EFFECTS OF PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR) INOCULUM ON INDIGENOUS MICROBIAL COMMUNITY STRUCTURE UNDER CROPPING SYSTEM

Pongdet Piromyou

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

Suranaree University of Technology

Academic Year 2010

ผลกระทบของ หัวเชื้อ PGPR ต่อโครงสร้างชุมชนจุลินทรีย์ท้องถิ่นในระบบแปลงปลูก

นายพงษ์เดช ภิรมย์อยู่

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

สาขาวิชาเทคโนโลยีชีวภาพ

มหาวิทยาลัยเทคโนโลยีสุรนารี

ปีการศึกษา 2553

EFFECTS OF PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR) INOCULUM ON INDIGENOUS MICROBIAL COMMUNITY STRUCTURE UNDER CROPPING SYSTEM

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

Thesis Examining Committee

_____
(Prof. Dr. Nantakorn Boonkerd)
Chairperson

_____
(Assoc. Prof. Dr. Neung Teaumroong)
Member (Thesis Advisor)

_____
(Dr. Achara Nuntagij)
Member

_____
(Dr. Panlada Tittabutr)
Member

_____
(Assoc. Prof. Dr. Piyada Tantasawat)
Member

_____
(Prof. Dr. Sukit Limpijumnong)
Vice Rector for Academic Affairs

_____
(Asst. Prof. Dr. Suwayd Ningsanond)
Dean of Institute of Agricultural Technology

พงษ์เดช ภิรมย์อยู่ : ผลกระทบของ หัวเชื้อ PGPR ต่อโครงสร้างชุมชนจุลินทรีย์ท้องถิ่นในระบบแปลงปลูก (EFFECTS OF PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR) INOCULUM ON INDIGENOUS MICROBIAL COMMUNITY STRUCTURE UNDER CROPPING SYSTEM) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.หนึ่ง เตียอำรุง, 86 หน้า

แบคทีเรียกลุ่ม PGPR (plant growth promoting rhizobacteria) มีบทบาทสำคัญในระบบการเกษตรโดยเฉพาะอย่างยิ่งคือ การใช้ในรูปปุ๋ยชีวภาพ การทดลองนี้มีวัตถุประสงค์เพื่อคัดเลือกแบคทีเรียกลุ่ม PGPR สำหรับข้าวโพดเลี้ยงสัตว์ (*Zea mays* L.) และผักคะน้า (*Brassica alboglabra*) และศึกษาผลกระทบของการใส่เชื้อแบคทีเรียกลุ่ม PGPR ต่อโครงสร้างชุมชนจุลินทรีย์ท้องถิ่น โดยแบคทีเรียสายพันธุ์ *Pseudomonas* sp. SUT19 และ *Brevibacillus* sp. SUT47 พบว่ามีประสิทธิภาพในการส่งเสริมการเจริญของข้าวโพดเลี้ยงสัตว์ได้มากกว่าจากการใช้หัวเชื้อที่จำหน่ายเป็นการค้าแล้วได้แก่ *Azotobacter* sp. และ *Azospirillum* sp. ได้ทำการตรวจสอบประสิทธิภาพในการส่งเสริมการเจริญของข้าวโพดอาหารสัตว์โดยทำการทดลองระดับกระถาง และระดับแปลงทดลอง พบว่าในการทดสอบระดับแปลงทดลองแบคทีเรีย *Pseudomonas* sp. SUT19 และ *Brevibacillus* sp. SUT47 ที่ใช้ร่วมกับปุ๋ยอินทรีย์ สามารถส่งเสริมการเจริญเติบโตของข้าวโพดเลี้ยงสัตว์ได้สูงกว่าทุกการทดลอง ผลการวิเคราะห์ DGGE (Denaturing Gradient Gel Electrophoresis) ร่วมกับการใช้ Principle Components Analysis (PCA) ของยีน 16s rDNA จากชุมชนจุลินทรีย์บริเวณรากข้าวโพดเลี้ยงสัตว์ ยืนยันได้ว่า แบคทีเรียสายพันธุ์ *Pseudomonas* sp. SUT19 และ *Brevibacillus* sp. SUT47 สามารถอาศัยอยู่ได้ในบริเวณรากพืชตลอดระยะเวลาที่ทำการทดลอง และโครงสร้างชุมชนจุลินทรีย์มีความแตกต่างกันน้อยมากในทุกตำรับการทดลอง ในการประเมินผลกระทบของแบคทีเรียกลุ่ม PGPR ทั้งสองสายพันธุ์ต่อความหลากหลายของสายพันธุ์จุลินทรีย์ในบริเวณรากพืชนั้น พบว่าจุลินทรีย์สายพันธุ์หลักในชุมชนของจุลินทรีย์ไม่ถูกรบกวนโดยการใส่เชื้อแบคทีเรียกลุ่ม PGPR ทั้งสองสายพันธุ์ แต่ในทางตรงกันข้ามปัจจัยหลักขึ้นกับระยะการพัฒนาของพืชเช่นกัน ในกรณีของระบบการปลูกผักคะน้า แบคทีเรียสายพันธุ์ *Bacillus* sp. SUT1 และ *Pseudomonas* sp. SUT19 ถูกคัดเลือกด้วยหลักการเดียวกันกับที่ทดสอบในข้าวโพดเลี้ยงสัตว์ ประสิทธิภาพในการส่งเสริมการเจริญของผักคะน้าได้ทดสอบทั้งในระดับกระถาง และแปลงทดลอง ผลการทดลองแสดงให้เห็นว่าแบคทีเรียกลุ่ม PGPR สายพันธุ์ *Bacillus* sp. SUT1 และ *Pseudomonas* sp. SUT19 ที่ใช้ร่วมกับปุ๋ยอินทรีย์สามารถเพิ่มชีวมวลของผักคะน้าได้สูงสุด เมื่อเปรียบเทียบกับตำรับควบคุมที่ไม่ใส่เชื้อ และในการวิเคราะห์การเปลี่ยนแปลงชุมชนจุลินทรีย์ให้กระจ่างมากขึ้นได้ใช้เทคนิค DGGE ร่วมกับการวิเคราะห์ด้วยวิธี PCA แสดงให้เห็นว่าโครงสร้างชุมชนของจุลินทรีย์ในดินบริเวณรากของคะน้า

และดินบริเวณแปลงปลูกคะน้าที่ไม่มีการปลูกพืช มีความแตกต่างกันไม่ชัดเจน ยิ่งไปกว่านั้นผลการทดลองแสดงให้เห็นว่าจุลินทรีย์สายพันธุ์หลักในโครงสร้างชุมชนของจุลินทรีย์ไม่ถูกรบกวนโดยการใส่เชื้อแบคทีเรียกลุ่ม PGPR แต่ในทางตรงกันข้าม ปัจจัยหลักขึ้นกับอายุพืชเช่นเดียวกับการทดลองในข้าวโพดเลี้ยงสัตว์ อย่างไรก็ตาม โครงสร้างชุมชนของจุลินทรีย์กลุ่มอาร์คีแบคทีเรียในดินบริเวณรากผักคะน้ามีความสัมพันธ์ที่ไม่ขึ้นกับพืช เมื่อเทียบกับจุลินทรีย์กลุ่มอื่น การศึกษาครั้งนี้พบว่า ไม่สามารถใช้แบคทีเรียกลุ่ม PGPR เพียงกลุ่มเดียวกับพืชทุกชนิดได้ ดังนั้นการคัดเลือกแบคทีเรียในกลุ่ม PGPR ที่จำเพาะต่อพืชเป้าหมายจึงเป็นในขั้นตอนแรกของการประยุกต์ใช้แบคทีเรียกลุ่มนี้ และในการศึกษาครั้งนี้พบว่า การใช้แบคทีเรียสายพันธุ์ *Pseudomonas* sp. SUT19 และ *Brevibacillus* sp. SUT47 สามารถนำไปเป็นหัวเชื้อสำหรับการปลูกข้าวโพดเลี้ยงสัตว์ และพบว่าจุลินทรีย์สายพันธุ์ *Bacillus* sp. SUT1 และ *Pseudomonas* sp. SUT19 มีความเหมาะสมกับผักคะน้าด้วยเช่นกัน

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2553

ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

PONGDET PIROMYOU : EFFECTS OF PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR) INOCULUM ON INDIGENOUS MICROBIAL COMMUNITY STRUCTURE UNDER CROPPING SYSTEM. THESIS ADVISOR : ASSOC. PROF. NEUNG TEAUMROONG, Dr. rer. nat. 86 PP.

FORAGE CORN/CHINESE KALE/PGPR/COMMUNITY STRUCTURE

Plant Growth Promoting Rhizobacteria (PGPR) play an important role in agricultural systems, especially as biofertilizer. The objectives of this study were to select effective PGPR for forage corn (*Zea mays* L.) and Chinese kale (*Brassica alboglabra*) cultivation and to investigate the effect of their inoculation on indigenous microbial community structure. The *Pseudomonas* sp. SUT 19 and *Brevibacillus* sp. SUT 47 were selected on the basis of their better forage corn growth promotion when compared with two commercialized PGPR strains i.e. *Azotobacter* sp. and *Azospirillum* sp. inoculation. The efficiency of the selected PGPR on forage corn growth promotion was evaluated both in pot and field trials. In field experiment, using strains *Pseudomonas* sp. SUT 19 and *Brevibacillus* sp. SUT 47 mixed with compost can promote the growth the best among all treatments. Denaturing Gradient Gel Electrophoresis (DGGE) fingerprints of 16S rDNA amplified from total community DNA from rhizosphere together with Principle Components Analysis (PCA) confirmed that our isolates existed in rhizosphere throughout this study. Also, the microbial community structures were found to be slightly different among all treatments. In order to evaluate whether both strains of PGPR have an effect on species diversity in rhizosphere, DNA sequencing of excised DGGE bands was conducted. The results demonstrated that dominant species in microbial community structure were not interfered by both strains of PGPR, but strongly influenced by plant

development. In case of Chinese kale cultivation system, the *Bacillus sp.* SUT1 and *Pseudomonas* sp. SUT 19 were selected for determining their efficiency in Chinese kale growth promotion in both pot and field experiments. The results showed that the selected PGPR mixed with compost were able to increase biomass of Chinese kale better in comparison to the uninoculated control. In order to determine the microbial community shifting more clearly, DGGE and PCA were carried out, it revealed that microbial community structure was not clearly different from microbial community in bulk soil. In addition, the community changes were not interfered by PGPR, whereas strongly influenced by plant age, which is similar to what found in forage corn experiment. However, archeobacterial community structure in Chinese kale root rhizosphere was found to be more root-independent than other microbial communities. This study demonstrated that there is no universal strain of PGPR for every plant species, thus selection of PGPR on the basis of host preference is needed in the first step of application. This study also recommends that *Pseudomonas* sp. SUT 19 and *Brevibacillus* sp. SUT 47 can be applied as PGPR inoculum for forage corn, and *Bacillus sp.* SUT1 and *Pseudomonas* sp. SUT 19 are appropriate for Chinese kale.

School of Biotechnology

Academic Year 2010

Student's Signature _____

Advisor's Signature _____

Co-advisor's Signature _____

Co-advisor's Signature _____

ACKNOWLEDGMENTS

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

I would like to take this opportunity to thanks: Assoc. Prof. Dr. Neung Teaumroong, my advisor, for his encouragement, valuable suggestion and always intelligent reading. He gave me full freedom in my work, while at the same time he was always most supportive. My deep appreciation in also expressed to my co-advisor, Dr. Panlada Tittaburt and Prof. Dr. Nantakorn Boonkerd.

I gratefully acknowledge Prof. Hiroo Uchiyama, for his excellent advice and allowing me to carry out the DGGE part of this research at the Tsukuba University.

Beside people making my life has funny and happy, I very much appreciate all those people who have made things work as well as all my friends in School of Biotechnology.

Finally, my warm thanks are dedicated to my parents, who have always believed in me and give me all the possible support during the good and difficult times. I always keep them in my mind.

Pongdet Piromyou

CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

bp	=	base pair
°C	=	degree celcius
CFU	=	colony forming unit
cm^3	=	centimeter
DNA	=	deoxynucleotide
dNTP	=	deoxynucleotide 5’ triphosphate
et al.	=	et alia (and other)
g	=	gram
h	=	hour
kg	=	kilogram
m	=	meter
mg	=	milligram
mm	=	millimeter
min	=	minute
ml	=	milliliter
mM	=	millimolar
µM	=	micromolar
ng	=	nanogram
nm	=	nanometer
µm	=	micrometer

LIST OF ABBREVIATIONS (Continued)

PCR	=	polymerase chain reaction
pmol	=	picomol
ppm	=	parts per million
RNA	=	ribonucleic acid
rRNA	=	ribosomal RNA
rpm	=	revolution per minute
s	=	second
µg	=	microgram
µl	=	microlitre
UV	=	ultraviolet
V	=	volt
W/V	=	weigth per volume

CHAPTER I

INTRODUCTION

1.1 Significant of this study

Nowadays, microorganisms play an important role in agricultural system, especially the group of bacteria called plant growth promoting rhizobacteria (PGPR). PGPR are widely studied because of their potential for plant production under three characteristics. Firstly, PGPR acting as biofertilizers provide nitrogen via nitrogen fixation reaction, which can subsequently be used by the plants. Secondly, phytostimulators can directly promote the growth of plant, usually by the production of plant hormones. Finally, biological control agents are able to protect plant via root system from phyto-pathogenic organisms. The application of PGPR in agricultural system as inoculants is being very attractive since it would substantially reduce the use of chemical fertilizers and pesticides as well as a growing number of PGPR is markets in the developed countries as EU and USA. With the use of PGPR gaining acceptance, numerous bacterial species have been isolated and their capacity to promote plant growth has been investigated. In the search for efficient PGPR strains with multiple attributes, various genera of bacteria show promising results. Thus, bacteria genera including *Azotobacter*, fluorescent *Pseudomonas* species, *Rhizobium* and *Bacillus* are widely used (Teaumroong et al., 2010). At Suranaree University of Technology, PGPR as *Azotobacter* and *Azospirillum* have been used in agricultural system and commercialized. This research was focued on selection of PGPR that can

promote growth of maize (*Zea mays indurata*) and Chinese kale (*Brassica alboglabra*) better than using *Azotobacter* sp. and *Azospirillum* sp. The initial step of this research was started with the screening of PGPR from each type of plants by determining root elongation. The top two strains of PGPR were collected and tested on the basis of inoculated into rhizosphere of target plants in order to confirm benevolence properties with plants. In addition, the ecological impact of PGPR on microbial community structure which is an important issue when attempting to better define usage conditions for these inoculants must concerned. Therefore, the effect of inoculant on microbial community structure of indigenous bacteria and fungi were demonstrated by using the Denaturing Gradient Gel Electrophoresis (DGGE) and Principle Component Analysis (PCA) techniques.

1.2 Research objectives

This research were aimed at selection the most effective PGPR strains as inocula and investigation of the effect of selected inoculant strains on microbial community structure in rhizosphere of maize (*Zea mays L.*) and Chinese kale (*Brassica alboglabra*).

CHAPTER II

LITERATURE REVIEWS

2.1 Roles of PGPR

Fertilizers are essential components of modern agriculture because they provide essential plant nutrients. However, overuse of fertilizer can cause negative environmental impacts. One potential way decrease unanticipated environmental impacts resulting from continued use of chemical fertilizer is inoculation with plant growth promoting rhizobacteria (PGPR). These bacteria exert beneficial effect on plant growth and development. These important mechanisms for these beneficial effects are briefly described as followed:

A. Biofertilizers: These groups of bacteria can facilitate plant nutrient uptake via different direct mechanisms such as nitrogen (N) fixation, solubilization of phosphate (P) and synthesis of siderophore for iron sequestration making nutrients more available to plants. Though a variety of nitrogen fixing bacteria so called biofertilizers like *Rhizobium*, *Azotobacter*, *Bacillus*, *Pseudomonas*, *Azospirillum* and *Acetobacter* has been isolated from the rhizosphere of various crops (Steenhoudt and Vanderleyden, 2006), interest in the beneficial nitrogen fixing growth promoting rhizobacteria-plant association has increased recently due to their potential effect for replacing chemical N-fertilizer (Vessey, 2003).

B. Phytostimulators: The promotion of plant growth regulators such as auxin, cytokinin and gibberellin by PGPR may also aid in growth and development of host plant species. *Azospirillum brasilense*, one of the most studied PGPR has been shown to improve growth development by the production of auxin, cytokinin and gibberellin. Inoculation of plants with this bacterium causes morphological changes, such as an increase in root surface area through the production of more root hair, which in turn enhance mineral uptake (Steenhoudt and Vanderleyden, 2006). In addition**,** PGPR include the strains in the genera *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Azotobacter*, *Bacillus*, *Beijeriakia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Rhizobium*, *Seratia*, etc. were also reported as phytohormone producer (Lucy et al., 2004).

C. ACC deaminase enzyme

Even though, ethylene is an important growth hormone, which is produced by almost all plants and mediates a wide range of different plant response and developmental processes. The higher concentrations of ethylene are inhibitory to plant growth. Any factor/stimulus which causes a change in the endogenous levels of ethylene in a plant results in modified growth and development. Recently, inoculation with specific bacteria has been shown to alter the endogenous levels of ethylene, which subsequently led to changes in the growth and development of inoculated plants (Glick et al., 1998).

The model description of the mode of action of PGPR containing 1-aminocyclopropane-1-carboxylate (ACC)-deaminase was precisely elaborated originally by Glick et al., (Glick et al., 1998). They comprehensively addressed the

question, how bacterial ACC deaminase having a low affinity for ACC, can effectively compete with the plant enzyme, ACC oxidase, which has a high affinity for the same substrate, with the result that the plant's endogenous ethylene concentration is reduced. They argued that the biological activity of PGPR relates to the relative amounts of ACC deaminase and ACC oxidase in the system under consideration. For PGPR to be able to lower plant ethylene levels, the ACC deaminase level should be at least 100- to 1,000-fold greater than the ACC oxidase level. This is likely to be the case, provided that the expression of ACC oxidase has not been induced. PGPR synthesize and secrete indole-3-acetic acid (IAA), which gets adsorbed on the seed or root surface of the plants (Fallik et al., 1994; Hong et al., 1991) from tryptophan and other small molecules present in seed or root exudates. Some of the newly synthesized IAA is taken up by the plant and, in conjunction with the endogenous plant IAA can further stimulate plant cell proliferation and elongation. In the meanwhile, IAA stimulates the activity of the enzyme ACC synthetase to convert S-adenosyl-L-mathionine SAM into ACC (Kende, 1993). According to the model outlined by Glick et al (Glick et al., 1998), a significant portion of ACC may be exuded from plant roots or seeds and taken up by the soil microbes or hydrolyzed by the vital microbial enzyme ACC deaminase to yield ammonia and α-ketobutyrate. The uptake and subsequently hydrolysis of ACC by microbes decreases the amount of ACC outside the plant. Furthermore, the equilibrium between the internal and the external ACC levels is maintained through exudation of more ACC into the rhizosphere. Soil microbial communities containing ACC deaminase activity cause plants to biosynthesize more ACC than the plant would otherwise need and stimulate ACC exudation from plant roots, while providing

microorganisms with a unique source of nitrogen (ACC), and consequently, the growth of microorganism containing ACC deaminase is accelerated in the close vicinities of plant roots as compared to the other soil microorganisms. By doing so, not only the ACC level is lowered within the plant but also the biosynthesis of the stress hormone ethylene is inhibited. A schematic representation of this model is shown in Fig. 2.1. Thus, a plant inoculated with bacteria containing ACC deaminase exhibits more root growth. In a number of studies, inoculation with PGPR containing ACC deaminase has been unequivocally shown to alter the endogenous levels of ethylene, which subsequently leads to changes in plant growth.

