	turbid	+++	2	circular	convex	entire	white
					irregular	convex	lobate	clear
SNR2-2	foam	turbid	+++	2	circular	convex	entire	white
					irregular	convex	lobate	clear
SNR4-3	-	turbid	+++	1	circular	convex	entire	white
RUL2-2	foam	slime, green	+++	2	circular	convex	entire	white
					irregular	convex	lobate	clear
RUR2-2	-	turbid	+++	2	circular	convex	entire	beige
					irregular	convex	lobate	clear
RUR4-2	subpellicle	turbid	++	2	circular	convex	entire	beige
					punotiform	convex	entire	orange

Abbreviations, First letter is growing stage of rice (S; seedling stage, V; vegetative stage, R; reproductive stage), second letter is soil type (F; fertilizer amendment, N; no-fertilizer experiment, U; undisturbed experiment), and third letter is rice part (R; root, S; stem, L; leaf)

+, little growth; ++, normal growth; +++, Very good growth

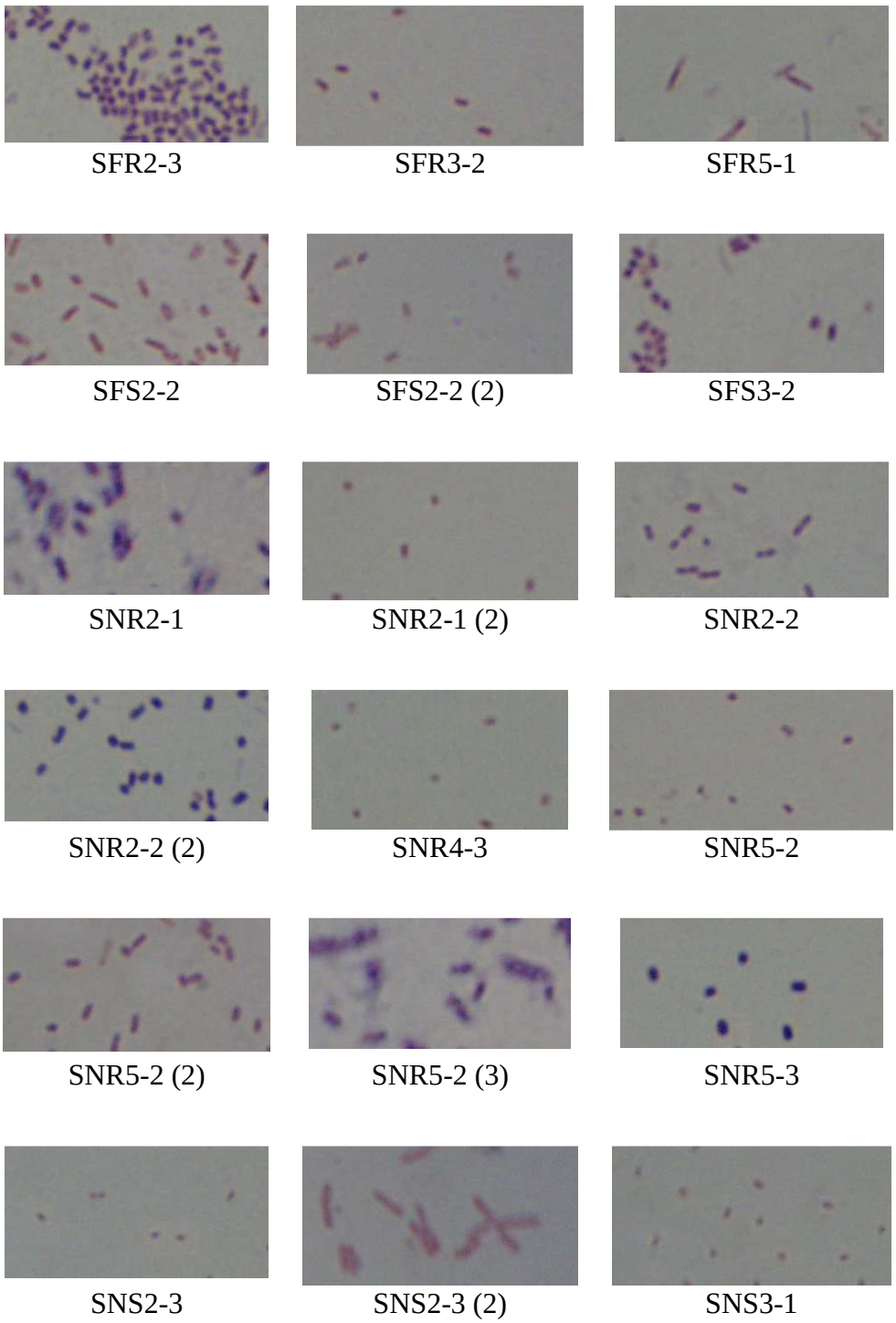
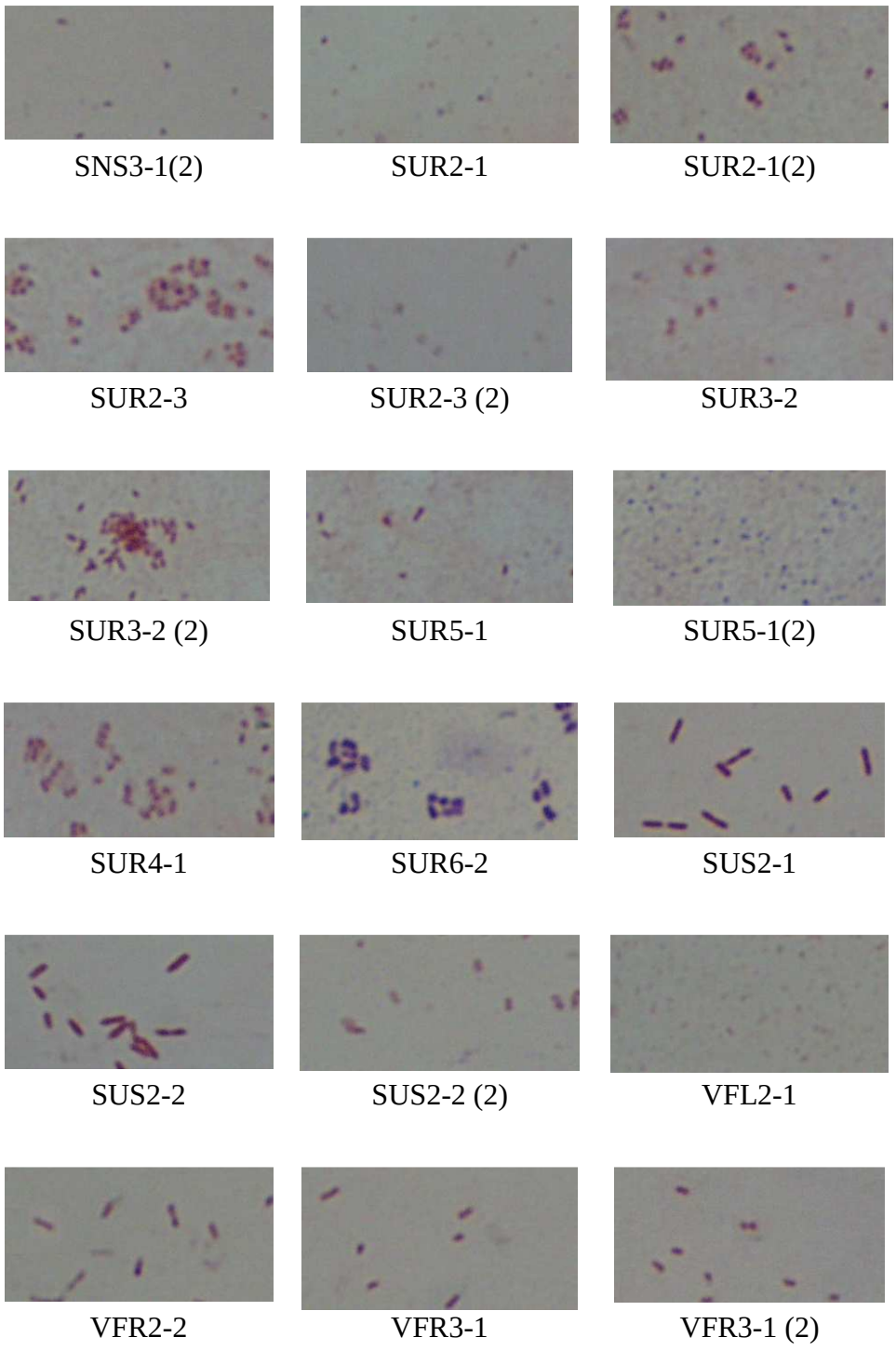