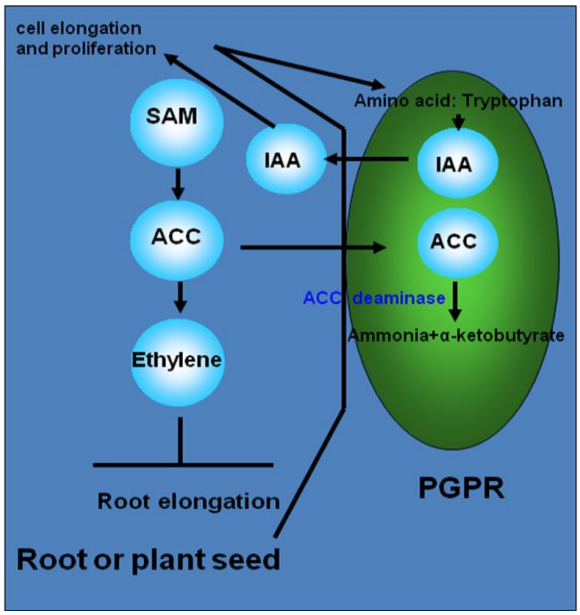


Figure 2.1 Model of action of bacterial ACC deaminase (Tarun et al., 1998)

D. Biological control: PGPR also enhance plant growth via suppression of phytopathogens by a variety of mechanisms such as antibiotics, fungal all wall-lysing enzymes or hydrogen cyanide which suppress the growth of fungal pathogens. Antagonistic microbe-microbe interactions mediated by Pseudomonas species are

major drivers in the biological control of phytopathogenic fungi in the rhizosphere and may indirectly benefit plant growth and survival (Winding *et al.,* 2004). The synthesis of molecules involves in antagonistic interactions and disease suppression such as the antibiotic 2, 4-diacetylphoroglucinal (2, 4-DAPG), pyoluteocin, etc (Costa et al., 2007).

E. Biofilm formation: Biofilms are defined as bacterial communities surrounded by a self-produced polymeric matrix, and reversibly attached to an inert or a biotic surface. After attachment to the surface, the bacteria multiply, and the communities acquire a three-dimensional structure. The major components of biofilm are typically water and bacterial cells. The next most component is a polysaccharide matrix composed of exopolysaccharide, which provides a physical barrier against antibiotic, host defense substances and protection against various environmental stresses. In general, cell aggregation involves natural polymers such as complex polysaccharides and polyaminoacids, which are excreted or exposed at cellular surfaces. These polymeric molecules are of sufficient length to form bridges between the microbial cells. The specificity, the high affinity, and the reversibility of microbial aggregation are not due to covalent bonding but to the rather highly selective affinity of complementary surfaces. The specificity of biological interactions derives from the stereochemical complementarity of molecular structures. The main forces involved in the cell-to-cell adhesion are hydrogen bonding, ionic interactions, Van der Waals forces, and even hydrophobic interactions, depending on the system. *Azospirillum* is one of PGPR which has been studied intensively. The mechanism of attachment of azospirilla to plant roots still remains unclear. A fibrillar material is observed in *Azospirillum*-root association, but its nature is still unknown. In vitro binding assays showed that root attachment by *Azospirillum* is a biphasic process. In a first step, the adhesion of

A. brasilense to wheat roots is mediated by an adhesion, closely associated with the polar flagellum. This kind of adsorption is relatively rapid (occurring within 2 h), weak, and reversible. Cells lacking the polar flagellum fail to adsorb to wheat roots, whereas purified polar flagella specifically adsorb onto the root surface. A second step of firm and irreversible anchoring, in which bacterial aggregates are also formed, is thought to be mediated by extracellular polysaccharides. establishment of the bacterial-root association. Cell aggregation could increase survival of *Azospirillum* cells under diverse stress conditions. This phenomenon may also be important during root colonization where cell aggregates are commonly observed. Although much evidence has accumulated during recent years indicating the involvement of extracellular polysaccharides and proteins in both cell aggregation and root attachment process, the precise mechanisms of these phenomena remain unexplained. The difficulty in elucidating these processes derives from their complexity, because it seems that they are mediated by various cell-surface components. Moreover, data from different works are sometimes contradictory. This can be partially explained by the high number of factors that affect these adhesion processes, such as strain variability, culture growth conditions, culture age, bacterium-plant interaction variability in the case of root attachment, physical and chemical conditions of the binding assays, and more.

2.2 Application of PGPR with forage corn and Chinese kale

Maize (*Zea may* L.) is one of five major crops in Thailand. In addition to rice, cassava, sugarcane, and rubber, maize occupies a major portion (about 33%) of Thai upland farmlands. The maize area began to decline and occupied only 7.3 million Rais (nearly 1.2 million ha) by 2002-03, with a production of around 4.5 million tons.

In 2000, 1.35 million ha were planted to maize, and of all upland farm households about 37% (740,000) cultivated maize. At present, Northern Thailand is the largest maize producing region, accounting for about 49% of the national acreage, followed by the Northeast Region with 26%. Fertilizer prices for maize were around 1,100-1,500 Bahts per 50-kg bag. The most common fertilizers used in maize production are urea (46-0-0), Triple 15 (15-15-15), and 16-20-0, which cost 1,340, 1,450 and 1,150 Bahts per 50-kg bag, respectively (www.doae.go.th/spp/mark/p16_31may45.htm). However, this has a heavy impact on the natural and human environment, as well as on human health, through the pollution of soils, waters, and the whole food supply chain. There are some applications of *Pseudomonas* spp. for improve growth and yield of maize in field (Baby *et al.*, 2006), *Bacillus subtilis* strain Kodiak R was quoted as that is highly effective for maize protection from the pathogens *Fusarium* and *Rhizoctonia* (Backman et al. 1994).

In case of vegetables, Chinese kale is one of ten favorable vegetables in Thailand. The production area of Chinese kale is around 4.3 % from vegetable production area (4,722.9 sq.km). Fertilizer prices for Chinese kale are around 1,400 Bahts per 50-kg bag. The most common fertilizers use in Chinese kale production are triple 16 (16-16-16) and 25-7-7, which cost 1,400 and 1,420 Bahts per 50-kg bag, respectively (www.doae.go.th/spp/mark/p16_31may45.htm). However, no report regards to application of PGPR with Chinese kale was found.

2.3 Applications of PGPR in agriculture

Researchers in the former Soviet Union and India conducted widespread tests in the early to the mid part of the 20th century studying the effects of PGPR on different crops. Though results from different experiments were not harmonized and were often inconsistent, up to 50 to 70% yield increases were reported. Inconsistency of results was due to a lack of quality in experimental design and analysis of results (Lucy et al., 2004). Moreover, during this time an understanding of the detailed mechanisms of plant growth promotion by rhizobacteria was largely unknown. Nevertheless, these field experiments provided clues concerning the optimal conditions for bacterial colonization and growth promotion of target crops. The results of some studies of the effect of free-living rhizobacteria on various crop plants are given in Table 2.1. Plant growth benefits due to the addition of PGPR include increases in germination rates, root growth, yield including grain, leaf area, chlorophyll content, magnesium content, nitrogen content, protein content, hydraulic activity, tolerance to drought, shoot and root weights, and delayed leaf senescence. Another major benefit of PGPR use is disease resistance conferred to the plant, sometimes known as 'biocontrol'.The use of PGPR to increase crop yield has been limited due to the variability and inconsistency of results between laboratory, greenhouse and field studies (Lucy et al., 2004). Soil is an unpredictable environment and an intended result is sometimes difficult to obtain (Bashan 1998). For example, in a study by Frommel et al, 1993 poor colonization of the PGPR on plant roots occurred at one site due to adverse conditions, including high *Verticillum* infection of the soil, low soil pH, high mean temperature, and low rainfall during the growing season. These undesirable growing conditions most likely contributed to

the low root colonization (Dobbelaere et al. 2001; Klein et al. 1990; Parke1991; Suslow and Schroth 1982). Climatic variability also has a large impact on the effectiveness of PGPR (Okon and Labandera-Gonzalez 1994) but sometimes unfavourable growth conditions in the field are to be expected as a normal functioning of agriculture. Increased yields obtained with wheat inoculated by Pseudomonas species in the growth chamber have also been observed in the field (Weller and Cook 1986). Even though there is a possibility of great variability in field results, if a positive effect of a PGPR is seen on a specific crop in greenhouse studies, there is a strong likelihood that those benefits will carry through to field conditions. Some reports that several related PGPR could promote growth of maize (*Zea may* L.) are Baudoin et al., 2009; Costa et al., 2005; El Zemrany et al., 2006; Kumar et al., 2007; Mar Vazquez et al., 2000; Shaharoona et al., 2006 etc.

Table 2.1 Examples of free-living plant growth promoting rhizobacteria tested on various crop types (Lucy et al., 2004).

Bacteria	Plant	Conditions	Results of addition of bacteria to plant	Reference
Azospirillum (local isolatesfrom Argentina)	Wheat, Maize	Field	- in wheat cultivars over seven seasons, increases of yield from 15 to 30 %, and increases in yield of 50-60% when fertilized - over six seasons, increases of maize yield from 15 to 25% observed, and with fertilization, yield increased up to 40%	Okon and Labandera-Gonzalzelze 1994
Azospirillum brasilense	Guinea grass Pearl millet, Digitaria decumbens	Field	- greater dry matter yield compared to uninoculated controls - approximately 40 kg/ha per year of nitrogen estimatedas saved due to inoculation	Smith et al. 1978
Azospirillum brasilense	Finger millet, Sorghum, Pearl millet	Field	- average of up to 15% yield increase for finger millet -for sorghum, average increase is 19% -in ten years of study, *Azospirillum* successful in signifitly increase yield in 60% of trials	Rao 1986

In case of Thailand, PGPR as *Azotobacter* sp. and *Azospirillum* sp. have been produced by Suranaree University of Technology (SUT) and mixed with good quality of organic fertilizer, thus so called bioorganic fertilizer (BOF). The applications of BOF were tested in different areas and plants. In order to compare the plant yields obtained by using BOF and chemical fertilizers in the field experiment, vegetables and rice plants were used. The results indicated that application of BOF could provide almost the same yield of rice as chemical fertilizer amendment. The use of together with chemical fertilizer (half of recommended amount of each) resulted in the highest yield. In case of vegetable cultivation, results demonstrated that the inoculation of the leguminous plants *Sesbania rostrata* cowpea (*Vigna unguiculata*) with the

appropriate rhizobial strains as green manures followed by plowing before BOF application could enhance the yield of Chinese kale. This demonstrates that application of green manure could enhance the effect of BOF to become more advantageous, thus confirming its replacement of chemical fertilizer application (Teaumroong et al., 2010).

2.4 Microbial community structure

The interaction between plants and microbes is essential for plant health and growth and should be considered when aiming to combine high yields with farming practices which are environmentally friendly. Interactions in the rhizosphere, the part of the soil which is highly influenced by the plant, are of central importance (Copenhagen, 1997). Composition, abundance and dynamics of the microbial community in the rhizosphere play an important role and may have a positive or negative influence on plant growth. Microbes are essential for the mobilisation of plant nutrients and may produce plant growth hormones which are important for plant development (Lynch, 1990). Other microorganisms act as biocontrol agents and protect the plants from phytopathogenic bacteria and fungi (Bashan and Holguin, 1998). In contrast plant pathogenic microbes can have a severe impact on plant health. Plants can influence the composition of the microbial rhizosphere community by several mechanisms. Root exudates (rhizodeposition) have been identified as the most important factor for the development of a typical rhizosphere microflora (Lynch and Whipps, 1990). A high percentage (30-60%) of the CO_2 bound by photosynthesis is released to the rhizosphere by roots, whereas the composition and amount is depending on the plant such as sugar, amino acids and

organic acids (Copenhagen, 1997). Therefore, potential effects of plant and PGPR of crops on the structural and functional diversity of bulk and rhizosphere microbial communities should be assessed under greenhouse and subsequently under field conditions. In addition, some previous reports, displayed the relationship between the plant-microbe interaction and the microbial community structure of varies plant host (Baudoin et al., 2002; Baudoin et al., 2003; Costa et al., 2006; Gomes et al., 2003; Marschner and Baumann, 2003; Yang and Crowley, 2000).

2.5 Approach for soil microbial community structure analysis

Although originating from plant-associated microenvironment, beneficial bacteria, if applied to plant roots in sufficient numbers, may perturb indigenous microbial populations and the important ecological functions associated therewith (Bankhead et al., 2004; Winding et al., 2004). Therefore, unwanted, unspecific actions of the introduced beneficial microorganism against non target organism have to be assessed. To this end, knowledge concerning the microbial ecology of the target habitats is necessary for reasonable risk assessment studies relating to the release of beneficial microorganism can be analyzed by common cultivation technique. Several DNA-based, cultivation-independent methods have been developed to overcome the limitation of cultivation techniques (Smalla, 2004).

Culture-independent methods have been used increasingly to study microbial communities and their activities in environmental sample, because the majority of microorganism cannot be cultivated on standard laboratory media (Amann et al., 1995). Alternative approaches, base on polymerase chain reaction (PCR)

amplification of 16s rRNA from DNA extracted from soil samples followed by used of DNA fingerprinting methods, such as DGGE (Denaturing gradient gel electrophoresis) and T-RFLP (Terminal restriction fragment length polymorphism) are new studying complex bacteria communities.

Denaturing gradient gel electrophoresis (DGGE) is an alternative DNA fingerprinting technique in which DNA fragments obtained after PCR amplification of target genes from complex microbial communities are separated according to their sequence (guanine + cytosine or G+C content) (Murray et al., 1996; Nakatsu, 2007). The separation of the different DGGE bands depends on the melting behavior of the PCR product and not on the size of the fragment. The advantage of this technique is that DGGE bands of interest can be excised from the gel and further analyzed by cloning and sequencing (Avrahami and Conrad, 2003; Nakatsu, 2007; Watanabe et al., 2006).

CHAPTER III

MATERIALS AND METHODS

3.1 Soil sampling and bacterial isolation

Bacterial strains were isolated from the root of forage corn (*Zea mays* L.) and Chinese kale (*Brassica alboglabra*) grown at different provinces i.e. Chiangmai (18°47’25”N/98°58’54”E), Lampang (18°17’25”N/99°30’25”E), Nakhon Sawan (15°41’0”N/100°7’0”E), Saraburi (14°51’0”N/100°91’0”E), and Nakhon Ratchasima (14°58’0”N/102°7’0”E) in Thailand. Plants were uprooted along with good amount of rhizosphere soil, brought immediately to the laboratory in polyethylene bags and air-dried within 2 h. The non-rhizosphere soil was removed from the roots then, dipping and gentle shaking in sterilized water under aseptic conditions for 5 min. This step was repeated and only root samples were collected. The root soil suspension was obtained by dipping the root and vigorously vortexing in 10 ml of 1 % sterilized tween 80 for 5 min. The obtained root suspension was then further diluted with ten-fold dilution technique prior to spread on LG (N-free) medium (10 g glucose, 0.41 g KH_2PO_4, 0.52 g K_2HPO_4, 0.2 g $CaCl_2$, 0.05 g Na_2SO_4, 0.1 g $MgSO_4.7H_2O$, 0.005 g $FeSO_4.7H_2O$, 0.0025 g $Na_2MoO_4.2H_2O$ per liter) (Lipman, 1904). The higher dilutions between 10^6-10^8 were focused in order to obtain bacterial isolates represented high density of root-adhering bacteria. The plates were incubated for 2 days at 28^{o}C and colonies showing morphological difference were collected for further analysis.

3.2 Selection of appropriate bacterial strains

Seeds of forage corn and Chinese kale were surface sterilized by soaking in 70% ethanol for 1 min followed by incubation in 1% sodium hypochlorite for 10 min. The bleach solution was suctioned off and the seeds were thoroughly rinsed with sterilized distilled water at least five times. The seeds were germinated on sterilized filter paper sheets in the Petri dish. Each seed was inoculated with ~ 10^8 CFU/ml of single rhizobacterial isolate. Seeds inoculated with sterilized 0.85% NaCl solution were used as control. The seeds were covered with other sterilized filter paper sheets and 10 ml of sterilized distilled water was added to each Petri dish to moist the filter paper sheets and allow the germination. The plates were incubated at room temperature for a week, and the root growth (root elongation and root weight) was examined. This was conducted as five replicates. The top ten bacterial isolates that can promote the root growth were selected.

3. 3 Acetylene reduction assay (ARA)

The N_2-fixing activity of the selected bacterial culture was examined by acetylene reduction assay (ARA). The reactions were carried out in a 21-ml test tube containing 7 ml of bacterial culture in LG (N-free) medium and incubated at 28^{o}C for 2 days (Hardy et al., 1968). Ten percentages (v/v) of gas phase in the headspace was replaced with acetylene and further incubated at 28^{o}C for 24 h. Ethylene production was measured by using gas chromatograph (GC) with a flame ionization detector and PE-Alumina column equipped, 50m x 0.32mm x 0.25µm (Perkin Elmer, USA). After completion of the ARA, the cells were predigested by adding 10% SDS (W/V) and

sonicated briefly. Total protein concentration of the cell suspension was determined according to Lowry's method (Lowry et al., 1951). One unit of nitrogenase enzyme refers to the activity to form 1 nmol of ethylene per hour under this condition. The activity of the enzyme was calculated as nmol of ethylene forming/h/mg of protein. Standard curve of ethylene was constructed by varied concentration of pure ethylene.

3.4 Indole-3-acetic acid (IAA) production

Production of indole-3-acitic acid (IAA) was colorimetrically determined as described by Fukuhara et. al. (1994). The isolates were grown in LG (N-free) broth medium supplemented with L-tryptophan (100 mg/L) at 28^{o}C. The supernatant of the stationary phase culture was obtained by centrifugation at 12,000 rpm for 15 min. IAA produced per ml culture was estimated by mixing 5 ml Salkowsky reagent (0.01 M $FeCl_2$ in $HClO_4$), followed by measuring the color changes at 530 nm. (Costacurta et al., 2006). Varied amounts of pure indole-3-acitic acid were used as standard.

3.5 ACC deaminase activity assay

Selected bacterial isolates were cultured in LG (N-free) medium at 28^{o}C for 2 days with shaking at 200 rpm until cell reached the early stationary phase. The cells were collected by centrifugation at 5,000 rpm for 5 min and washed twice with minimal medium (Penrose and Glick, 2003). Cell pellets were suspended in 15 ml of minimal medium supplemented with 1 mM ACC (1-aminocyclopropane-1-carboxylate), and further incubated at 28^{o}C for 24 hours with shaking at 200 rpm to induce ACC deaminase enzyme production. ACC-deaminase activity was measured

as described by Penrose and Glick (2003).

3.6 Microtiter plate biofilm production assay

Each selected strains were grown in 10 ml of LB medium at 28^{o}C overnight. Biofilm formation assays were performed with LB medium. Overnight cultures in LB were transferred (0.1 ml) to 10 ml of LB and vortexed. After vortexing, 100 µl volumes were transferred into eight Poly vinyl chloride (PVC) microtiter plate wells per strain. Plates were made in duplicate, incubated, and covered at 12 h. Each plate included eight wells of LB without selected strains as control wells.

The cell turbidity was monitored using a microtiter plate reader (Bio-Rad), at an optical density at 595 nm (OD_{595}). After 12 h incubation period, medium was removed from wells and microtiter plate wells were washed five times with sterile distilled water to remove loosely associated bacteria. Plates were air dried for 45 min and each well was stained with 150 µl of 1% crystal violet solution in water for 45 min. After staining, plates were washed with sterile distilled water five times. At this point, biofilms were visible as purple rings formed on the side of each well. The quantitative analysis of biofilm production was performed by adding 200 µl of 95% ethanol to destain the wells. One hundred microliters from each well was transferred to a new microtiter plate and the level (OD) of the crystal violet present in the destaining solution was measured at 595 nm (O'Toole and Kolter, 1998).

3.7 GFP-tagging of Pseudomonas strains

Plasmid DNA (pUCP26-gfp) for electroporation was purified using the Qiagen plasmid purification kit (Qiagen) as recommended by the manufacturer. Approximately 1 μg DNA of each plasmid was used for the electroporation.

An important aim of this experiment was to obtain GFP-tagged strains that could be used for root colonization detection on root surface. To show that the *gfp* vectors were useful for this purpose, rhizosphere experiments were carried out with *Pseudomonas* sp. SUT19::*gfp*. Forage corn and Chinese kale seeds were surface-sterilized as previously described and germinated on moist filter paper for approximately 16 h. The seeds were coated with *Pseudomonas* sp. SUT19::*gfp* and planted in sterilized soil. The seedlings were incubated at 30°C with a light/dark cycle 16/8 h. After 7 days, the plants were harvested. The fixative soil was removed by vortexing the roots in 0.95% NaCl for 10 s, after which the roots were transferred to fresh 0.95% NaCl and stored at 4°C before analyze. GFP was monitored using a confocal laser scanning microscope and detection of GFP of wavelengths above 590 nm from root surface (de Kievit, 2009).

3.8 16S rRNA gene analysis

The PGPR isolates SUT1, SUT 19 and SUT 47 were identified by cloning and nearly sequencing of the 16S rRNA gene. The chromosomal DNA were extracted (Prakamhang et al., 2009) and used as a DNA template in PCR reactions. 16S rRNA gene was amplified by using the primers pair (Ovreas et al., 1997). The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany).

The amplicons were ligated into the pGEM®-T Easy Vector System (Promega, USA) and then further transformed into *Escherchia coli* DH5∞ competent cells, following the manufacturer's protocol. Cells were grown overnight at 37 °C on Petri plates containing S-gal®/LB agar blend (Sigma–Aldrich) supplemented with 100 µg ml^{-1} ampicillin (Sigma–Aldrich). White colonies (transformants) were picked randomly from the plates for colony PCR using the SP6 and T7 primers (Promega). Twenty-five microliter PCR reactions containing 0.1 U $µl^{-1}$ GoTaq® DNA Polymerase (Promega), 1× PCR buffer and 1.5 mM $MgCl_2$ supplied with the enzyme, 0.2 mM dNTPs, 0.2 µM of each primer were performed using an PERKIN ELMER, GeneAmp PCR System 2400 under the following reaction conditions: 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 48 °C for 45 s, and 72 °C for 45 s, and a final extension step at 72 °C for 10 min. PCR products were evaluated by running a small volume of product in an agarose gel. DNA sequencing was performed by MACROGEN company (Korea). The DNA sequences were generated and the most closely related sequences were obtained from the NCBI database.

3.9 Plant experiment

3.9.1 Leonard jar experiment

Plastic jar (383 cm^3) was filled with sand and a half strength Hoagland solution (150 ml) (Hoagland and Arnon, 1950) was applied through a wick to provide nutrients to plants. The whole apparatus was autoclaved (25 min at 121^oC) prior to the transplantation of seedlings. Surface-disinfected forage corn and Chinese kale seeds were germinated on sterilized filter sheets in Petri dish. Uniformly germinated

seeds of forage corn and Chinese kale were transplanted to the plastic jar containing sand under aseptic conditions. One milliliter of selected 2-day-old inoculum (10^8 CFU ml^{-1}) was applied to the seedling, 2 days after transplanting. This was conducted as three replicates per single bacterial isolate. Plants were grown under controlled environmental conditions of 28 ± 2^oC on 16/8 hours day/night cycle. The shoot and root were harvested after one month of planting. The isolates SUT 19 and SUT 47 displayed the highest forage corn biomass when compared with commercial strains (*Azotobacter* sp. and *Azospirillum* sp.). And the isolates SUT 1 and SUT 19 displayed the highest Chinese kale biomass when compared with commercial strains (*Azotobacter* sp. and *Azospirillum* sp.), then isolated strains were collected for further analysis. In addition, to enumerate the number of root colonizing cell, root sample were collected and vigorously vortexing in 20 ml of 1% sterilized tween 80 for 5 min. The obtained suspension was then diluted with ten-fold dilution technique prior to spread on LG (N-free) medium. The plates were incubated for 2 days at 30^oC before colony counting.

3.9.2 Pot and field experiments

Both pot and field experiments of forage corn were conducted from January 2009 to March 2009 as first crop (average rainfall = 40 mm and temperature range 27.1 to 38^oC) and July 2009 to September 2009 as second crop average (rainfall = 150 mm and temperature range 23 to 32^oC). For the pot experiments, the same soil samples used in field experiment from Suranaree University of Technology farm (14^{o}59’0”N/102^{o}7’0”E) were collected, air-dried, sieved (2-mm/10-mesh) and analyzed for physico-chemical characteristics before filling the pots. The soil was clay

loam having pH 7.26; electrical conductivity (EC), 192.5 µS cm^{-1}; available phosphate (Bray II), 65.73 ppm; potassium (NH_4OAc), 180 ppm; and organic matter (Walkley and Black, 1934), 1.64 %. The selected bacterial isolates (SUT 19 and SUT 47) and commercial strains were inoculated with and without the compost. The compost was applied at one ton per ha to both pot and field experiments. The compost was analyzed for physico-chemical characteristics before applied into pots and field. The analyses of compost were ; pH 8.26; electrical conductivity (EC), 4.18 µS cm^{-1}; phosphorus (wet digestion), 2.87 %; potassium (wet digestion), 0.95 %; nitrogen (Kjeldahl method) (Bremner, 1996), 1.03 %; and organic matter (Walkley and Black, 1934), 15.01 %. The forage corn seeds were sown in soil filled pots (12 kg soil per pot) receiving nutrient inputs of N, P and K at 120, 75 and 50 kg rai^{-1} in the form of urea, diammonium phosphate and muriate of potash, respectively. The forage corn was inoculated (10^8 CFU $seed^{-1}$) with the isolated strains and commercial strains (*Azotobacter* sp. and *Azospirillum* sp.) after a day and five days of sawing. The pots were arranged in completely randomized design with four replications at ambient light. The plants were harvested after two, five and eight weeks. The field experiments were conducted along with university farm agronomic practices (watering around 1.5 mm day^{-1}). Seeds of forage corn were sown with a single row seed drill keeping row to row distance of 25.0 cm. Each experiment was conducted in randomized complete block design (RCBD) with four replications. The forage corn was harvested after two, five and eight weeks after sowing. Data of plant biomass were recorded and the samples from each rhizosphere soil were collected for PGPR microbial community analyses.

Both pot and field experiments of Chinese kale were conducted from September to November 2009 as first crop (average rainfall = 69 mm and temperature range 22to 35^{o}C) and April to June 2010 as second crop average (rainfall = 46.6 mm and temperature range 25.5 to 38^{o}C). For the pot experiments, the same soil samples used in field experiment from famer farm at Nakhon Ratchasima (14°58'0"N/102°7'0"E) were collected, air-dried, sieved (2-mm/10-mesh) and analyzed for physico-chemical characteristics before filling the pots. The soil was clay loam having pH 6.55; electrical conductivity (EC), 205.3 µS cm^{-1}; available phosphate (Bray II), 105.55 ppm; potassium (NH_4OAc), 235 ppm; and organic matter (Walkley and Black, 1934), 1.39 %. The selected bacterial isolates (SUT 1 and SUT 19) and commercial strains were inoculated with and without the compost. The compost was applied a ton per ha to both pot and field experiments. The compost was analyzed for physico-chemical characteristics as same with forage corn experiment. The Chinese kale seeds were sown in soil filled pots (5 kg soil per pot) receiving nutrient inputs of N, P and K at 70, 50 and 50 kg rai^{-1} in form of urea, diammonium phosphate and muriate of potash, respectively. The Chinese kale was inoculated (10^8 CFU seed^{-1}) with the isolated strains and commercial strains (*Azotobacter* sp. and *Azospirillum* sp.) after a day and five days of sawing. The pots were arranged randomly with four replications at ambient light. The plants were harvested after three, five and seven weeks. The field experiments were conducted along with famer farm agronomic practices (watering around 3 mm day^{-1}). Seeds of Chinese kale were sown with a single row seed drill keeping row to row distance of 7.0 cm. Each experiment was conducted in randomized complete block design (RCBD) with four replications. The forage corn was harvested after three, five and seven weeks after

sowing. Data of plant biomass were recorded and the samples from each rhizosphere soil were collected for PGPR microbial community analyses.

3.10 Total community DNA isolation and PCR amplification of universal 16s rRNA of eubacterial, archeobacterial and fungal genes fragment and DGGE analysis

DNA extraction was performed using the Ultra Clean Soil DNA kit (MoBio Laboratories, Solana Beach, Califonia, USA). A portion of 0.25 g of forge corn and Chinese kale rhizosphere was processed according to the protocol provided by the manufacturer with an additional bead-beating step using as cell homogenizer (Braun, Melsungen, Germany) to achieve a harsh cell lysis. Amplification of eubacterial 16S rRNA gene was performed using universal primers PBA338F (5'-ACT CCT ACG GGA GGC AGC AG-3') and PRUN518R (5'-ATT ACC GCG GCT GCT GG-3') which yielded products of approximately 200 base pairs (Ovreas et al., 1997). The GC-clamps (5'-CCC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GCC G-3') (Costa et al., 2005) was added to the 5'end of the forward primer. The PCR reaction contained 50 ng of DNA from soil samples, 0.5 µmol of each primer, 0.2 mM dNTP, 1x PCR buffer, 3 mM $MgCl_2.2H_2O$ and 0.05 U Taq DNA polymerase (Promega, USA). The thermal cycler were performed using an PERKIN ELMER, GeneAmp PCR System 2400 under the following reaction conditions: 94^{o}C for 5 min (1 cycle), 94^{o}C for 30 sec, 55^{o}C for 30 sec, 72^{o}C for 30 sec (35 cycles) and final 72^{o}C for 10 min (1 cycle).

Archaeal 16S rRNA genes were amplified by using the forward primer PARCH340F (5'-CCC TAC GGG G(C/T)G CA(G/C) CAG -3') and a reverse primer PRAH519R (5'-TTA CCG CGG C(G/T)G CTG-3') which yielded products of approximately 200 base pairs (Moeseneder et al., 1999). The GC-clamp (Costa et al., 2005) was added to the 5'end of the forward primer. The PCR reaction contained 50 ng of DNA from soil sample, 0.5 µmol of each primer, 0.2 mM dNTP, 1x PCR buffer, 3 mM $MgCl_2.2H_2O$ and 0.05 U Taq DNA polymerase (Promega, USA). The PCR amplifications were performed in the following condition: 5 min at 94^oC, followed by 30 cycles of 95^oC for 45 seconds, 53.5^oC for 45 seconds, and 72^oC for 2 min, and a final extension step at 72^oC for 10 min.

The PCR products of eubacteria and archeobacteria were separately subjected to DGGE analyses. PCR product (50 µl) was loaded onto 10% (w/v) polyacrylamide (Acrylamide : Bisacrylamide ratio, 37.5:1) gel in 1.0 strength Tris-acetate- EDTA (TAE, pH 8.5) buffer. The polyacrylamide gel was prepared with a denaturing gradient ranging from 30% to 70%. DGGE was performed at 60^oC. The electrophoresis was run for 12 h at 120 V. Subsequently, the gel was stained with SYBR Green solution and documented on gel documentation and analysis.

The fungus-specific primers NS1 (5'-GTA GTC ATA TGC TTG TCT C-3') and FR1 (5'-AIC CAT TCA ATC GGT AIT-3') were used for amplification of 18S rRNA gene fragments (1,650 bp) (Oros-Sichler et al., 2006). The reaction mixture (50 µl) consisted of 1 µl of template DNA (ca. 20 ng), Stoffel buffer (10 mM KCl, 10 mM Tris-HCl [pH 8.3]), 0.2 mM deoxynucleoside triphosphates, 3.75 mM $MgCl_2$, 2% (w/v) dimethyl sulfoxide, 0.2 M concentration of each primer (NS1 and FR1-GC), and 2 U of *Taq* DNA polymerase (Stoffel fragment; Applied Biosystems, Foster City,

Calif.). A GC-rich sequence (5'-CCC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GCC G-3') was attached to the primer FR1 to prevent complete melting of PCR products during separation in the denaturating gradient gel. Dimethyl sulfoxide was added to the reaction mixture to improve specificity and facilitate the amplification of GC-rich templates (Oros-Sichler et al., 2006). After 8 min of denaturation at 94°C, 35 thermal cycles of 30 s at 94°C, 45 s at 48°C, and 3 min at 72°C were performed, followed by an extension step at 72°C for 10 min. DGGE analysis was performed as previously described with a denaturing gradient of 18 to 43% denaturant. Aliquots of PCR samples (50 µl) were applied to the DGGE gel, and DGGE was performed in 1 X Tris-acetate- EDTA (TAE) buffer at 58°C with constant voltage of 180 V for 16 h. The gel was stained with SYBR Green solution and documented on gel documentation and analysis.

3.11 Cloning and sequencing

The microbial community composition in DGGE gel was analyzed by cloning and partial sequencing of the 16S rDNA and 18S rDNA genes. Interested bands from DGGE gel were used as a DNA template in PCR reactions as followed by Prakamhang et al. (Prakamhang et al., 2009). 16S rDNA and 18S rDNA genes were amplified by using the primers pair PRBA338F-PRUN518R, PRAH519R-PARCH340F, and NS1-FR1 (Ovreas et al., 1997) for eubacteria, archeobacteria and fungi, respectively. The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). The amplicons were ligated into the pGEM®-T Easy Vector System (Promega, USA) and then further transformed into *E.coli* DH5∞ competent cells, following the manufacturer's protocol. Cells were

grown overnight at 37 °C on Petri plates containing S-gal®/LB agar blend (Sigma–Aldrich) supplemented with 100 µg ml^{-1} ampicillin (Sigma–Aldrich). White colonies (transformants) were picked randomly from the plates for colony PCR using the SP6 and T7 primers (Promega, USA). Twenty-five microliter PCR reactions containing 0.1 U $µl^{-1}$ GoTaq® DNA Polymerase (Promega, USA), 1× PCR buffer and 1.5 mM $MgCl_2$ supplied with the enzyme, 0.2 mM dNTPs, 0.2 µM of each primer were performed using an PERKIN ELMER, GeneAmp PCR System 2400 under the following reaction conditions: 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 48 °C for 45 s, and 72 °C for 45 s, and a final extension step at 72 °C for 10 min. PCR products, DNA sequencing and analysis were conducted as described above.

3.12 Statistical analyses

The experimental data were statistically analyzed according to Stell et al. (1980), and means were compared by Duncan's Multiple Range Test (Duncan, 1955). The cluster analysis and dendrogram generation were carried out by the NTSYSpc (2.2, Exeter Software, USA) (Rohlf, 2000). The cluster analysis was performed according to the presence and absence of bands occurred in DGGE gels. The presence or absence of a nucleic acid band at the same height in each lane was marked with a 1 or 0, respectively. The similarities between the DGGE patterns were analyzed using the pearson correlation coefficient, and displayed graphically as a dendrogram based on UPGMA algorithms (unweighted pair group method with arithmetic averages). NTSYSpc version 2.2 was also used to perform principle components analysis (PCA) to perform multiple dimensions of microbial community structure.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Forage corn experiment

4.1.1 Effect of inoculum size on root colonization and plant biomass

Since *Azotobacter* sp. and *Azospirillum* sp. have been commercialized as PGPR inocula for various crops cultivation by Suranaree University of Technology (SUT) (Teaumroong et al., 2010). Thus aim of this study was to select the appropriate PGPR strains which provide better plant promoting efficiency than both of commercial strains. In this study, forage corn was selected as crop representative. In order to obtain the most abundant root-adhering bacteria, the roots of forage corn were used as source of PGPR isolation. The bacteria isolated from higher dilution between 10^6 to 10^8 folds were collected. The top two (SUT 19 and SUT 47) bacterial strains from 153 isolated strains were selected on the basis of their efficiency to promote better forage corn growth in Leonard's jar condition. Subsequently, the inoculation size of strains SUT 19 and SUT 47 on forage corn was determined before applied as inocula. The effect of bacterial inoculum size on the root colonization and forage corn biomass was summarized in Table 4.1. Even the low amount of bacterial cells at 10^3 CFU ml^{-1} $seed^{-1}$ was applied, the number of bacterial root colonization could reach to 10^6 CFU g root dry $weight^{-1}$. This again confirms the benefit of bacterial isolation approach which is conducted on the principle bacteria appeared in

higher dilution represented of high number of root-adhering bacteria (as mentioned in Materials and Methods). In addition, all of the 4 bacterial strains demonstrated higher root colonization efficiency when inoculum size was increased. The inoculum size in the range of 10^5-10^7 CFU ml^{-1} showed that SUT 47 was able to colonize root of forage corn higher than other strains, whereas root colonization of SUT 19 was not significantly different when compared with *Azotobacter* sp. and *Azospirillum* sp. In comparison, the plant biomass of plant inoculated with the isolates SUT 19 and SUT 47 at population number at 10^4 CFU ml^{-1} was 36.3 % and 41.6 %, respectively, which is higher than with the uninoculated control plant. Moreover, the ability to promote plant growth of isolate SUT 47 was higher than that of other inoculated strains at 10^4 CFU ml^{-1} (0.72 g of plant dry weight). Besides, inoculum size at 10^6 CFU ml^{-1} displayed that all inoculated strains promoted the plant growth significantly better than the control. Most of the PGPR strains could promote plant biomass at inoculum level 10^4 to 10^6 CFU ml^{-1} $seed^{-1}$ except *Azotobacter* sp. Generally, PGPR inoculants in this experiment that were inoculated at 10^4 CFU ml^{-1} $seed^{-1}$ raised the level of bacterial root colonization up to 10^7 CFU g root dry $weight^{-1}$. And this level could promote higher forage corn biomass than other degree of inoculum size. However, the inoculum size at 10^4 CFU ml^{-1} only showed good results in sterilized condition, therefore, when PGPR strains of this size was applied in the field, higher number of PGPR is needed. As experienced by El Zemrany et al., 2006 (El Zemrany et al., 2006b), the inoculum level of *Azospirillum lipoferum* CRT1 at 2.8 x 10^7 CFU per seed of maize in large-scale application could be recovered to be as high as 10^8 CFU g $root^{-1}$ at 35 days after planting.

Table 4.1 Effect of PGPR inoculum size on plant biomass and root colonization of forage corn in Leonard's jar experiment.

Treatments	Dilutions (CFU ml^{-1})					
	10^3	10^4	10^5	10^6	10^7	10^8
	Root colonization (CFU g root dry $weight^{-1}$)					
Control	-	-	-	-	-	-
Azotobacter sp.	$4.0x10^6 \pm 8.9x10^{5\ b}$	$8.3x10^7 \pm 1.6x10^{7\ a}$	$2.4x10^8 \pm 1.4x10^{8\ b}$	$8.0x10^8 \pm 1.0x10^{8\ b}$	$3.0x10^9 \pm 1.2x10^{9\ a}$	$3.7x10^9 \pm 6.7x10^{8\ a}$
Azospirillum sp.	$9.0x10^6 \pm 4.2x10^{6\ a}$	$3.7x10^7 \pm 5.3x10^{6\ c}$	$4.9x10^7 \pm 8.0x10^{6\ c}$	$1.4x10^8 \pm 4.3x10^{7\ c}$	$5.6x10^8 \pm 1.4x10^{8\ b}$	$4.8x10^9 \pm 3.6x10^{9\ a}$
SUT 19	$4.0x10^6 \pm 4.6x10^{5\ b}$	$5.2x10^7 \pm 1.2x10^{6\ b}$	$6.5x10^6 \pm 1.1x10^{6\ c}$	$3.7x10^7 \pm 3.1x10^{6\ c}$	$1.2x10^8 \pm 9.0x10^{7\ b}$	$2.5x10^9 \pm 1.6x10^{9\ ab}$
SUT 47	$7.0x10^6 \pm 1.4x10^{6\ ab}$	$4.0x10^7 \pm 2.3x10^{7\ c}$	$5.5x10^8 \pm 2.4x10^{8\ a}$	$9.5x10^8 \pm 2.0x10^{8\ a}$	$3.3x10^9 \pm 1.5x10^{9\ a}$	$4.1x10^9 \pm 1.7x10^{9\ a}$
	Total plant biomass (g plant dry weight)					
Control	$0.41 \pm 0.04^{\ a}$	$0.42 \pm 0.06^{\ b}$	$0.35 \pm 0.08^{\ b}$	$0.40 \pm 0.07^{\ b}$	$0.41 \pm 0.05^{\ a}$	$0.36 \pm 0.09^{\ a}$
Azotobacter sp.	$0.49 \pm 0.13^{\ a}$	$0.37 \pm 0.23^{\ b}$	$0.47 \pm 0.11^{\ ab}$	$0.53 \pm 0.13^{\ ab}$	$0.55 \pm 0.10^{\ a}$	$0.43 \pm 0.06^{\ a}$
Azospirillum sp.	$0.45 \pm 0.06^{\ a}$	$0.46 \pm 0.15^{\ ab}$	$0.55 \pm 0.11^{\ a}$	$0.63 \pm 0.09^{\ a}$	$0.54 \pm 0.17^{\ a}$	$0.50 \pm 0.13^{\ a}$
SUT 19	$0.47 \pm 0.07^{\ a}$	$0.60 \pm 0.08^{\ a}$	$0.57 \pm 0.12^{\ a}$	$0.64 \pm 0.16^{\ a}$	$0.58 \pm 0.12^{\ a}$	$0.54 \pm 0.09^{\ a}$
SUT 47	$0.60 \pm 0.19^{\ a}$	$0.72 \pm 0.14^{\ a}$	$0.49 \pm 0.05^{\ ab}$	$0.64 \pm 0.12^{\ a}$	$0.58 \pm 0.07^{\ a}$	$0.49 \pm 0.15^{\ a}$

Within a column for each dilution of plant biomass and root colonization, the data were separately investigated. Means followed by different letter are significantly different at 0.05 probability level according to least significant difference (LSD) test.