Figure 16 Endophytic diazotroph bacteria cell morphology



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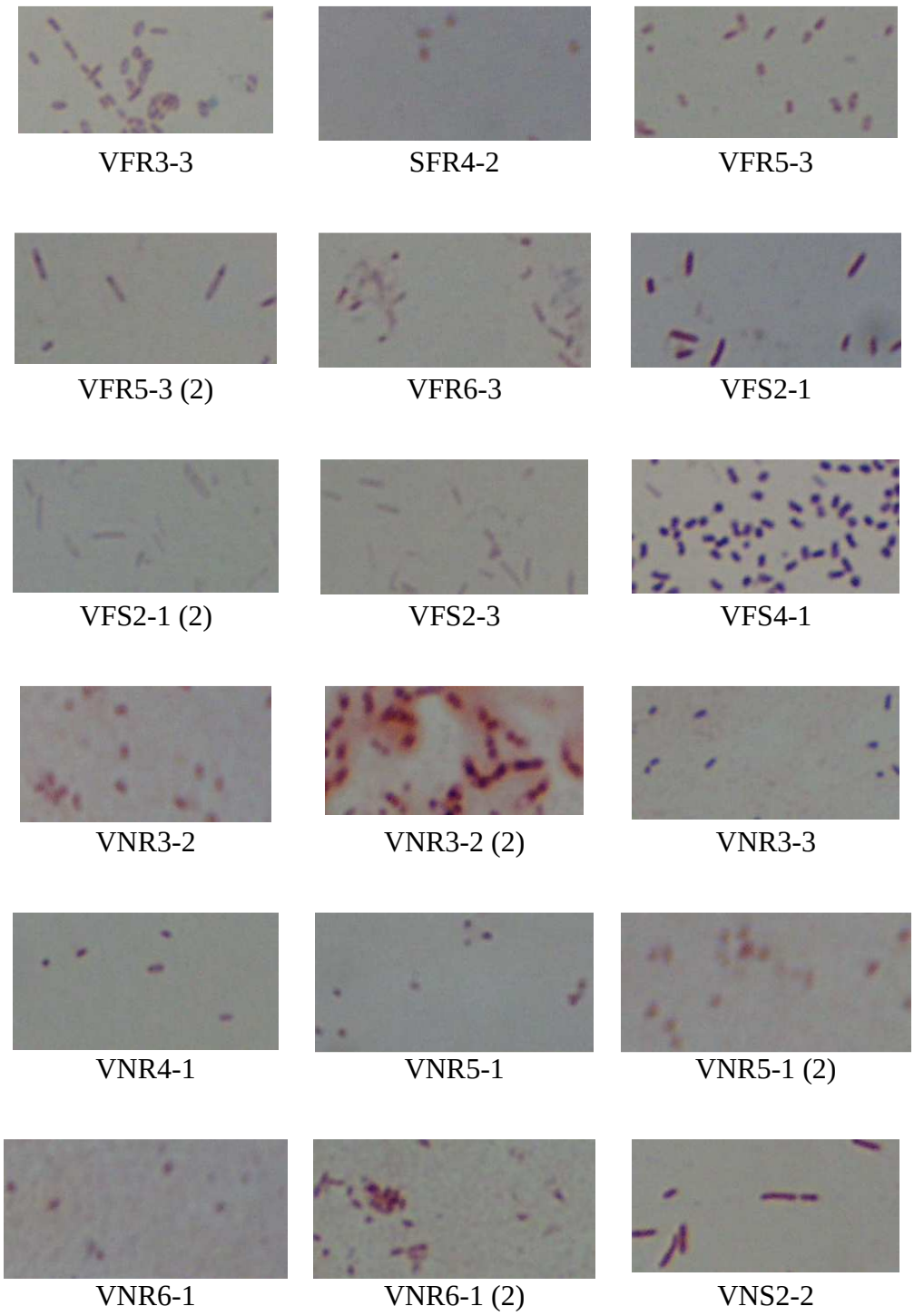

Figure 16 Endophytic diazotroph bacteria cell morphology

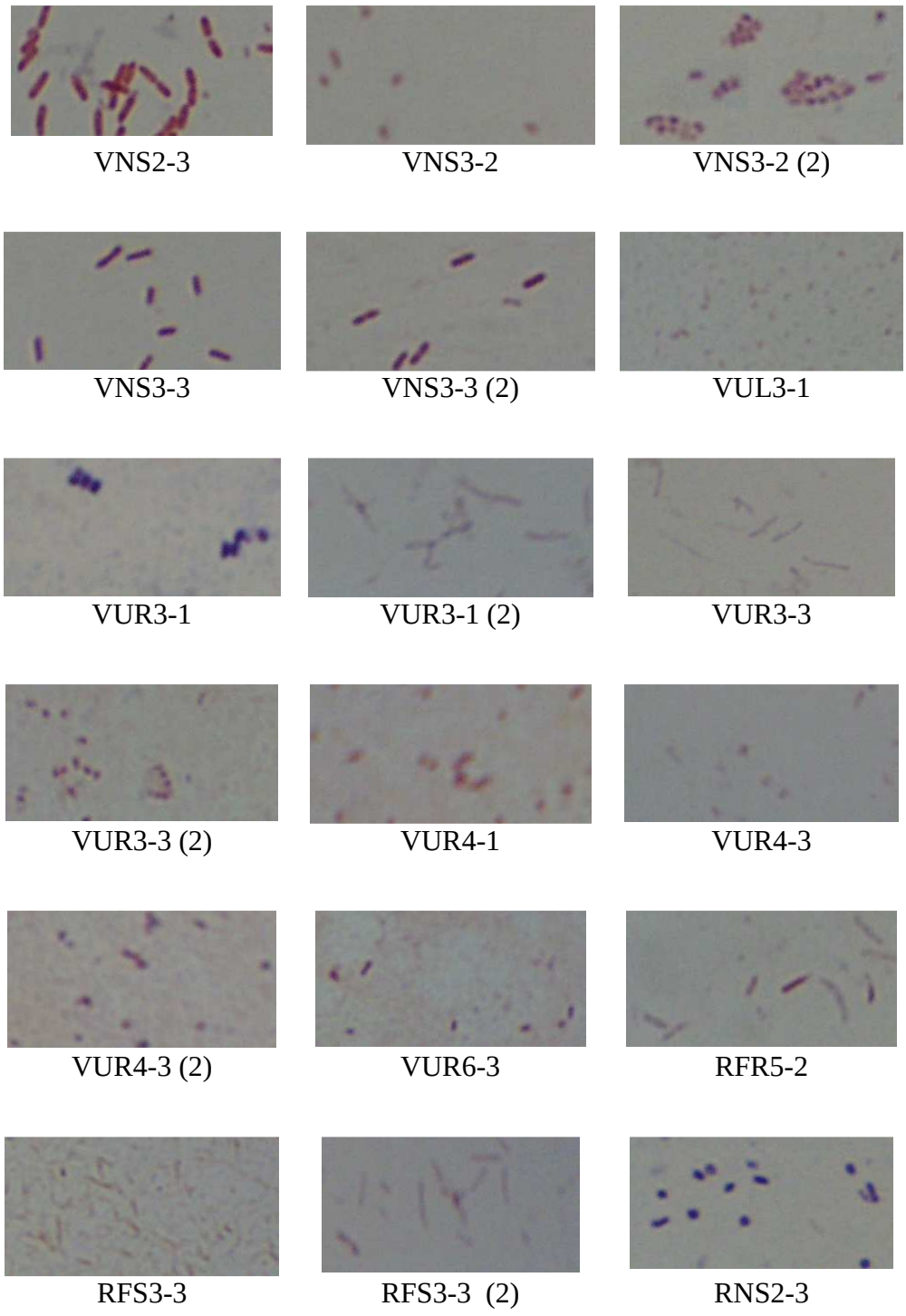

Figure 16 Endophytic diazotroph bacteria cell morphology

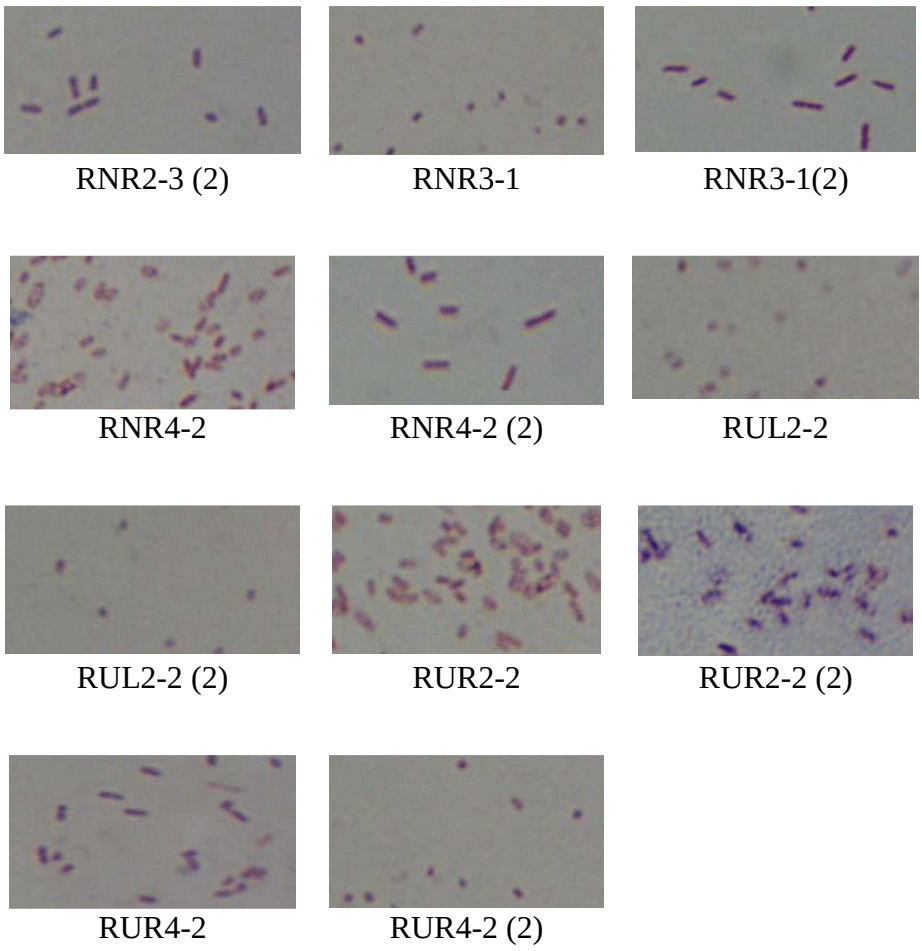

Figure 16 Endophytic diazotroph bacteria cell morphology

Table 11 N_2-fixing activities and biochemical characteristics of endophytic diazotroph bacteria isolates from rice

	SFR2-3	SFR3-2	SFR4-2	SFR5-1	SFS2-2	SFS3-2	SNR2-1	SNR2-2
N_2-fixing activity	254.78	162.8	290.37	527.44	2,201.67	3,817.33	936.84	585.34
Carbon source								
Xylose	++	++	+++	+	++++	+	++	+++
Lactose	+	+	+	++	++++	+	+	+
Glycerol	++	++	+++	+	+	+	++	+++
Fructose	+++	++++	+++	++	++++	++	+	++
Arabinose	++	+	+++	++++	+	+	+	+
Rhamnose	+	+++	-	+	++	+	+	+
Glucose	+++	+++	+++	+++	++	++	+	+
Manitol	++	+++	++++	+++	++	+	+	+
Sorbitol	++	+++	-	-	++	+	+	++
Sucrose	-	++++	++++	+	+++	++	+	++
Myo-Inositol	++++	+	++	++++	+	++	+	+++
Mannose	-	++	++	-	+++	++	+	+
Tartaric acid	-	+	+	-	+	++	+	+
Fumaric acid	-	++	+	-	++	++	+	++