4.1.2 Characterization of selected PGPR

The abilities of plant growth promotion such as N_2-fixation ability, IAA production and ACC-deaminase activity of the tested strains were determined. The isolates SUT 19 and SUT 47 showed less efficiency of nitrogen fixation than *Azotobacter* sp. and *Azospirillum* sp. while IAA production was not significantly different among all bacteria tested. However, strains SUT 19 and SUT 47 performed the activity of ACC-deaminase enzyme at 0.25 and 0.19 µmol of a-ketobutyrate mg protein^{-1} h^{-1}, respectively while this property could not be detected in the *Azotobacter* sp. and *Azospirillum* sp. Moreover, only isolate SUT 19 had the ability to solubilize inorganic phosphate as well as displayed the biofilm formation higher than other strains. Based on 16S rRNA sequence analysis, the isolates SUT 19 and SUT 47 were closely related to *Pseudomonas* sp. and *Brevibacillus* sp. with homology 98 % (HM446471) and 97% (HM453885), respectively (Table 4.2).

Table 4.2 Identification and characterization of PGPR for forage corn.

Treatments	Characterization				
	ARA	**IAA**	**ACC-daminase activity**	**P-solubilization**	**Biofilm Formation**
***Azotobacter* sp.**	0.30±0.09 ^{a}	0.14±0.10 ^{ab}	0.00±0.00 ^{b}	-	0.72±0.13 ^{b}
***Azospirillum* sp.**	0.60±0.10 ^{a}	0.08±0.10 ^{b}	0.00±0.00 ^{b}	-	0.65±0.12 ^{b}
***Pseudomonas* sp. SUT19**	0.07±0.07 ^{b}	0.16±0.14 ^{ab}	0.25±0.19 ^{a}	+	1.44±0.18 ^{a}
***Brevibacillus* sp. SUT47**	0.11±0.03 ^{ab}	0.19±0.17 ^{a}	0.19±0.16 ^{ab}	-	0.55±0.20 ^{b}

ARA unit= nmole of acetylene mg protein^{-1} day^{-1}, ACC-deaminase activity unit = µmol of a-ketobutyrate mg protein^{-1} h^{-1}, IAA unit = µM mg protein^{-1}, + = can solubilize P and - = cannot solubilize P. Different letters in the same column indicate a significant different among treatments ($P \leq 0.05$).

Even the isolates SUT 19 and SUT 47 can fix atmospheric nitrogen with lower amount than the commercial strains (*Azotobacter* sp. and *Azospirillum* sp.) but when comparing all tested isolates with the *Rhizobium*-legume symbiosis, all of PGPR strains in this study still have lower nitrogen fixing ability (O'Gara and Shanmugam, 1976). Recently, Adesemoye et al, 2010 confirmed that PGPR as *Bacillus amyloliquefaciens* and *Bacillus pumilis* which can fix nitrogen able to increase plant N uptake from fertilizer via other mechanisms but not with their own nitrogen fixing capability. This indicated that plant growth promotion is caused by some other factors rather than nitrogen fixation. Whereas one advantage of these isolates may that they can survive in N-deprived condition. Therefore, the other factors such as phytohormones production, phosphate solubilization and ACC-deaminase would be the key factors for plant growth promotion.

The mechanism most often invoked to explain the direct effects of plant growth promoting bacteria on plants is the production of phytohormones, including auxins such as indole acetic acid or IAA (Patten and Glick, 1996; Patten and Glick, 2002). We found that the efficiency to produce plant hormone (IAA) of the strains *Pseudomonas* sp. SUT 19 and *Brevibacillus* sp. SUT 47 was not significantly different when compared to *Azotobacter* sp., in spite of their different plant biomass. However, lower amount of IAA produced by *B. subtilis* 101 could promote more tomato biomass than higher amount of IAA from *Azospirillum brasilense* Sp245 (Felici et al., 2008). Thus, in this case, IAA might not play as an important role for forage corn growth promotion.

Interestingly, the isolate *Pseudomonas* sp. SUT 19 and *Brevibacillus* sp. SUT 47 were found to produce ACC-deaminase enzyme. These results imply that the ACC

deaminase may help to promote the forage corn growth. The ACC-deaminase can cleave the plant ethylene precursor ACC, and thereby lower the level of ethylene in a developing or stressed plant (Glick et al., 1998; Jacobson et al., 1994). The inoculation with rhizobacterial strains containing ACC-deaminase activity significantly promoted root, shoot and other growth contributing parameters of wheat at all salinity levels both under axenic and pot condition (Zahir et al., 2009). However, organisms with higher levels of ACC deaminase activity, which is from 0.3 to 0.4 µmol a-ketobutyrate mg protein^{-1} h^{-1}, do not necessarily promote root elongation of *Brassica campestris* to any greater extent than the strains that contain less enzyme activity (Patten and Glick, 2002). Therefore, the role of ACC-deaminase activity of *Pseudomonas* sp. SUT 19 and *Brevibacillus* sp. SUT 47 on forage corn growth promotion should be further elucidated. As widely recognized that soil inoculation with phosphate solubilizing bacteria (PSB) can improve solubilization of fixed soil phosphates and applied phosphates resulting in higher crop yields (Chen et al., 2006), the highest forage corn biomass caused by *Pseudomonas* sp. SUT 19 inoculation may possibly be promoted via phosphate solubilization trait. In addition, some previous reports displayed the root-associated pseudomonas have been studied extensively, and many of these promote the growth of host plants or are used as biocontrol agents (Guido and Bloemberg, 2001).

In case of biofilm production, *Pseudomonas* sp. SUT 19 was found to produce in highest amount among tested strains. The species of *Pseudomonas* form dense biofilms on both abiotic and biotic surfaces, and are a primary model in biofilm research. The *Pseudomonas putida* can respond rapidly to the present of root exudates in soil, converging at root colonization sites and establishing stable biofilm (Espinosa-

Urgel et al., 2002). The plant-growth- promoting pseudomonas have been reported to discontinuously colonize the root surfaces (Bloemberg et al., 2000). *Azospirillum brasilense* and related species are motile heterotrophic proteobacteria that interact with roots of a variety of cereals such as wheat and maize, and often promote the growth of their host plant (Burdman et al., 2000). *A. brasilense* is a free-living nitrogen fixer, its ability to promote plant growth seems to be related to stimulation of root proliferation, rather than providing fixed nitrogen to the plant. The bacteria colonize root elongation zones and root hairs, forming dense biofilms (Assmus et al., 1995). Besides the gram-positive microbes also effectively colonize the rhizophere and are well represented in soil populations (Boureau et al., 2004). Therefore, biofilm formation from our strains could increase root-microbe association. Nevertheless, the role of biofilm formation of *Pseudomonas* sp. SUT19 and *Brevibacillus* sp. SUT47 on forage corn growth promotion should be further elucidated.

4.1.3 The effect of PGPR on plant biomass in pot and field experiments

In this experiment, compost was amended since nutrient available in compost might support the growth of PGPR during the less developed root period. The results showed no effect of compost on shoot and root dry weight in all treatments. Inoculation of forage corn with selected PGPR strains in pot and field experiments for both crops (January to March 2009 and July to September 2009) resulted in a visible increase in root and shoot development, especially during the establishment of the plant. The results of effect of PGPR on plant biomass in pot/field experiments in first crop showed in Table 4.3. The analysis of shoot and root dry weights using the F-test revealed that inoculation of PGPR resulted in a significant ($P \leq 0.05$) increase in the biomass of roots and shoot as compared to uninoculated controls.

Table 4.3 The effect of PGPR on plant biomass in pot/field experiment.

Treatments	January 2009 – March 2009					
	Pot experiments				Field experiments	
	Shoot dry weight (g)		Root dry weight (g)		Shoot dry weight (g)	
	2nd week	5th week	2nd week	5th week	2nd week	5th week
Control	1.04±0.30 b	11.52±3.15 b	0.63±0.15 a	1.24±0.10 f	1.04±0.21 a	8.69±3.78 b
Compost	1.02±0.10 ab	13.54±1.79 b	0.65±0.21 a	1.59±0.09 def	1.02±0.14 a	10.09±2.17 ab
Azotobacter sp.	0.96±0.17 ab	14.56±3.25 b	0.65±0.10 a	1.37±0.19 f	0.96 ±0.08a	10.87±1.04 ab
Azotobacter sp. + Compost	1.06±0.19 a	15.28±4.58 b	1.75±0.35 a	1.48±0.11 ef	1.06±0.14 a	12.61±2.39 a
Azospirillum sp.	1.00±0.32 ab	13.78±1.57 b	0.74±0.11 a	2.02±0.11 bcd	1.00±0.18 a	9.97±1.60 ab
Azospirillum sp. + Compost	1.07±0.24 ab	14.27±1.29 b	0.78±0.23 a	2.31±0.10 abc	1.07±0.28 a	9.76±1.67 ab
SUT19	0.85±0.13 ab	14.09±2.27 b	0.71±0.23 a	1.89±0.13 cde	0.85±0.26 a	11.49±1.37 ab
SUT19 + Compost	0.92±0.18 ab	16.87±3.50 ab	0.91±0.52 a	2.77±0.26 a	0.92±0.16 a	12.95±2.37 a
SUT47	1.01±0.32 ab	15.67±1.27 b	0.96±0.19 a	1.55±0.33 def	1.01±0.17 a	9.47±2.17 ab
SUT47 + Compost	1.14±0.17 a	22.15±2.88 a	0.90±0.40 a	2.44±0.53 ab	1.14±0.18 a	11.27±2.47 a
F- test	*	*	ns	**	ns	*

Mean values within a column followed by different letters were significantly different according to the DUNCAN's test, P≤0.05 (*), P≤0.01 (**), ns = non significant

Table 4.4 Effect of PGPR on plant biomass in pot/field experiment.

Treatments	July 2009 – September 2009								
	Pot experiments						Field experiments		
	Shoot dry weight (g)			Root dry weight (g)			Shoot dry weight (g)		
	2nd week	5th week	8th week	2nd week	5th week	8th week	2nd week	5th week	8th week
Control	0.59±0.26 [b]	11.81±0.57 [d]	29.61±2.64 [b]	0.35±0.08 [b]	2.32±0.54 [d]	11.29±2.93 [c]	0.83±0.34 [a]	9.80±1.89 [e]	215.62±29.66 [c]
Compost	0.77±0.16 [ab]	13.72±0.91 [cd]	30.65±6.63 [ab]	0.45±0.10 [ab]	3.04±0.32 [bcd]	14.93±1.43 [abc]	0.95±0.20 [a]	11.80±2.70 [de]	261.07±37.87 [bc]
Azotobacter sp.	0.81±0.32 [ab]	16.45±3.49 [cd]	33.27±7.46 [ab]	0.5±0.07 [ab]	3.01±0.53 [cd]	15.95±1.73 [abc]	1.04±0.20 [a]	12.94±3.38 [cde]	260.00±12.78 [bc]
Azotobacter sp. + Compost	0.89±0.19 [ab]	23.07±2.48 [ab]	34.05±6.97 [ab]	0.66±0.16 [a]	3.51±1.23 [bcd]	17.43±1.51 [ab]	1.09±0.16 [a]	18.92±3.14 [ab]	273.18±33.13 [bc]
Azospirillum sp.	0.80±0.31 [ab]	16.92±4.21 [cd]	33.68±5.34 [ab]	0.61±0.22 [ab]	2.86±0.36 [d]	14.99±1.57 [abc]	1.05±0.19 [a]	17.09±0.23 [bc]	242.83±63.42 [bc]
Azospirillum sp. + Compost	0.92±0.30 [ab]	24.35±3.92 [a]	34.36±10.26 [ab]	0.63±0.11 [ab]	3.60±0.75 [bcd]	16.38±2.43 [ab]	1.20±0.22 [a]	20.26±4.42 [ab]	276.42±53.13 [abc]
SUT19	0.77±0.22 [ab]	20.25±4.88 [abc]	31.94±4.54 [ab]	0.59±0.25 [ab]	5.05±0.21 [abc]	13.54±3.67 [bc]	0.97±0.28 [a]	16.39±2.67 [bcd]	282.04±21.26 [abc]
SUT19 + Compost	0.90±0.33 [ab]	24.25±4.26 [a]	38.35±7.13 [a]	0.70±0.24 [a]	5.82±1.11 [a]	19.44±0.74 [a]	1.06±0.31 [a]	24.15±3.71 [a]	341.13±91.56 [a]
SUT47	0.82±0.23 [ab]	17.57±3.42 [bcd]	31.04±1.09 [ab]	0.49±0.06 [ab]	3.70±0.48 [bcd]	14.59±1.27 [abc]	0.99±0.24 [a]	16.17±1.38 [bcd]	275.73±16.22 [abc]
SUT47 + Compost	0.95±0.27 [a]	25.04±6.89 [a]	35.55±7.65 [ab]	0.65±0.22 [a]	5.55±2.12 [ab]	19.33±2.74 [a]	1.11±0.25 [a]	24.61±3.17 [a]	301.15±23.66 [ab]
F- test	*	**	*	*	**	**	ns	**	*

[1]Mean values within a column followed by different letters were significantly different according to the DUNCAN's test, P≤0.05 (*), P≤0.01 (**), ns = non significant

For the results in pot experiment, shoot biomass performed at 2nd week (Table 4.4) showed no significant differences in all treatments, whereas at 5th week, *Pseudomonas* sp. SUT 19, *Brevibacillus* sp. SUT 47 and *Azospirillum* sp. amended with compost were able to increase shoot dry weight of forage corn when compared to the compost amendment alone (*Pseudomonas* sp. SUT 19 with compost: 43%, *Brevibacillus* sp. SUT 47 with compost: 45%, and *Azospirillum* sp. with compost: 44%, respectively). However, both commercial strains and isolated strains (*Pseudomonas* sp. SUT 19, *Pseudomonas* sp. SUT 19 with compost and *Brevibacillus* sp. SUT 47 with compost) were able to promote growth of forage corn significantly better than uninoculated control. Especially at 8th week after planting, only *Pseudomonas* sp. SUT 19 amended with compost demonstrated the ability to promote higher growth of forage corn than uninoculated control. In case of root dry weight, the results showed that at 2 weeks after planting, a commercial strain (*Azotobacter* sp.) and isolated strains (*Pseudomonas* sp. SUT 19 and *Brevibacillus* sp. SUT 47) amended with compost significantly increased the root dry weight of forage corn when compared to uninoculated control. At 5 weeks after planting, the isolates *Pseudomonas* sp. SUT 19, *Pseudomonas* sp. SUT 19 with compost, and *Brevibacillus* sp. SUT 47 with compost gave similar results better in promoting growth of forage corn than uninoculated control. Moreover, *Pseudomonas* sp. SUT 19 amended with compost more positively affects the root dry weight of forage corn when compared to compost amendment alone. At 8 weeks after planting, all of inoculated strains amended with compost enhanced forage corn growth significantly better than uninoculated control except compost amendment alone.

The results in field experiment displayed only the effect of PGPR on shoot

biomass because of limitation of root sampling. The tendency of shoot biomass was similar to the pot experiment. At 2 weeks after planting, the effects from all of inoculated strains treatments were not significantly different when compared with uninoculated control. Whereas, at 5 weeks after planting, all of inoculated strains were significantly different with uninoculated control except *Azotobacter* sp. treatment. Nevertheless, commercial strains (*Azotobacter* sp., *Azospirillum* sp. and *Azospirillum* sp. with compost) and selected strains (*Pseudomonas* sp. SUT 19 with compost and *Brevibacillus* sp. SUT 47 with compost) showed high efficiency to promote the forage corn growth when compared with compost amendment alone. At the 8 weeks after planting, the ability to promote forage corn growth of the isolate *Pseudomonas* sp. SUT 19 and *Brevibacillus* sp. SUT 47 amended with compost was higher than that of uninoculated control at 59.4 % and 60.1 %, respectively. In addition, the isolate *Pseudomonas* sp. SUT 19 amended with compost is the best among all treatments in terms of promoting growth of forage corn. In addition, the results from first crop cultivation (Table 4.3) were also similar to the second crop eventhough the plantation period was conducted only 5 weeks. Our results are in accordance with some reports that *Azospirillum lipoferum* CRT1 could promote growth of maize (*Zea may* L.) (Baudoin et al., 2009; Costa et al., 2005; El Zemrany et al., 2006a; Kumar et al., 2007; Mar Vazquez et al., 2000; Shaharoona et al., 2006).

4.1.4 Comparative PCR-DGGE and PCA analyses of microbial populations

The effect of PGPR inoculation on soil microbial community structure in forage corn rhizosphere in both pot and field experiments was evaluated using

PCR-DGGE approach. DGGE fingerprints confirmed that our isolates are able to establish in the forage corn rhizosphere throughout the plantation period (Fig. 4.1A) and the DGGE patterns obtained from rhizosphere in first crop (Fig 4.4) are similar to the second crop (Fig 4.1 and 4.2). The community structures of all eubacteria, archeobacteria and fungi from bulk soil showed absolutely different DGGE patterns when compared to other treatments. Both of eubacterial DGGE patterns obtained from pot (Figs. 4.1A) and field (Figs. 4.2A) consisted of a large amount of bands representing huge variety of ribotype at rhizosphere soil. The DGGE fingerprints of archeobacteria in both pot and field showed that the amounts of bands slightly increase along with plant age. Each treatment of DGGE patterns consisted of a few stronger bands and a large number of fainter bands representing less dominant ribotypes, whereas the relative abundance of several ribotypes was enhanced in the 8 weeks after planting (Figs. 4.1B and 4.2B). The DGGE fingerprints of fungi were similar to the archeobacterial fingerprint in terms of the amount of bands, which slightly increased when time of plantation was increased (Figs. 4.1C and 4.2C). However, the results from DGGE analysis revealed that relative abundance of eubacterial, archeobacterial and fungal populations in the rhizosphere of forage corn strongly shifted during plant growth.

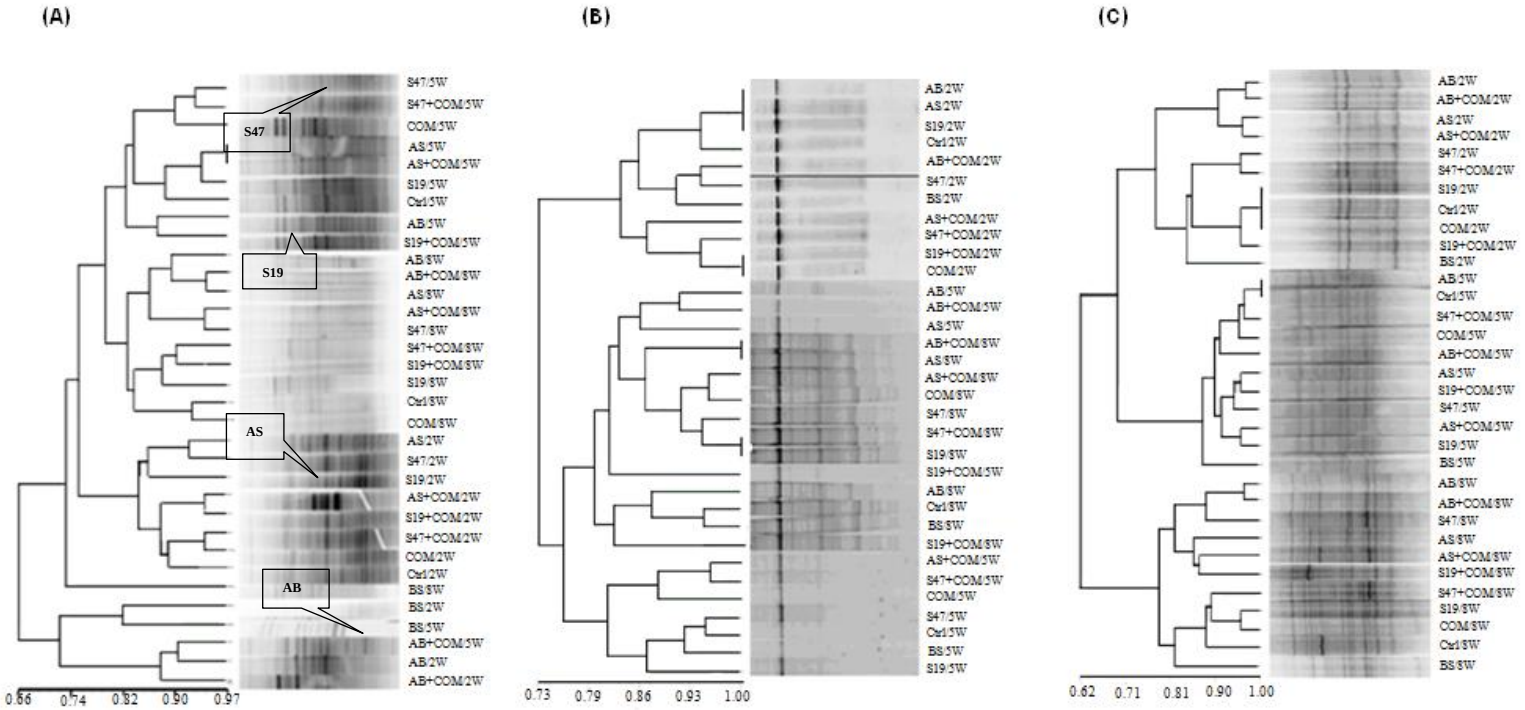


Figure 4.1 Community structure of soil microorganism in 2nd-crop from pot experiment. Dendrograms of soil microorganism based on PCR-DGGE bands. (A) Bacterial community structure; (B) Archeobacterial community structure; (C) Fungal community structure. Arrows indicate the inoculated treatments; (AB) *Azotobacter* sp.; (AS) *Azospirillum* sp.; (S19) *Pseudomonas* sp. SUT19; (S47) *Brevibacillus* sp. SUT47; (COM) compost; (Ctrl) control; (BS) bulk soil; (2, 5, 8) weeks after inoculation.

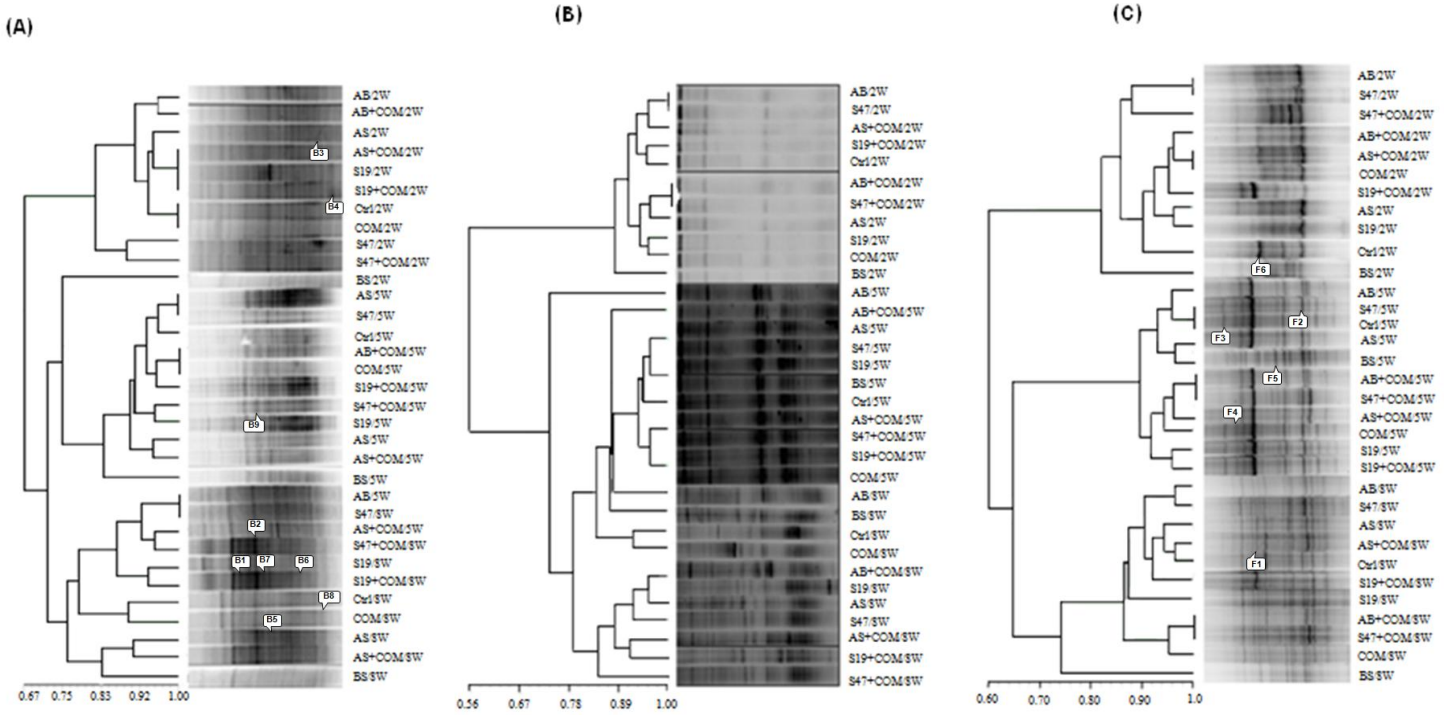
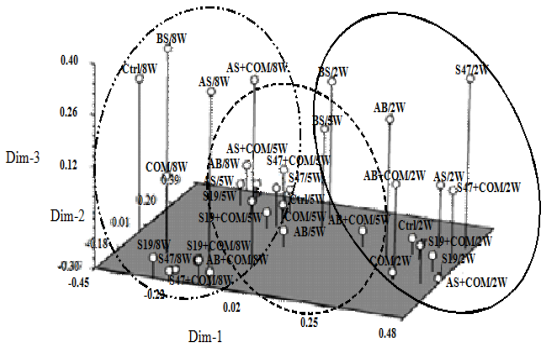


Figure 4.2 Community structure of soil microorganism in 2nd-crop from field experiment. Dendrograms of soil microorganism based on PCR-DGGE bands. (A) Bacterial community structure; (B) Archeobacterial community structure; (C) Fungal community structure. Letters indicate the inoculated treatments; (AB) *Azotobacter* sp.; (AS) *Azospirillum* sp.; (S19) *Pseudomonas* sp. SUT19; (S47) *Brevibacillus* sp. SUT47; (COM) compost; (Ctrl) control; (BS) bulk soil; (2, 5, 8) weeks after inoculation.

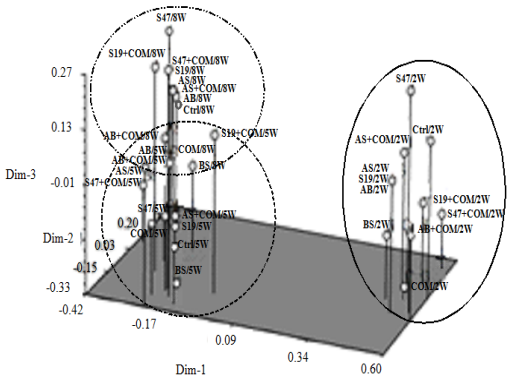
In order to determine the microbial community shifting more clearly, PCA was used to demonstrate multidimensional relationships derived from portions of the DGGE fingerprints. The results in pot experiment revealed that eubacterial community structure was separated from eubacterial community in the bulk soil (Fig. 4.3A). Whereas, the compost amendments did not have any influence on the eubacterial community structure. In addition, it was clearly demonstrated that the state of plant growth showed great influence on the bacterial community structure. In the case of archeobacterial community in pot experiment, it was revealed that the community structure of bulk soil was absolutely separated from rhizosphere soil (Fig. 4.3B). For fungal community structure, the community changes at 5^{th} and 8^{th} week were grouped together and community structure in bulk soil of each time was separated from rhizosphere soil (Fig. 4.3C).

The results in field experiments showed that eubacterial community structure from rhizosphere was different from that of eubacterial community structure of bulk soil (Fig. 4.3D). In addition, when considering archeobacterial and fungal community structure, it was found that the community changes were strongly influenced by plant age (Fig. 4.3E and 4.3F). Since the aim of setting pot experiment was to observe the effect of in soil volume might bring about changes in microbial community structure. The results obtained from PCA analysis demonstrated that the each archeobacterial and fungal community structure at 5^{th} and 8^{th} week was not as clearly shifted as in DGGE. This might be due to higher rhizosphere/bulk soil ratio cause more active soil or special soil properties than in the field. For the PCA results from field experiment conducted between January to March 2009 showed the similar results with the crop in July to September 2009 (Fig. 4.4).

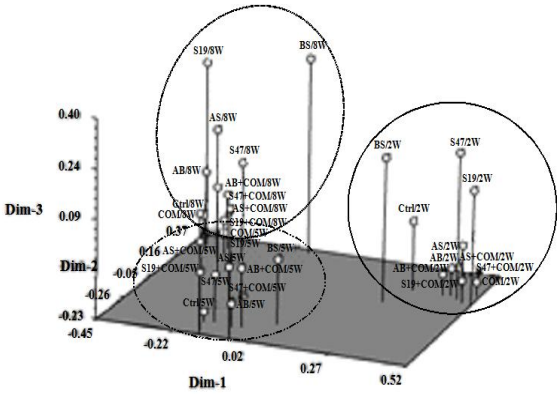
(A)


Dim-3
Dim-2
Dim-1
BS/8W
Ctrl/8W
AS+COM/8W
AS/8W
BS/2W
S47/2W
AB/2W
COM/8W
AB/8W
S47+COM/8W
S47/8W
AB+COM/2W
AS/2W
S47+COM/2W
S19/5W
S19+COM/5W
COM/5W
AB+COM/5W
Ctrl/2W
S19+COM/2W
S19/2W
AB/5W
S19/8W
S19+COM/8W
AB+COM/8W
COM/2W
AS+COM/2W

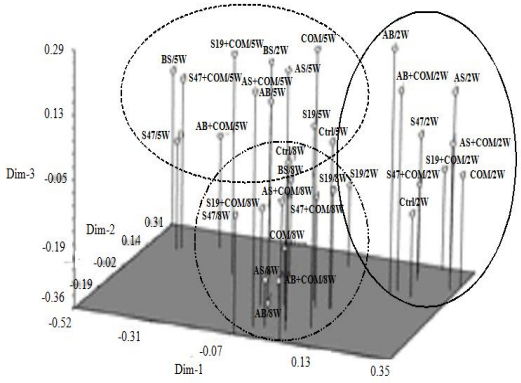
(B)


Dim-3
Dim-2
Dim-1
S47/8W
S19+COM/8W
S47+COM/8W
S19/8W
AS/8W
AB/8W
Ctrl/8W
AB+COM/8W
S19+COM/5W
AB/5W
COM/8W
AS/5W
AB+COM/5W
BS/8W
S47+COM/5W
S47/5W
AS+COM/5W
S19/5W
COM/5W
Ctrl/5W
BS/5W
S47/2W
Ctrl/2W
AS+COM/2W
AS/2W
S19/2W
AB/2W
S19+COM/2W
S47+COM/2W
BS/2W
AB+COM/2W
COM/2W

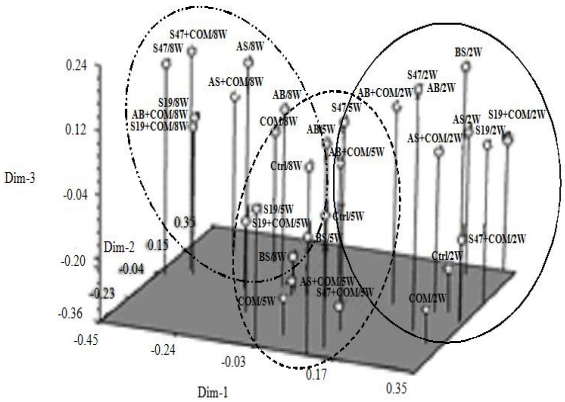
(C)


Dim-3
Dim-2
Dim-1
S19/8W
BS/8W
AS/8W
S47/8W
AB/8W
AB+COM/8W
S47+COM/8W
AS+COM/8W
Ctrl/8W
COM/8W
S19+COM/8W
S19/5W
AS+COM/5W
S19+COM/5W
AS/5W
BS/5W
AB+COM/5W
S47/5W
S47+COM/5W
Ctrl/5W
AB/5W
BS/2W
S47/2W
S19/2W
Ctrl/2W
AS/2W
AB/2W
AS+COM/2W
AB+COM/2W
S47+COM/2W
S19+COM/2W
COM/2W

(D)


Dim-3
Dim-2
Dim-1
S19+COM/5W
BS/2W
COM/5W
AB/2W
BS/5W
S47+COM/5W
AS+COM/5W
AS/5W
AB/5W
AB+COM/2W
AS/2W
S19/5W
Ctrl/8W
S47/5W
AB+COM/5W
S47/2W
Ctrl/5W
AS+COM/2W
BS/5W
S19/2W
S19+COM/2W
S47+COM/2W
COM/2W
AS+COM/8W
S19+COM/8W
S47/8W
Ctrl/2W
S47+COM/8W
COM/8W
AS/8W
AB+COM/8W
AB/8W

(E)


Dim-3
Dim-2
Dim-1
S47+COM/8W
S47/8W
AS/8W
AS+COM/8W
BS/2W
S47/2W
AB/8W
AB+COM/2W
AB/2W
S19/8W
AB+COM/8W
S19+COM/8W
S47/5W
AS/2W
S19+COM/2W
COM/8W
AB/5W
S19/2W
AS+COM/2W
AB+COM/5W
Ctrl/8W
S19/5W
S19+COM/5W
Ctrl/5W
AS/5W
S47+COM/2W
BS/8W
Ctrl/2W
AS+COM/5W
S47+COM/5W
COM/5W
COM/2W

(F)

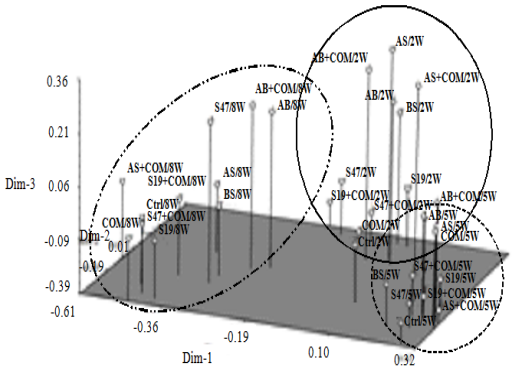

Dim-3
Dim-2
Dim-1
AS/2W
AB+COM/2W
AS+COM/2W
AB+COM/8W
AB/2W
AB/8W
S47/8W
BS/2W
AS+COM/8W
S19+COM/8W
AS/8W
S47/2W
S19/2W
BS/8W
S19+COM/2W
AB+COM/5W
Ctrl/8W
S47+COM/2W
S47+COM/8W
COM/8W
AB/5W
S19/8W
COM/2W
AS/5W
Ctrl/2W
COM/5W
S47+COM/5W
BS/5W
S19/5W
S47/5W
S19+COM/5W
AS+COM/5W
Ctrl/5W

Figure 4.3 The community analysis of 2nd-crop experiment derived tree-dimentional plot based on the first three principal coordinates from a principal corrordonate analysis (PCA) of maize rhizosphere. (A) PCA of eubacteria in pot experiment; (B) PCA of archeobacteria in pot experiment; (C) PCA of fungi in pot experiment; (D) PCA of eubacteria in field experiment; (E) PCA of archeobacteria in field experiment; (F) PCA of fungi in field experiment. Letters indicate the inoculated treatments; (Ctrl) control; (AB) *Azotobacter* sp.; (AS) *Azospirillum* sp.; (COM) compost; (S19) *Pseudomonas* sp. SUT19; (S47) *Brevibacillus* sp. SUT47; (BS) bulk soil; (2, 5, 8) weeks after inoculation; —— -, ····· , and ····· show a trend of 2, 5, and 8 week, respectively after inoculation are different from each other.

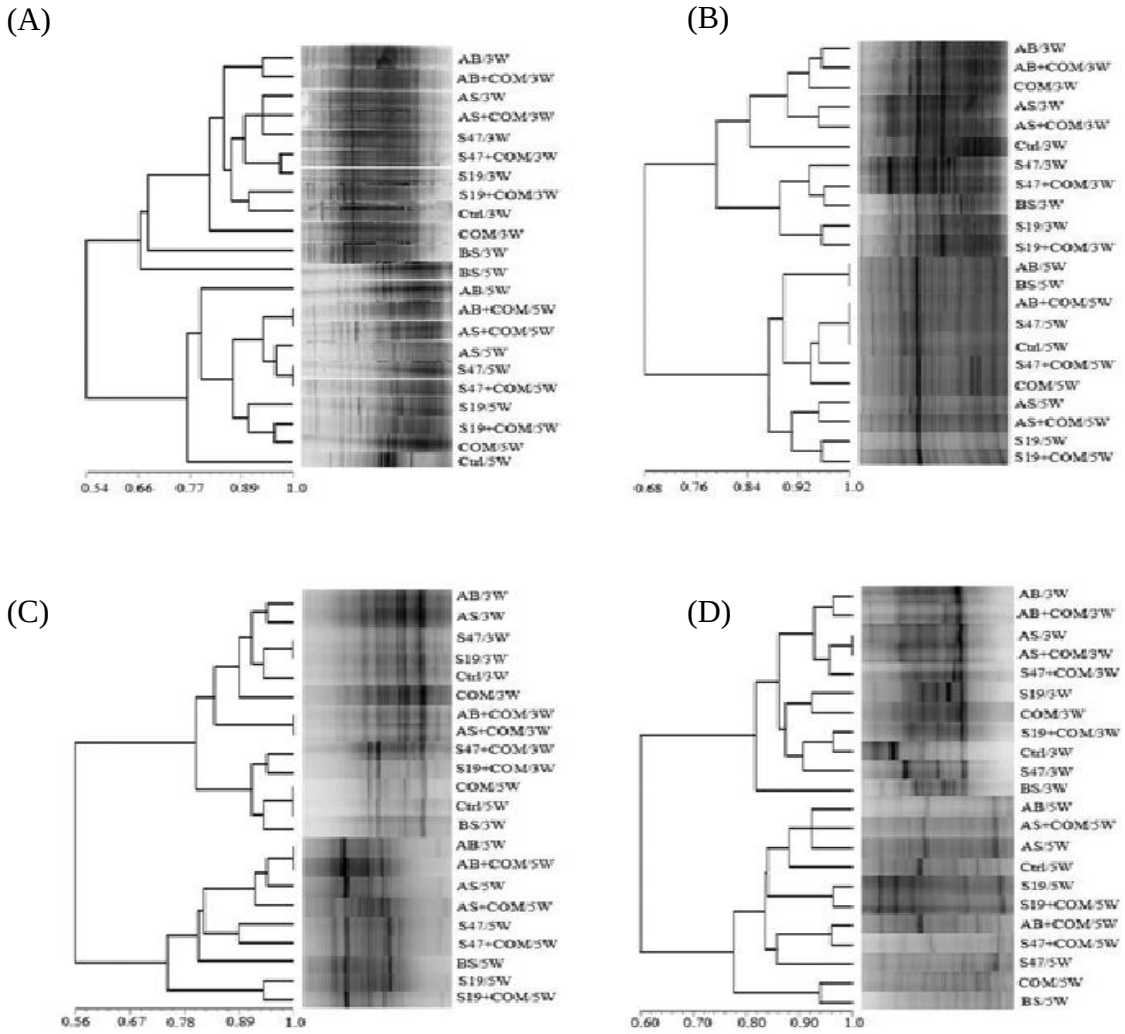


Figure 4.4 Community structure of soil microorganism in 1st-crop from pot and field experiment. Dendrograms of soil microorganism based on PCR-DGGE bands. (A) Eubacterial community structure from pot experiment; (B) Eubacterial community structure from field experiment; (C) Fungal community structure from pot experiment; (D) Fungal community structure from field experiment. Letters indicate the inoculated treatments; (AB) *Azotobacter* sp.; (AS) *Azospirillum* sp.; (S19) *Pseudomonas* sp. SUT19; (S47) *Brevibacillus* sp. SUT47; (COM) compost; (Ctrl) control; (BS) bulk soil; (2, 5) weeks after inoculation.