Cellulase	-	-	+	++	+	++	++++	++
Pectinase	+	-	+++	++	+++	+	+	+
IAA	-	+	-	+++	++++	-	++++	++
Antibiotic resistant								
Kanamycin	S	R	S	S	S	R	S	R
Tetracyclin	S	S	S	S	S	S	S	S
Chloramphenicol	S	S	S	S	S	S	S	R
Streptomycin	S	R	S	S	R	S	S	S
Ampicillin	R	R	R	R	R	R	R	S
Erythromycin	S	S	S	S	R	R	S	S
Spectinomycin	S	R	S	S	S	R	S	S

Abbreviations, First letter is growing stage of rice (S; seedling stage, V; vegetative stage, R; reproductive stage), second letter is soil type (F; fertilizer amendment, N; no-fertilizer experiment, U; undisturbed experiment), and third letter is rice part (R; root, S; stem, L; leaf)

N_2-fixing activity = Ethylene concentration (nmol C_2H_4 $h^{-1}tube^{-1}$)

Duplicate plates were used for each test isolate

+, little growth; ++, normal growth; +++, Very good growth; -, No growth

R, resistant; S, sensitive

Table 11 N_2-fixing activities and biochemical characteristics of endophytic diazotroph bacteria isolates from rice (Continued)