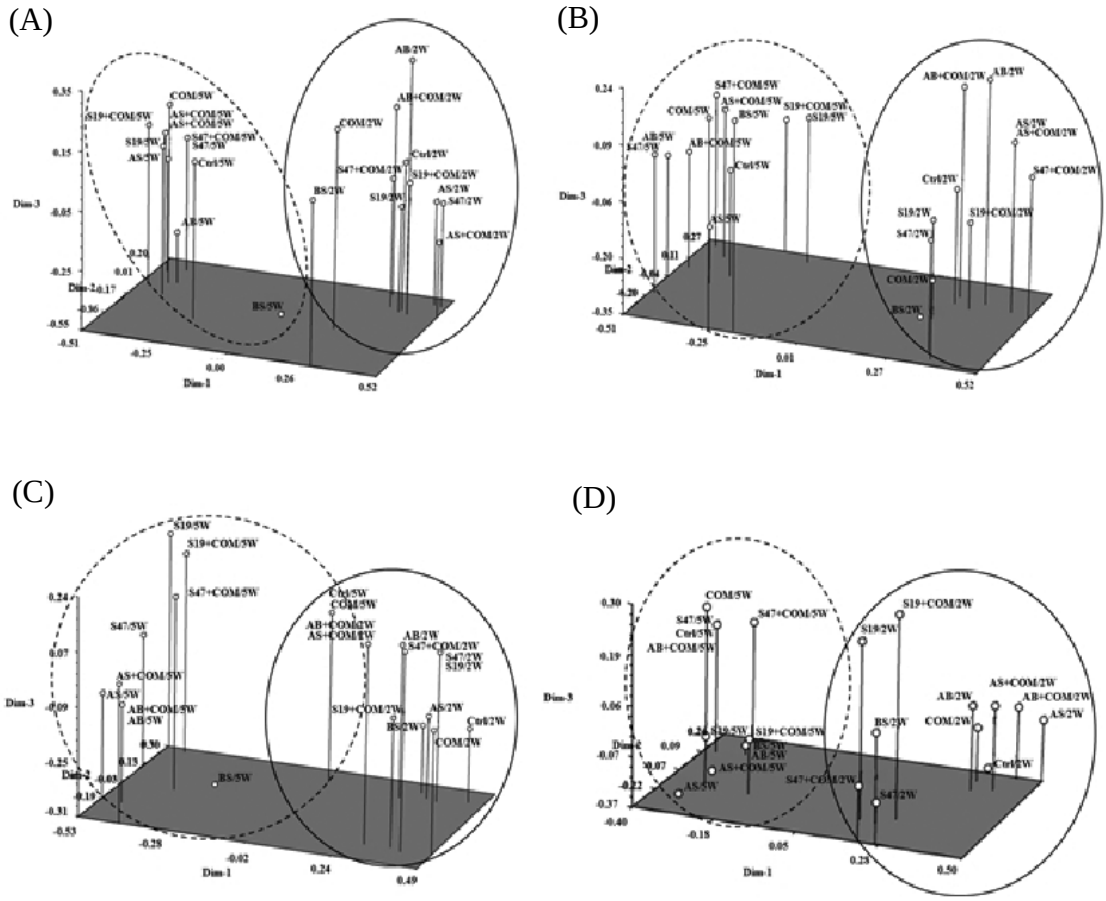


Figure 4.5 The community analysis in 2nd-crop experiment derived tree-dimentional plot based on the first three principal coordinates from a principal coordinate analysis (PCA) of maize rhizosphere. (A) PCA of eubacteria in pot experiment; (B) PCA of fungi in pot experiment; (C) PCA of eubacteria in field experiment; (D) PCA of fungi in field experiment. Letters indicate the inoculated treatments; (Ctrl) control; (BS) bulk soil; (AB) *Azotobacter* sp.; (AS) *Azospirillum* sp.; (COM) compost; (S19) *Pseudomonas* sp. SUT19; (S47) *Brevibacillus* sp. SUT47; (2, 5) weeks after inoculation; -, and ····· show a trend of 2 and 5 week, respectively after inoculation are different from each other.

To understand the ecological impact of PGPR on microbial community structure is an important issue when attempting to better define usage conditions for these inoculants. The DGGE fingerprints displayed that compost did not have any influence on microbial community structure. Our result correspond to Inbar et al. (2005) who found similar response of microbial community structure that was detected when compost was applied to soil at high levels of compost. However, all stages of plant growth showed the distinct profile characteristics. This also implies that plant age is a major factor influencing rhizobacterial community structure (Castro-Sowinski et al., 2007). The biological processes in the rhizosphere are strongly influenced by plant root exudates, which consist of easily degradable organic compounds that might attract and stimulate microbial growth (Walker et al., 2003). In addition, changing of root morphology and root exudation driving maize development might dictate the community patterns of eubacteria and fungi (Gomes et al., 2001). Some previous studies also report that the plant development is a major cause for microbial community changes in rhizosphere soil (Herschkovitz et al., 2005a; Herschkovitz et al., 2005b).

4.1.5 DGGE analysis of forage corn rhizosphere microbial community

Some of DGGE bands in Fig, 4.1A and 4.1C were excised and subsequently sequenced (Fig. 4.1A,4.1C and Table 4.5). The major eubacterial groups from forage corn rhizosphere soil in field experiment were gamma proteobacteria and members of the uncultured bacteria. Root associated bacterial population was diverse but some species was certainly appeared in all plant development such as bacterial species closely related to *Enterobacter* sp. and uncultered cyanobacterium (B2 and B7, respectively). Both species of uncultured bacteria and *Paenibacillus* sp. (B3 and

B4, respectively) appeared only at 2nd week of planting. Only Uncultured Firmicutes bacterium (B9) was found at 5th week of planting and disappeared at 8th week. Several species were found at only 8th week of planting such as uncultured bacteria and uncultured *Chloroflexi bacterium* (B1, B5, B6, and B8, respectively). Some previous studies also report that the *Enterobacter* sp. can colonize root and promote growth of maize in pot experiment (Sheng et al., 2008). The result displayed that the *Enterobacter* sp. appeared in all stages of plant development. These results implied that the *Enterobacter* sp. might be indigenous species in SUT farm soil. In addition, *Enterobacter* sp. 12J1 could promote growth of maize and reduce pyrene contamination in soil sample (Sheng et al., 2008). *P. polymyxa* (Da Mota et al., 2008) and cyanogenic bacteria (Owen and Zdor, 2001) are also widely recognized as PGPR since they could produce IAA and hydrogen cyanide (HCN), respectively

The fungal population in field experiment showed various species on fungal community structure in rhizosphere soil. The *Thanatephorus cucumeris* was found at all stages of plant development (F2). This result implies that *T. cucumeris* is indigenous fungus in SUT farm soil. The *Basipetospora chlamydospora*, *Madurella* sp., and *Ceratobasidium* sp. (F3, F4, and F5, respectively) appeared only at 5th week of planting and *Psathyrella spadicea* (F6) persisted only 2nd week of planting. The species of *Coriolopsis gallica* (F1) appeared only at 8th week of planting. *T. cucumeris* (anamorph *Rhizoctonia solani*) is a soilborne basidiomycete that occurs worldwide and causes economically important diseases to a large variety of vegetable and field crops (Julián et al., 1999; Justesen et al., 2003). In the Philippines, this fungus causes banded leaf and sheath blight in maize (Pascual et al., 2001). However, there no any reports from SUT farm regard to this disease. Most of the sampling

fungal sequences in this study belong to basidiomycete genera (Carbajo et al., 2002; Hietala et al., 2003; Murray and Burpee, 1984; Sllgiyama and Ogawa, 2004; Vašutová, 2008) except *Madurella* sp. is ascomycota (Ahmed et al., 2003). They also were reviewed as general soil fungi. The results also demonstrated that our inoculated PGPR do not mainly interfere fungal community. The DGGE fingerprint revealed that the effect of PGPR inoculation was much less pronounced in the plant growth development. Although, the exact mechanism of maize-microbe and microbe-microbe interactions remain to be further explored.

Table 4.5 Some bacterial and fungal taxa detected by DGGE from the rhizosphere of forage corn.

Clone	Most closely related taxa	Similarity %[a]	Accession number[1]	Present in sample (week after planting)
B1	Uncultured bacterium (HM327849.1)	98	HM453876	8
B2	*Enterobacter* sp. (FJ593851.1)	100	HM453877	2, 5, 8
B3	Uncultured bacterium (HM269092.1)	100	HM453878	2
B4	*Paenibacillus* sp. (EU362183.1)	100	HM453871	2
B5	Uncultured bacterium (AB483850.1)	99	HM453879	8
B6	Uncultured bacterium (AB485554.1)	97	HM453880	8
B7	Uncultured *cyanobacterium* (FN646729.1)	100	HM453881	2, 5, 8
B8	Uncultured *Chloroflexi* bacterium (CU922904.1)	99	HM453882	8
B9	Uncultured Firmicutes bacterium (FM252749.1)	92	HM453883	5
F1	*Coriolopsis gallica* (AY336772.1)	99	HM453873	8
F2	*Thanatephorus cucumeris* (DQ917659.1)	98	HM446472	2, 5, 8
F3	*Basipetospora chlamydospora* (AB024046.1)	98	HM446473	5
F4	*Madurella* sp. (EU815932.1)	98	HM453875	5
F5	*Ceratobasidium* sp. (AY757266.1)	97	HM453874	5
F6	*Psathyrella spadicea* (DQ465340.1)	97	HM453872	2

[1] Percent similarity and accession number of sequences with first closest match and closest match with named sequences with a percent similarity limit of 90% from the GenBank database.

4.2 Chinese kale experiment

4.2.1 Effect of inoculum size on root colonization and plant biomass

In order to obtain the most abundant root-adhering bacteria, the roots of Chinese kale were used as source of PGPR isolation. The bacteria isolated from higher dilution between 10^6 to 10^8 folds were collected. The top two (SUT 1 and SUT 19) bacterial strains from 70 isolated strains were selected on the basis of their efficiency to promote better Chinese kale growth in Leonard's jar condition. Subsequently, the inoculation size of strains SUT 1 and SUT 19 on Chinese kale was determined before applied as inocula. The effect of bacterial inoculum size on the root colonization and Chinese kale biomass was summarized in Table 4.6. Even the low amount of bacterial cells at 10^3 CFU ml^{-1} $seed^{-1}$ was applied, the number of bacterial root colonization could reach to 10^6-10^7 CFU g root dry $weight^{-1}$. This again confirms the benefit of bacterial isolation approach which is conducted on the principle bacteria appeared in higher dilution represented of high number of root-adhering bacteria (as mentioned in Materials and Methods). In addition, all of the 4 bacterial strains demonstrated higher root colonization efficiency when inoculum size was increased. The tendency of inoculum size in the range of 10^5-10^6 CFU ml^{-1} showed that SUT 19 was able to colonize root of Chinese kale higher than other strains, whereas root colonization of SUT 1 was not significantly different when compared with *Azotobacter* sp. and *Azospirillum* sp. In comparison, the plant biomass of plant inoculated with the commercial strains and isolates SUT 1 and SUT 19 at population number only 10^6 CFU ml^{-1} is higher than the uninoculated control plant. Generally,

PGPR inoculants in this experiment that were inoculated at 10^6 CFU ml^{-1} $seed^{-1}$ raised the level of bacterial root colonization up to 10^6-10^8 CFU g root dry $weight^{-1}$. And this level could promote higher Chinese kale biomass than other degree of inoculum size. However, the inoculum size at 10^6 CFU ml^{-1} only showed good results in sterilized condition, therefore, when PGPR strains was applied in the field, higher number of PGPR (> 10^6 CFU/ml^{-1}) is needed.

Table 4.6 Effect of inoculum size on root colonization and plant biomass**.**

Treatments	Dilutions (CFU ml^{-1})					
	10^3	10^4	10^5	10^6	10^7	10^8
	Root colonization (CFU g root dry $weight^{-1}$)					
Control	-	-	-	-	-	-
Azotobacter sp.	$4.1x10^6 \pm 1.0x10^{6\ b}$	$5.1x10^7 \pm 3.8x10^{7\ ab}$	$1.9x10^8 \pm 1.7x10^{8\ b}$	$3.1x10^8 \pm 2.1x10^{8\ ab}$	$3.5x10^8 \pm 2.1x10^{8\ ab}$	$6.9x10^8 \pm 4.4x10^{8\ a}$
Azospirillum sp.	$8.4x10^6 \pm 4.5x10^{6\ b}$	$3.5x10^7 \pm 2.2x10^{7\ ab}$	$5.1x10^7 \pm 1.2x10^{7\ b}$	$1.5x10^8 \pm 4.4x10^{7\ b}$	$5.9x10^8 \pm 1.6x10^{8\ a}$	$7.5x10^8 \pm 4.1x10^{8\ a}$
SUT 1	$4.3x10^6 \pm 8.0x10^{5\ b}$	$7.3x10^6 \pm 3.2x10^{6\ b}$	$8.6x10^6 \pm 3.2x10^{6\ b}$	$2.9x10^7 \pm 1.6x10^{6\ b}$	$6.1x10^7 \pm 4.1x10^{7\ b}$	$7.5x10^7 \pm 3.9x10^{7\ b}$
SUT 19	$6.9x10^7 \pm 2.4x10^{7\ a}$	$4.7x10^8 \pm 1.5x10^{8\ a}$	$5.1x10^8 \pm 2.5x10^{8\ a}$	$6.3x10^8 \pm 3.9x10^{8\ a}$	$6.6x10^8 \pm 3.9x10^{8\ a}$	$1.3x10^9 \pm 5.4x10^{8\ a}$
	Total plant biomass (g plant dry weight)					
Control	$0.028 \pm 0.006^{\ b}$	$0.045 \pm 0.018^{\ a}$	$0.034 \pm 0.018^{\ b}$	$0.029 \pm 0.006^{\ b}$	$0.034 \pm 0.007^{\ b}$	$0.032 \pm 0.008^{\ a}$
Azotobacter sp.	$0.057 \pm 0.023^{\ ab}$	$0.056 \pm 0.012^{\ a}$	$0.068 \pm 0.023^{\ a}$	$0.063 \pm 0.019^{\ a}$	$0.068 \pm 0.023^{\ a}$	$0.059 \pm 0.029^{\ a}$
Azospirillum sp.	$0.069 \pm 0.018^{\ a}$	$0.047 \pm 0.015^{\ a}$	$0.062 \pm 0.014^{\ ab}$	$0.055 \pm 0.022^{\ a}$	$0.061 \pm 0.005^{\ ab}$	$0.041 \pm 0.022^{\ a}$
SUT 1	$0.067 \pm 0.015^{\ a}$	$0.056 \pm 0.022^{\ a}$	$0.070 \pm 0.018^{\ a}$	$0.056 \pm 0.008^{\ a}$	$0.055 \pm 0.018^{\ ab}$	$0.051 \pm 0.013^{\ a}$
SUT 19	$0.054 \pm 0.029^{\ ab}$	$0.068 \pm 0.013^{\ a}$	$0.050 \pm 0.008^{\ ab}$	$0.069 \pm 0.013^{\ a}$	$0.062 \pm 0.020^{\ ab}$	$0.044 \pm 0.018^{\ a}$

Within a column for each dilution of plant biomass and root colonization, the data were separately investigated. Means followed by different letter are significantly different at 0.05 probability level according to least significant difference (LSD) test.

4.2.2 Characterization of selected PGPR

The abilities of plant growth promotion such as N-fixation ability, IAA production and ACC-deaminase activity of the tested strains were determined. The isolates SUT 1 and SUT 19 showed less efficiency of nitrogen fixation than *Azotobacter* sp. and *Azospirillum* sp. while IAA production was not significantly different among all bacteria tested. However, strains SUT 1 and SUT 19 performed the activity of ACC-deaminase enzyme at 0.20 and 0.26 µmol of a-ketobutyrate mg protein^{-1} h^{-1}, respectively while this property could not be detected in the *Azotobacter* sp. and *Azospirillum* sp. Moreover, only isolate SUT 19 had the ability to solubilize inorganic phosphate as well as displayed the biofilm formation higher than other strains. Based on 16S rRNA sequence analysis, the isolates SUT 1 and SUT 19 were closely related to *Bacillus* sp. and *Pseudomonas* sp. with homology 97 % and 98 % respectively (Table 4.7). Even the isolates SUT 1 and SUT 19 can fix atmospheric nitrogen with lower amount than the commercial strains (*Azotobacter* sp. and *Azospirillum* sp.) but when comparing all tested isolates with the Rhizobium-legume symbiosis, all of PGPR strains in this study still have lower nitrogen fixing ability (O'Gara and Shanmugam, 1976). This indicated that plant growth promotion is caused by some other factors rather than nitrogen fixation. Whereas one advantage of these isolates may that they can survive in N-deprived condition. Therefore, the other factors such as phytohormones production, phosphate solubilization, ACC-deaminase and biofilm formation would be the key factors for plant growth promotion.

Table 4.7 Identification and characterization of PGPR.

Treatments	Characterization				
	ARA	**IAA**	**ACC-daminase activity**	**P-solubilization**	**Biofilm Formation**
Azotobacter sp.	0.30±0.10 [b]	0.14±0.11	0.00±0.00 [b]	-	0.72±0.13 [b]
Azospirillum sp.	0.64±0.10 [a]	0.08±0.11	0.00±0.00 [b]	-	0.65±0.12 [b]
Bacillus sp. SUT1	0.24±0.11 [c]	0.13±0.15	0.20±0.14 [ab]	-	0.18±0.03 [c]
Pseudomonas sp. SUT19	0.08±0.07 [d]	0.17±0.15	0.26±0.19 [a]	+	1.44±0.18 [a]

ARA unit= nmole of acetylene mg protein^{-1} day^{-1}, ACC-daminase activity unit = µmol of a-ketobutyrate mg protein^{-1} h^{-1}, IAA unit = µM mg protein^{-1}, + = can solubilize P and - = cannot solubilize P. Different letters in the same column indicate a significant different among treatments ($P \leq 0.05$).

The mechanism most often invoked to explain the direct effects of plant growth promoting bacteria on plants is the production of phytohormones, including auxins such as indole acetic acid or IAA (Patten and Glick, 1996; Patten and Glick, 2002). We found that the efficiency to produce plant hormone (IAA) of the strains *Bacillus* sp. SUT 1 and *Pseudomonas* sp. SUT 19 was not significantly different when compared to both commercial strains, in spite of their different plant biomass. However, lower amount of IAA produced by *Bacillus subtilis* 101 could promote more tomato biomass than higher amount of IAA from *Azospirillum brasilense* Sp245 (Felici et al., 2008). Thus, in this case, IAA might not play as an important role for Chinese kale growth promotion. However, some other phytohormones such as gibberellins are not negligible. Recently, (Kang et al.) reported that gibberellins produced by *Burkholderia* sp. KCTC 11096 BP is one of the key factor for cucumber growth promotion. Interestingly, the isolate *Bacillus* sp. SUT 1 and *Pseudomonas* sp. SUT 19 were found to produce ACC-deaminase enzyme. These results imply that the

ACC deaminase may help to promote the Chinese kale growth. The inoculation with rhizobacterial strains containing ACC-deaminase activity significantly promoted root, shoot and other growth contributing parameters of wheat at all salinity levels both under axenic and pot condition (Zahir et al., 2009). However, organisms with higher levels of ACC deaminase activity, which is from 0.3 to 0.4 µmol a-ketobutyrate mg protein^{-1} h^{-1}, do not necessarily promote root elongation of *Brassica campestris* to any greater extent than the strains that contain less enzyme activity (Patten and Glick, 2002). Therefore, the role of ACC-deaminase activity of *Bacillus* sp. SUT 1 and *Pseudomonas* sp. SUT 19 on Chinese kale growth promotion should be further elucidated. In addition, As widely recognized that soil inoculation with phosphate solubilizing bacteria (PSB) can improve solubilization of fixed soil phosphates and applied phosphates resulting in higher crop yields (Chen et al., 2006). In case of biofilm formation *Pseudomonas* sp. SUT 19 could produce in highest amount when compared with other tested strains. This factor might also be one of critical factor for plant growth promotion. Interestingly, *Pseudomonas* sp. SUT 19 showed the best results in term of forage corn and Chinese kale growth promotions.