	SNR4-3	SNR5-2	SNR5-3	SNS2-3	SNS3-1	SUR2-1	SUR2-3	SUR3-2
N_2-fixing activity	287.9	245.16	142.85	3,735.68	5,840.07	481.58	644.32	487.24
Carbon source								
Xylose	-	-	+++	+	+	+	-	-
Lactose	+	-	++++	+++	++	+	-	++
Glycerol	+++	-	++	++	++	++	-	+
Fructose	+++	-	+++	+	+	+++	+	+++
Arabinose	++	+	+++	+	+	++	+	++++
Rhamnose	-	++	-	+	+	+	+	-
Glucose	+++	+	+++	++	+	+++	++	+++
Manitol	+++	+	++++	++	+	+++	++	++
Sorbitol	++	++	+++	+	+	+++	++	++
Sucrose	-	+++	++++	+	++	++++	++++	-
Myo-Inositol	+++	+	-	+	+	++++	++	+++
Mannose	++	+	++	+	+	-	+	++
Tartaric acid	-	+	+	+	+	-	+	-
Fumaric acid	+	+	+	++	++	-	++	+
Cellulase	+++	++	+	++	++	+	++	+++
Pectinase	-	+	+++	+++	-	++	-	-
IAA	++	-	-	++	-	++	+++	++
Antibiotic resistant								
Kanamycin	S	S	S	R	S	S	S	S
Tetracyclin	S	S	S	S	S	S	S	S
Chloramphenicol	S	S	S	S	S	S	S	S
Streptomycin	S	S	S	R	S	S	R	S
Ampicillin	R	R	R	R	S	R	R	R
Erythromycin	S	R	S	S	S	S	S	S
Spectinomycin	S	S	S	S	S	S	S	S

Abbreviations, First letter is growing stage of rice (S; seedling stage, V; vegetative stage, R; reproductive stage), second letter is soil type (F; fertilizer amendment, N; no-fertilizer experiment, U; undisturbed experiment), and third letter is rice part (R; root, S; stem, L; leaf)

N_2-fixing activity = Ethylene concentration (nmol C_2H_4 $h^{-1}tube^{-1}$)

Duplicate plates were used for each test isolate

+, little growth; ++, normal growth; +++, Very good growth; -, No growth

R, resistant; S, sensitive

Table 11 N_2-fixing activities and biochemical characteristics of endophytic diazotroph bacteria isolates from rice (Continued)

	SUR5-1	SUR5-3	SUR6-2	SUS2-1	SUS2-2	VFL2-1	VFR2-2	VFR3-1
N_2-fixing activity	149.2	2,136.40	1,662.14	2,905.17	217.32	315.12	2,657.38	565.7
Carbon source								
Xylose	-	-	-	+++	+++	-	++	+++
Lactose	++++	+++	++	++	+	++	++	++
Glycerol	++	-	++	++	++	+++	+++	++++
Fructose	+++	++	+	++	+++	+++	+++	++
Arabinose	++	+	+	+	++++	++	+	+
Rhamnose	+	+	+	+++	-	+	++++	+
Glucose	+++	+	+++	++	++	+++	++	+++
Manitol	++++	+	+++	++	+++	++	++++	++
Sorbitol	-	-	++	++	+++	-	++++	++
Sucrose	+	+	++++	++++	-	-	++++	++
Myo-Inositol	+	+	++	++	++++	+++	+	++
Mannose	-	++	+++	+++	++	++	+++	++
Tartaric acid	+	+	+	+	-	+	++	+
Fumaric acid	+	++	++	++	-	+	++	++
Cellulase	++++	+	+	+++	+	++	++	++++
Pectinase	+	+	+++	-	+++	++	+	+++
IAA	+++	++++	+++	+	-	++	+	+
Antibiotic resistant								
Kanamycin	S	S	R	R	S	S	R	R
Tetracyclin	S	S	S	S	S	S	S	S
Chloramphenicol	S	S	R	R	S	S	R	S
Streptomycin	S	S	S	S	S	S	R	S
Ampicillin	R	R	S	S	R	R	R	R
Erythromycin	S	S	R	S	S	S	R	S
Spectinomycin	S	S	S	R	S	S	R	S

Abbreviations, First letter is growing stage of rice (S; seedling stage, V; vegetative stage, R; reproductive stage), second letter is soil type (F; fertilizer amendment, N; no-fertilizer experiment, U; undisturbed experiment), and third letter is rice part (R; root, S; stem, L; leaf)

N_2-fixing activity = Ethylene concentration (nmol C_2H_4 $h^{-1}tube^{-1}$)

Duplicate plates were used for each test isolate

+, little growth; ++, normal growth; +++, Very good growth; -, No growth

R, resistant; S, sensitive

Table 11 N_2-fixing activities and biochemical characteristics of endophytic diazotroph bacteria isolates from rice (Continued)

	VFR3-3	VFR5-3	VFR6-3	VFS2-1	VFS2-3	VFS4-1	VNR3-2	VNR3-3
N_2-fixing activity	1,842.81	3,859.50	4,270.64	181.24	3,583.45	295.24	316.52	355.84
Carbon source								
Xylose	++	++++	++	+++	-	++++	++	+
Lactose	++++	+	++++	+	++++	++	+	+
Glycerol	+	++	+	+++	++	++++	++	++
Fructose	+++	++	++	++++	+++	++++	++++	+
Arabinose	+	+	+	++	+	+	+	+
Rhamnose	++++	+	++	+	+++	+	+	+
Glucose	+	+	++++	++++	++	+++	++++	+
Manitol	+	++++	+	++++	++	+++	++++	+
Sorbitol	+	+	+	+++	++	++	++	+
Sucrose	++++	+++	++++	++	++++	++++	++	+
Myo-Inositol	+++	+	+	+++	++++	+++	+++	+
Mannose	+++	++	++	+	++++	+++	+	+
Tartaric acid	+	++++	+	+	+	+	+	+
Fumaric acid	++	++	++	++	++	++	++	+
Cellulase	+	++++	++	++	+++	++	++	++
Pectinase	-	+	-	-	-	+	+++	-
IAA	+++	+++	-	-	-	++++	-	++
Antibiotic resistant								
Kanamycin	S	S	S	S	S	S	S	R
Tetracyclin	S	S	S	S	S	S	S	S
Chloramphenicol	S	S	S	S	S	S	S	S
Streptomycin	S	S	S	R	R	S	R	R
Ampicillin	R	R	R	R	R	S	R	R
Erythromycin	R	S	S	R	R	R	R	R
Spectinomycin	R	S	S	S	R	R	R	S

Abbreviations, First letter is growing stage of rice (S; seedling stage, V; vegetative stage, R; reproductive stage), second letter is soil type (F; fertilizer amendment, N; no-fertilizer experiment, U; undisturbed experiment), and third letter is rice part (R; root, S; stem, L; leaf)

N_2-fixing activity = Ethylene concentration (nmol C_2H_4 $h^{-1}tube^{-1}$)

Duplicate plates were used for each test isolate

+, little growth; ++, normal growth; +++, Very good growth; -, No growth

R, resistant; S, sensitive

Table 11 N_2-fixing activities and biochemical characteristics of endophytic diazotroph bacteria isolates from rice (Continued)

	VNR4-1	VNR5-1	VNR6-1	VNS3-2	VNS3-3	VUL3-1	VUR3-1	VUR3-3
N_2-fixing activity	53.45	56.89	132.13	759.22	926.84	817.25	458.86	635.04
Carbon source								
Xylose	-	-	-	+	-	+++	-	+++
Lactose	++	++++	+	++	++	++	+	+++
Glycerol	+	+	+	+	+	++	+	+++
Fructose	+	+++	+++	++	+	+++	+++	++++
Arabinose	+	++	+++	+	+	++++	+++	+
Rhamnose	+	-	+	+++	+	+	-	+++
Glucose	+	+++	+++	+++	+	+++	+++	+++
Manitol	+	++	++++	+++	+	++	++++	+++
Sorbitol	-	++	-	+++	-	+++	++	+++
Sucrose	+	++	++++	++++	+	++	+++	+++
Myo-Inositol	+	++	++++	++	+	+	+++	+
Mannose	+	-	-	+++	+	++	-	++++
Tartaric acid	+	-	-	++	+	+	-	+
Fumaric acid	++	-	+	+++	++	-	+	++
Cellulase	+	+++	+++	++	+	+	+++	+++
Pectinase	+	-	+	-	+	+++	-	+++
IAA	++	-	+++	+++	++++	-	++	-
Antibiotic resistant								
Kanamycin	S	S	S	R	S	S	S	S
Tetracyclin	S	S	S	S	S	S	S	S
Chloramphenicol	S	S	S	S	S	S	S	S
Streptomycin	S	S	S	R	S	S	S	S
Ampicillin	R	R	R	R	S	R	R	R
Erythromycin	S	S	S	S	R	S	S	S
Spectinomycin	S	S	S	R	S	S	S	S

Abbreviations, First letter is growing stage of rice (S; seedling stage, V; vegetative stage, R; reproductive stage), second letter is soil type (F; fertilizer amendment, N; no-fertilizer experiment, U; undisturbed experiment), and third letter is rice part (R; root, S; stem, L; leaf)

N_2-fixing activity = Ethylene concentration (nmol C_2H_4 $h^{-1}tube^{-1}$)

Duplicate plates were used for each test isolate

+, little growth; ++, normal growth; +++, Very good growth; -, No growth

R, resistant; S, sensitive

Table 11 N_2-fixing activities and biochemical characteristics of endophytic diazotroph bacteria isolates from rice (Continued)