4.2.3 The effect of PGPR on plant biomass in pot and field experiment

In this experiment, compost was mixed under the same purpose as conducted with forage corn. The results showed no significant effect of compost on plant biomass in all treatments. Inoculation of Chinese kale with selected PGPR strains in pot and field experiments for both crops (September to November 2009 and April to June 2010) resulted in a visible increase in plant development, especially

during the establishment of the plant. The results of effect of PGPR on plant biomass in pot/field experiments in first crop (raining season) showed in Table 4.8. The analysis of plant biomass using the F-test revealed that inoculation of PGPR resulted in a significant ($P \leq 0.05$) increase in the biomass as compared to uninoculated controls (Table 4.8).

Table 4.8 The effect of PGPR on plant biomass in pot and field experiments in raining season.

Treatments	September - November 2009					
	Plant dry weight (g)					
	Pot experiments			Field experiments		
	3rd week	5th week	7th week	3rd week	5th week	7th week
Control	0.031(0.006)^{b}	1.67(5.58)^{d}	3.40(0.87)^{b}	0.032(0.010)^{b}	2.01(0.49)^{b}	3.72(0.82)^{c}
Compost	0.035(0.006)^{ab}	2.13(0.59)^{bcd}	3.82(1.12)^{ab}	0.036(0.007)^{ab}	2.17(1.47)^{b}	4.28(0.80)^{bc}
Azotobacter sp.	0.037(0.001)^{ab}	2.12(0.80)^{bcd}	4.14(0.64)^{ab}	0.037(0.013)^{ab}	2.99(0.73)^{b}	4.64(0.58)^{b}
Azotobacter sp. + Compost	0.040(0.004)^{ab}	3.21(0.91)^{ab}	4.86(0.33)^{a}	0.041(0.003)^{ab}	3.48(1.81)^{a}	4.08(1.13)^{b}
Azospirillum sp.	0.036(0.008)^{ab}	2.12(0.84)^{bcd}	3.87(0.68)^{ab}	0.037(0.003)^{ab}	2.80(1.07)^{b}	3.73(0.68)^{b}
Azospirillum sp. + Compost	0.045(0.010)^{ab}	2.82(0.20)^{bc}	4.49(0.55)^{ab}	0.040(0.010)^{ab}	3.42(0.91)^{a}	4.84(1.17)^{b}
SUT1	0.044(0.009)^{ab}	2.20(0.80)^{bcd}	4.11(0.65)^{ab}	0.042(0.005)^{ab}	2.85(0.60)^{b}	4.73(0.98)^{b}
SUT1 + Compost	0.050(0.013)^{a}	3.93(0.28)^{a}	4.98(0.36)^{a}	0.049(0.010)^{ab}	4.66(1.96)^{a}	6.05(0.36)^{a}
SUT19	0.040(0.013)^{ab}	2.05(0.83)^{cd}	3.98(0.35)^{ab}	0.042(0.016)^{ab}	2.99(1.01)^{b}	4.80(1.03)^{b}
SUT19 + Compost	0.041(0.015)^{ab}	2.84(0.15)^{bc}	4.92(0.66)^{a}	0.043(0.008)^{a}	4.50(1.08)^{a}	6.10(0.27)^{a}
F- test	*	*	*	*	*	*

[1]Mean values within a column followed by different letters were significantly different according to the DUNCAN's test, $P \leq 0.05$ (*)

For the results in pot experiment, shoot biomass performed at 3rd week showed no significant differences in all treatments, whereas at 5th week, *Bacillus* sp. SUT 1 amended with compost was able to increase plant biomass of Chinese kale when compared to the compost amendment alone. However, commercial strains and isolated strains (*Azotobacter* sp. with compost, *Azospirillum* sp. with compost, *Bacillus* sp. SUT 1 with compost and *Pseudomonas* sp. SUT 19 with compost) were able to promote growth of Chinese kale significantly better than uninoculated control. Especially at 7th week after planting, only *Azotobacter* sp. with compost, *Bacillus* sp.

SUT 1 with compost and *Pseudomonas* sp. SUT 19 amended with compost demonstrated the ability to promote higher growth of Chinese kale than uninoculated control. At 5 weeks after planting of field experiment, the both commercial strains and isolated strains (*Bacillus* sp. SUT 1 with compost and *Pseudomonas* sp. SUT 19 with compost) gave similar results better in promoting growth of Chinese kale than uninoculated control and compost amendment alone. At 7 weeks after planting, both of inoculated strains amended with compost enhanced Chinese kale growth significantly better than all treatments.

The results of effect of PGPR on plant biomass of Chinese kale in pot/field experiments in second crop (summer season) showed in Table 4.9. In this experiment, the fertilizer was reduced 50% from recommended rate to confirm the efficiency of selected strains in lower amount of plant nutrients. The results in pot experiment displayed the tendency of plant biomass was similar to the first crop (full rate of fertilizer). At 3 and 5 weeks after planting, the effects from all of inoculated strains treatments were not significantly different when compared with uninoculated control. Whereas, at 7 weeks after planting, selected strains (*Bacillus* sp. SUT 1 amended with compost and *Pseudomonas* sp. SUT 19 amended with compost) were significantly different from uninoculated control. Nevertheless, selected strains (*Pseudomonas* sp. SUT 19 with compost) showed high efficiency to promote the Chinese kale growth when compared with compost amendment alone. At the 7 weeks after planting, the ability to promote Chinese kale growth of the isolate *Bacillus* sp. SUT 1 and *Pseudomonas* sp. SUT 19 mixed with compost was higher than that of uninoculated control.

Table 4.9 The effect of PGPR on plant biomass in pot and field experiments in summer season.

Treatments	April - June 2010					
	Plant dry weight (g)					
	Pot experiments			Field experiments		
	3rd week	5th week	7th week	3rd week	5th week	7th week
Control	0.030(0.016)[a]	0.92(0.45)[a]	1.16(0.42)[c]	0.029(0.008)[a]	0.98(0.49)[a]	1.95(1.06)[b]
Compost	0.030(0.018)[a]	0.90(0.69)[a]	1.58(1.07)[bc]	0.029(0.007)[a]	1.01(0.54)[a]	2.28(0.80)[b]
Azotobacter sp.	0.029(0.006)[a]	1.18(0.93)[a]	2.18(1.28)[abc]	0.033(0.009)[a]	1.28(0.90)[a]	2.73(0.52)[ab]
Azotobacter sp. + Compost	0.030(0.004)[a]	1.22(0.89)[a]	2.36(0.42)[abc]	0.034(0.004)[a]	1.31(0.98)[a]	3.08(1.14)[ab]
Azospirillum sp.	0.027(0.009)[a]	1.18(1.05)[a]	2.13(0.37)[abc]	0.029(0.005)[a]	1.27(0.91)[a]	2.74(0.69)[ab]
Azospirillum sp. + Compost	0.038(0.006)[a]	1.23(0.83)[a]	2.25(0.23)[abc]	0.031(0.006)[a]	1.30(0.97)[a]	2.85(1.18)[ab]
SUT1	0.036(0.008)[a]	1.24(1.06)[a]	2.08(0.71)[abc]	0.037(0.006)[a]	1.33(0.93)[a]	2.74(0.99)[ab]
SUT1 + Compost	0.038(0.011)[a]	1.33(1.02)[a]	2.85(1.18)[ab]	0.039(0.011)[a]	1.44(0.86)[a]	3.72(0.88)[a]
SUT19	0.034(0.009)[a]	1.20(0.94)[a]	1.99(0.36)[abc]	0.035(0.012)[a]	1.33(0.87)[a]	3.05(0.84)[ab]
SUT19 + Compost	0.033(0.012)[a]	1.27(0.96)[a]	2.95(0.67)[a]	0.036(0.005)[a]	1.56(0.70)[a]	3.67(0.49)[a]
F- test	ns	ns	*	ns	ns	*

[1]Mean values within a column followed by different letters were significantly different according to the DUNCAN's test, P≤0.05 (*), ns = non significant

In addition, the isolate *Pseudomonas* sp. SUT 19 amended with compost is the best among all treatments in terms of promoting growth of Chinese kale. This is again confirmed that our selected PGPR could promote growth of Chinese kale even when amount of fertilizers was reduced as well as higher temperature in summer.

4.2.4 Comparative PCR-DGGE and PCA analyses of microbial populations

The effect of PGPR inoculation on soil microbial community structure in Chinese kale rhizosphere in both pot and field experiments was evaluated using PCR-DGGE approach. DGGE fingerprints confirmed that our isolates are able to establish in the Chinese kale rhizosphere throughout the plantation period (Fig. 4.6A) and the DGGE patterns obtained from rhizosphere in the first crop are similar to the second crop (Fig. 4.8). The community structures of all eubacteria, archeobacteria and fungi

from bulk soil showed absolutely different DGGE patterns when compared to other treatments. Both of eubacterial DGGE patterns obtained from pot and field consisted of a large amount of bands representing huge verity of ribotype at rhizosphere soil (Figs. 4.6A and 4.7A). The DGGE fingerprints of archeobacteria in both pot and field showed the amounts of bands which was slightly increased along with the plant age. Each treatment of DGGE patterns consisted of a few stronger bands and a large number of fainter bands representing less dominant ribotypes, whereas the relative abundance of several ribotypes was enhanced in the 7 weeks after planting (Figs. 4.6B and 4.7B). The DGGE fingerprints of fungi were similar to the archeobacterial fingerprint in terms of the amount of bands, which slightly increased when time of plantation was increased (Figs. 4.6C and 4.7C). However, the results from DGGE analysis revealed that relative abundance of eubacterial, archeobacterial and fungal populations in the rhizosphere of Chinese kale strongly shifted during plant growth.

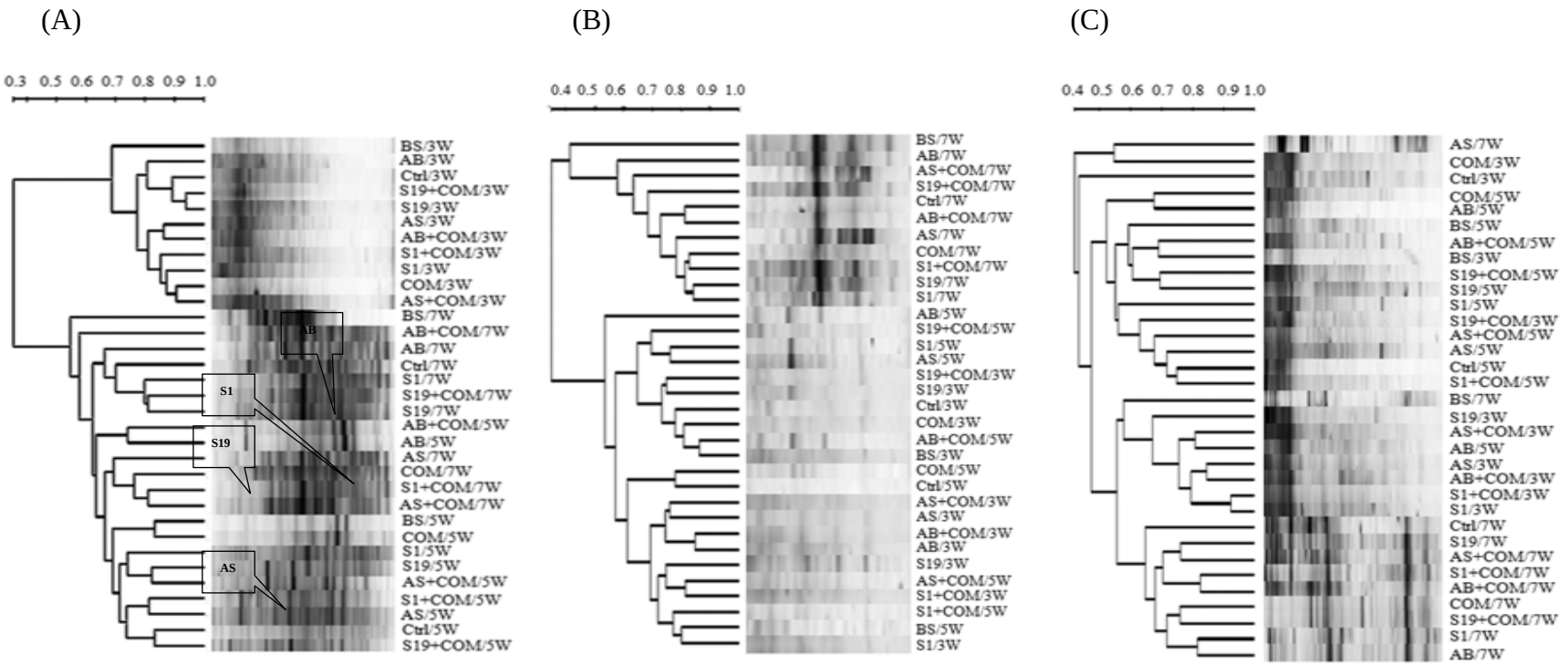


Figure 4.6 Community structure of soil microorganism from pot experiment. Dendrograms of soil microorganism based on PCR-DGGE bands. (A) Eubacterial community structure; (B) Archeobacterial community structure; (C) Fungal community structure. Letters indicate the inoculated treatments; (AB) *Azotobacter* sp.; (AS) *Azospirillum* sp.; (S1) *Bacillus* sp. SUT 1; (S19) *Pseudomonas* sp. SUT 19; (COM) compost; (Ctrl) control; (BS) bulk soil; (3, 5, 7) weeks after inoculation.

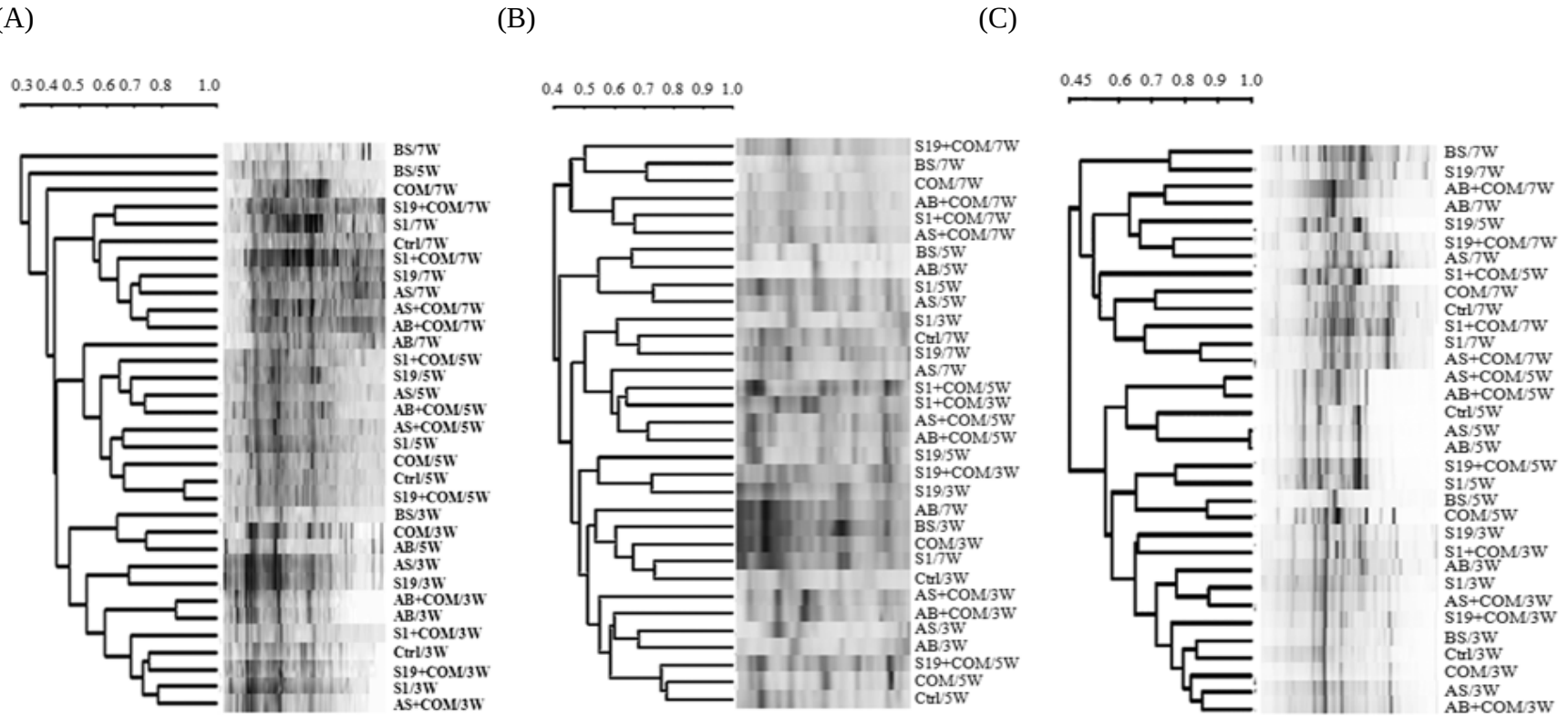


Figure 4.7 Community structure of soil microorganism from field experiment. Dendrograms of soil microorganism based on PCR-DGGE bands. (A) Eubacterial community structure; (B) Archeobacterial community structure; (C) Fungal community structure. Letters indicate the inoculated treatments; (AB) *Azotobacter* sp.; (AS) *Azospirillum* sp.; (S1) *Bacillus* sp. SUT 1; (S19) *Pseudomonas* sp. SUT 19; (COM) compost; (Ctrl) control; (BS) bulk soil; (3, 5, 7) weeks after inoculation

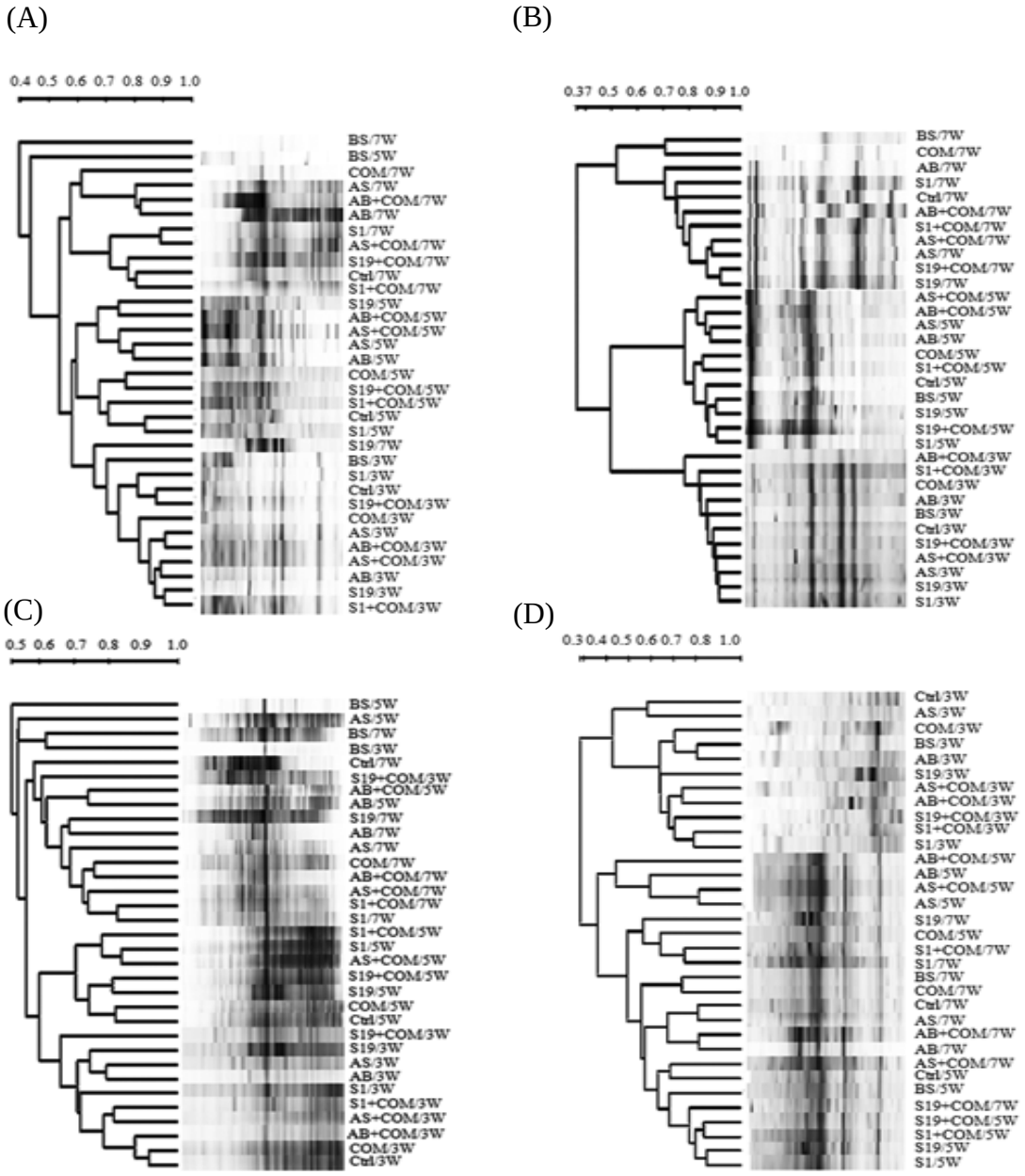
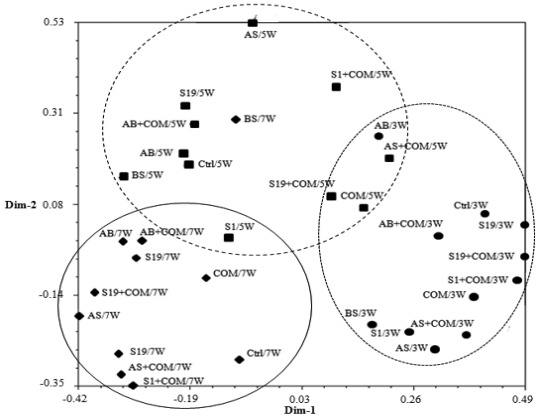


Figure 4.8 Community structure of soil microorganism from pot/field experiment. Dendrograms of soil microorganism based on PCR-DGGE bands. (A) Eubacterial community structure from pot experiment; (B) Fungal community structure from pot experiment; (C) Eubacterial community structure from field experiment; (D) Fungal community structure from field experiment. Letters indicate the inoculated treatments; (AB) *Azotobacter* sp.; (AS) *Azospirillum* sp.; (S1) *Bacillus* sp. SUT 1; (S19) *Pseudomonas* sp. SUT 19; (COM) compost; (Ctrl) control; (BS) bulk soil; (3, 5, 7) weeks after inoculation.