	VUR4-1	VUR4-3	VUR6-3	RFR5-2	RFS3-3	RNR3-1	RNR4-2	RNS2-3
N_2-fixing activity	1,069.52	937.09	1,436.29	1,276.70	991.75	529.31	43.4	911.3
Carbon source								
Xylose	++	++	+++	++	++	-	++	+
Lactose	+++	++	+++	++++	++++	+	+++	++
Glycerol	+	+++	++	+	++	+++	+	+
Fructose	+++	+	+++	+++	++	+++	+++	+
Arabinose	+	+	++	++++	+	+++	++	+
Rhamnose	+	++	-	+	+	-	+	+
Glucose	++++	++	+++	+++	+	+++	+++	+
Manitol	+++	++	++	++	+	++	++	+
Sorbitol	++	++	-	-	+	-	-	+
Sucrose	++++	++	+++	-	++++	+	+++	+++
Myo-Inositol	++	+	++	++++	+	++	+	+
Mannose	+++	+++	+++	-	++	+++	+++	+
Tartaric acid	+	+	+	-	+	+	-	+
Fumaric acid	++	++	+	+	++	-	+	++
Cellulase	+++	++	+++	++	++	+++	+	+
Pectinase	-	+++	+	-	-	-	+	++++
IAA	-	++++	+++	-	-	++	-	++
Antibiotic resistant								
Kanamycin	S	R	S	S	S	S	S	R
Tetracyclin	S	S	S	S	S	S	S	S
Chloramphenicol	S	R	S	S	S	S	S	R
Streptomycin	R	S	S	S	R	S	S	S
Ampicillin	R	R	R	R	R	R	R	R
Erythromycin	S	S	S	S	S	S	S	S
Spectinomycin	S	R	S	S	S	S	S	S

Abbreviations, First letter is growing stage of rice (S; seedling stage, V; vegetative stage, R; reproductive stage), second letter is soil type (F; fertilizer amendment, N; no-fertilizer experiment, U; undisturbed experiment), and third letter is rice part (R; root, S; stem, L; leaf)

N_2-fixing activity = Ethylene concentration (nmol C_2H_4 $h^{-1}tube^{-1}$)

Duplicate plates were used for each test isolate

+, little growth; ++, normal growth; +++, Very good growth; -, No growth

R, resistant; S, sensitive

Table 11 N_2-fixing activities and biochemical characteristics of endophytic diazotroph bacteria isolates from rice (Continued)

	RUL2-2	**RUR2-2**	**RUR4-2**
N_2-fixing activity	**949.24**	**328.93**	**557.97**
Carbon source			
Xylose	+	-	-
Lactose	++++	-	+
Glycerol	+	-	++
Fructose	++	-	+++
Arabinose	+	+	++++
Rhamnose	+	-	+
Glucose	++	+	+++
Manitol	++	-	+++
Sorbitol	+	-	++
Sucrose	+++	+	+
Myo-Inositol	-	-	++++
Mannose	++	-	-
Tartaric acid	+	++	-
Fumaric acid	++	++	+
Cellulase	++	++	+++
Pectinase	-	++	-
IAA	++++	-	++
Antibiotic resistant			
Kanamycin	R	S	S
Tetracyclin	R	S	S
Chloramphenicol	R	S	S
Streptomycin	R	S	S
Ampicillin	R	R	R
Erythromycin	S	S	S
Spectinomycin	S	S	S

Abbreviations, First letter is growing stage of rice (S; seedling stage, V; vegetative stage, R; reproductive stage), second letter is soil type (F; fertilizer amendment, N; no-fertilizer experiment, U; undisturbed experiment), and third letter is rice part (R; root, S; stem, L; leaf)

N_2-fixing activity = Ethylene concentration (nmol C_2H_4 $h^{-1}tube^{-1}$)

Duplicate plates were used for each test isolate

+, little growth; ++, normal growth; +++, Very good growth; -, No growth

R, resistant; S, sensitive

BIOGRAPHY

Miss Janpen Prakamhang was born on October 20th, 1975 in Buriram, Thailand. She graduated with the Bachelor Degree of Animal Production Technology, Suranaree University of Technology in 1998. During her Master Degree enrollment in the School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology (2004-2006), she presented research work in the The 6th National Symposium on Graduate Research, October, 13 -14, 2006, Chulalongkorn University, Bangkok, Thailand (Poster presentation; in “Microbial Communities of Rice Endophytic Diazotroph Bacteria”). Second Proceedings of the International Conference on Integration of Science and Technology for Sustainable Development (ICIST) “Biological Diversity, Food and Agricultural Technology”, April, 26-27, 2007, King Mongkut’s Institute of Technology Ladkrabang (KMITL), Thailand (Oral presentation; in "Microbial Community and their *nif* Gene Expression of Rice Endophytic Diazotroph Bacteria”)