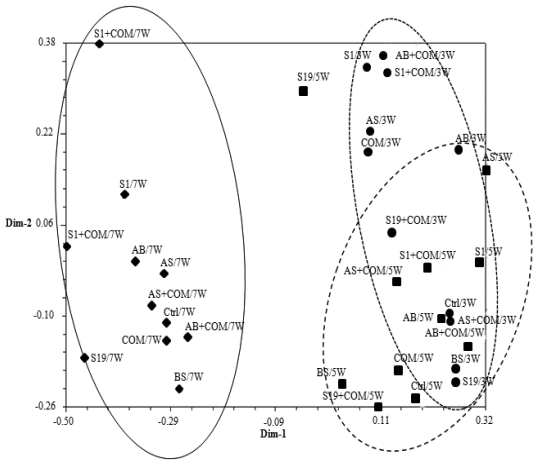
In order to determine the microbial community shifting more clearly, PCA was used to demonstrate multidimensional relationships derived from portions of the DGGE fingerprints. The results in pot experiment revealed that eubacterial community structure was slightly separated from eubacterial community in the bulk soil (Fig. 4.9A). Whereas, the compost amendments did not have any influence on the eubacterial community structure. In addition, it was clearly demonstrated that the state of plant growth showed great influence on the bacterial community structure. In the case of archeobacterial community in pot experiment, it was revealed that the community structure of bulk soil was not absolutely separated from rhizosphere soil (Fig. 4.9B). For fungal community structure, the community changes at 3^{rd} and 5^{th} week were grouped together and community structure in bulk soil of each time was not clearly separated from rhizosphere soil (Fig. 4.9C).

The results in field experiments showed that eubacterial community structure from rhizosphere was slightly different from that of eubacterial community structure of bulk soil (Fig. 4.9D). In addition, when considering fungal community structure, it was found that the community changes were strongly influenced by plant age (Fig.4.9F). Since the aim of setting pot experiment was to observe the effect of plant intensive care such as watering, the effects of more roots developed in soil volume might bring about changes in microbial community structure. The results obtained from PCA analysis demonstrated that the each archeobacterial community structure at 3^{rd}, 5^{th} and 7^{th} week was not as clearly shifted as analyzed by PCA (Fig.4.9E). For the DGGE and PCA results from field experiment conducted between September to November 2009 showed the similar results with the crop in April to June 2010 (Fig 4.10).

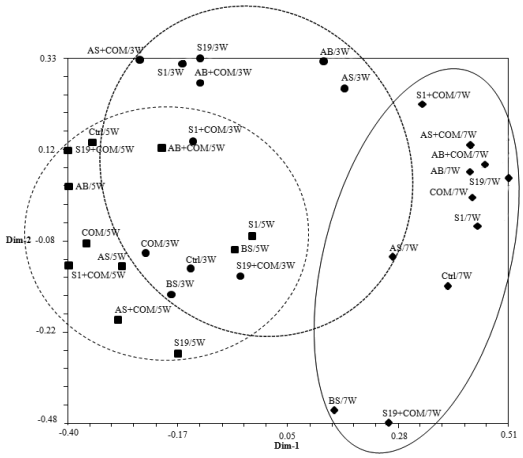
(A)


AS/5W
S1+COM/5W
S19/5W
AB+COM/5W
BS/7W
AB/3W
AS+COM/5W
AB/5W
Ctrl/5W
BS/5W
S19+COM/5W
COM/5W
Ctrl/3W
AB+COM/3W
S19/3W
AB/7W
AB+COM/7W
S1/5W
S19/7W
S19+COM/3W
COM/7W
S1+COM/3W
S19+COM/7W
COM/3W
AS/7W
BS/3W
AS+COM/3W
S1/3W
AS/3W
S19/7W
Ctrl/7W
AS+COM/7W
S1+COM/7W
Dim-2
Dim-1
0.53
0.31
0.08
-0.14
-0.35
-0.42
-0.19
0.03
0.26
0.49

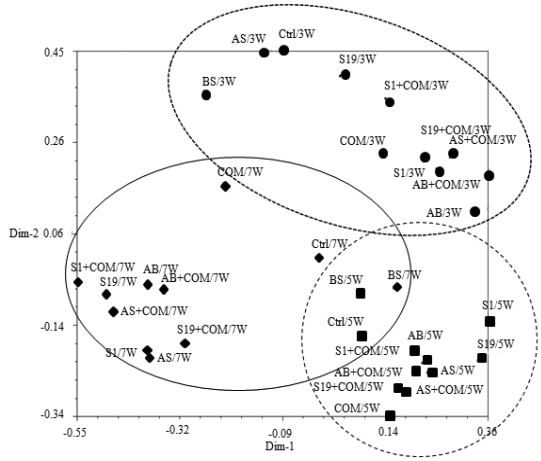
(B)


S1+COM/7W
S1/3W
AB+COM/3W
S1+COM/3W
S19/5W
AS/3W
COM/3W
AB/3W
AS/3W
S1/7W
S19+COM/3W
S1+COM/7W
AB/7W
AS/7W
S1+COM/5W
S1/5W
AS+COM/5W
AS+COM/7W
Ctrl/3W
Ctrl/7W
AB/5W
AS+COM/3W
AB+COM/7W
AB+COM/5W
COM/7W
COM/5W
BS/3W
S19/7W
BS/7W
BS/5W
S19/3W
Ctrl/5W
S19+COM/5W
Dim-2
Dim-1
0.38
0.22
0.06
-0.10
-0.26
-0.50
-0.29
-0.09
0.11
0.32

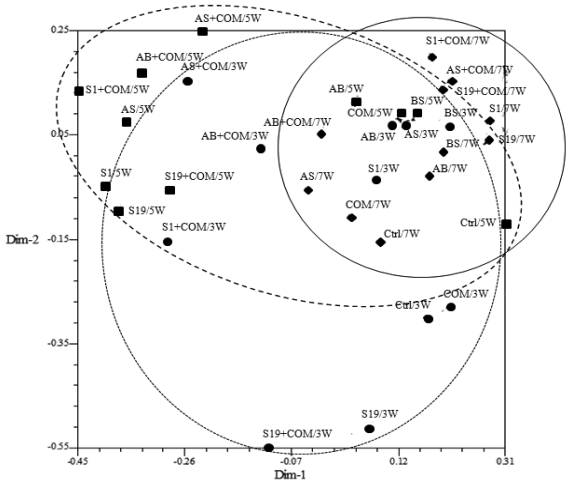
(C)


AS+COM/3W
S19/3W
AB/3W
S1/3W
AB+COM/3W
AS/3W
S1+COM/7W
Ctrl/5W
S1+COM/3W
AS+COM/7W
S19+COM/5W
AB+COM/5W
AB+COM/7W
AB/7W
S19/7W
AB/5W
COM/7W
S1/7W
COM/5W
S1/5W
COM/3W
BS/5W
AS/7W
AS/5W
Ctrl/3W
Ctrl/7W
S1+COM/5W
S19+COM/3W
BS/3W
AS+COM/5W
S19/5W
BS/7W
S19+COM/7W
Dim-2
Dim-1
0.33
0.12
-0.08
-0.22
-0.48
-0.40
-0.17
0.05
0.28
0.51

(D)


AS/3W
Ctrl/3W
S19/3W
BS/3W
S1+COM/3W
S19+COM/3W
COM/3W
AS+COM/3W
S1/3W
COM/7W
AB+COM/3W
AB/3W
Ctrl/7W
S1+COM/7W
AB/7W
BS/5W
BS/7W
S19/7W
AB+COM/7W
S1/5W
AS+COM/7W
Ctrl/5W
S19+COM/7W
AB/5W
S1/7W
AS/7W
S1+COM/5W
S19/5W
AB+COM/5W
AS/5W
S19+COM/5W
AS+COM/5W
COM/5W
Dim-2
Dim-1
0.45
0.26
0.06
-0.14
-0.34
-0.55
-0.32
-0.09
0.14
0.36

(E)


AS+COM/5W
S1+COM/7W
AB+COM/5W
AS+COM/7W
AB+COM/3W
S1+COM/5W
AB/5W
S19+COM/7W
BS/5W
AS/5W
COM/5W
S1/7W
AB+COM/7W
AB/3W
AS/3W
BS/7W
AB+COM/3W
S19/7W
S1/5W
S19+COM/5W
AS/7W
S1/3W
AB/7W
S19/5W
COM/7W
S1+COM/3W
Ctrl/5W
Ctrl/7W
Ctrl/3W
COM/3W
S19/3W
S19+COM/3W
Dim-2
Dim-1
0.25
0.05
-0.15
-0.35
-0.55
-0.45
-0.26
-0.07
0.12
0.31

(F)

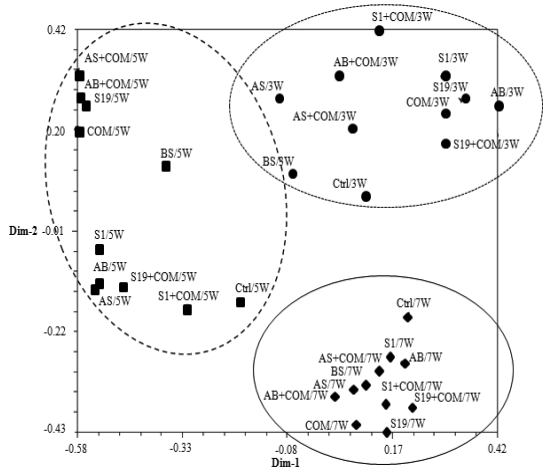

S1+COM/3W
AS+COM/5W
AB+COM/3W
S1/3W
AB+COM/5W
S19/3W
AS/3W
AB/3W
S19/5W
COM/3W
AS+COM/3W
COM/5W
S19+COM/3W
BS/5W
BS/5W
Ctrl/3W
S1/5W
AB/5W
S19+COM/5W
AS/5W
S1+COM/5W
Ctrl/5W
Ctrl/7W
S1/7W
AS+COM/7W
AB/7W
BS/7W
AS/7W
S1+COM/7W
AB+COM/7W
S19+COM/7W
COM/7W
S19/7W
Dim-2
Dim-1
0.42
0.20
-0.01
-0.22
-0.43
-0.58
-0.33
-0.08
0.17
0.42

Figure 4.9 The community analysis derived two-dimentional plot based on the first two principal coordinates from a principal coordinate analysis (PCA) of Chinese kale rhizosphere. (A) PCA of eubacteria in pot experiment; (B) PCA of archeobacteria in pot experiment; (C) PCA of fungi in pot experiment; (D) PCA of eubacteria in field experiment; (E) PCA of archeobacteria in field experiment; (F) PCA of fungi in field experiment. Letters indicate the inoculated treatments; (AB) *Azotobacter* sp.; (AS) *Azospirillum* sp.; (COM) compost; (S1) *Bacillus* sp. SUT 1; (S19) *Pseudomonas* sp. SUT 19; (Ctrl) control; (BS) bulk soil; (2, 5, 8) weeks after inoculation; ——— , ------ , and ····· show a trend of 2, 5, and 8 week, respectively after inoculation are different from each other.

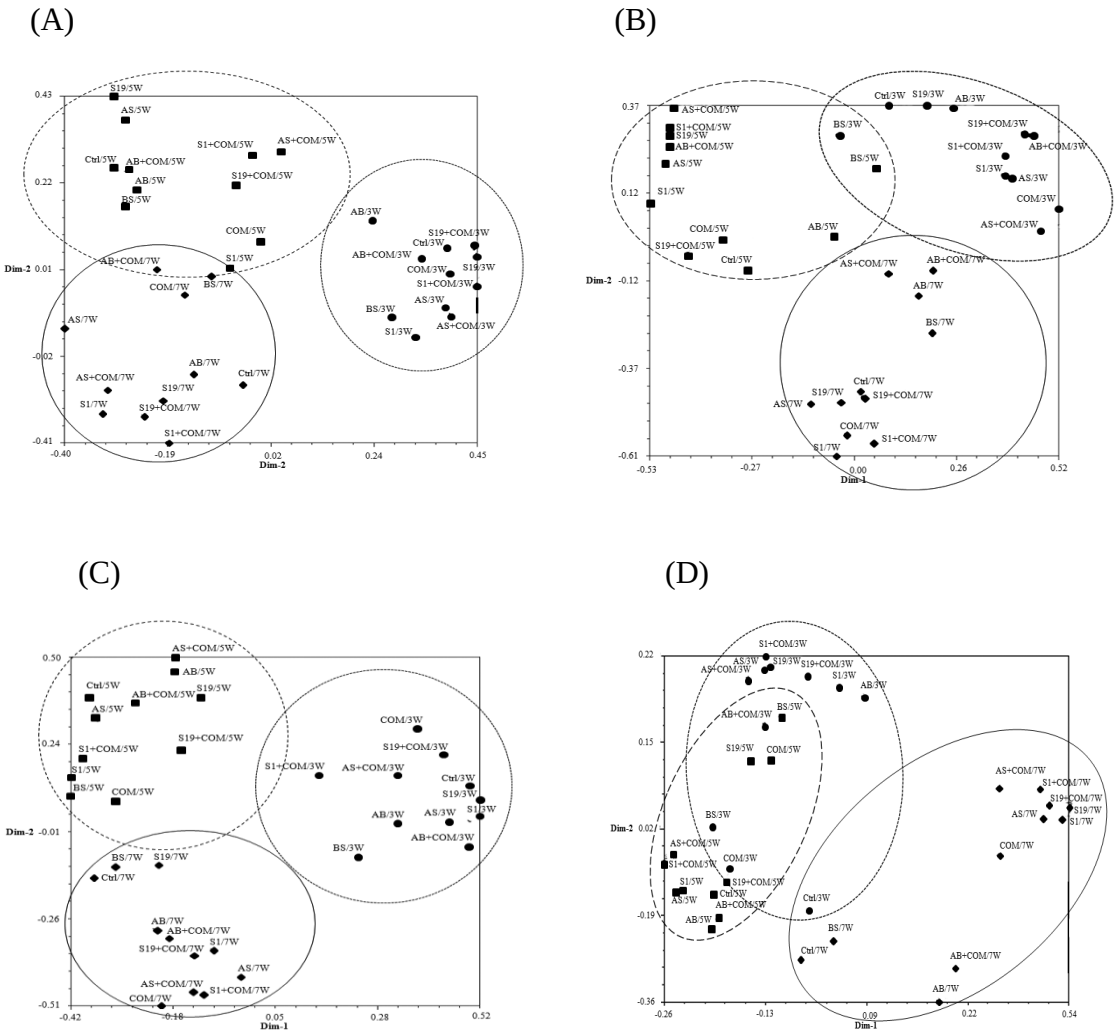


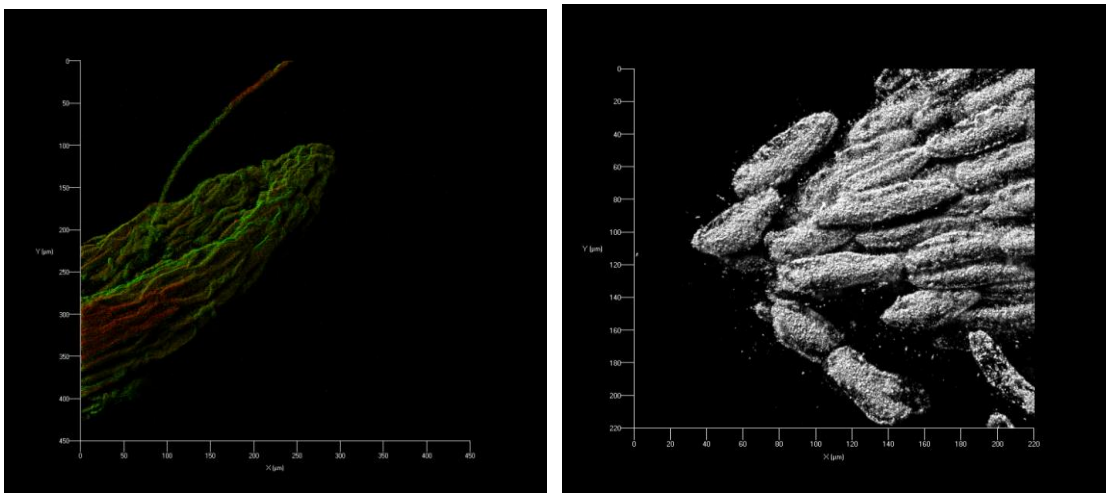
Figure 4.10 The community analysis derived two-dimentional plot based on the first two principal coordinates from a principal corrordonate analysis (PCA) of Chinese kale rhizosphere. (A) PCA of eubacteria in pot experiment; (B) PCA of fungi in pot experiment; (C) PCA of eubacteria in field experiment; (D) PCA of fungi in field experiment. Letters indicate the inoculated treatments; (AB) *Azotobacter* sp.; (AS) *Azospirillum* sp.; (COM) compost; (S1) *Bacillus* sp. SUT 1; (S19) *Pseudomonas* sp. SUT 19; (Ctrl) control; (BS) bulk soil; (2, 5) weeks after inoculation; and show a trend of 2 and 5 week, respectively after inoculation are different from each other.

However, from both experiments conducted with forage corn and Chinese kale, archeobacteria community structure in forage corn was clearly dictated by age of plant but community shift in Chinese kale was not observed. In addition, both amounts of fertilizer and temperature could not affect the shifting pattern of eubacterial and fungal community structure.

To understand the ecological impact of PGPR on microbial community structure is an important issue when attempting to better define usage conditions for these inoculants. The DGGE fingerprints displayed that compost did not have any influence on microbial community structure. However, all stages of plant growth showed the distinct profile characteristics. This also implies that plant age is a major factor influencing rhizobacterial community structure (Castro-Sowinski et al., 2007). The biological processes in the rhizosphere are strongly influenced by plant root exudates, which consist of easily degradable organic compounds that might attract and stimulate microbial growth (Walker et al., 2003). Some previous studies also reported that the plant development is a major cause for microbial community changes in rhizosphere soil (Herschkovitz et al., 2005a; Herschkovitz et al., 2005b)

Since *Pseudomonas* sp. SUT19 showed promising results in term of root colonization and plant growth promotion in both forage corn and Chinese kale, therefore *Pseudomonas* sp. SUT19 was tagged with GFP in order to investigate ability of root colonization. The result from *Pseudomonas* sp. SUT19::*gfp* confirmed the efficiency of root colonization of forage corn and Chinese kale (Figure 4.11). The root was rapidly colonized by *Pseudomonas* sp. SUT19::*gfp* cell after 7 days of inoculation.

(A) (B)



(C) (D)

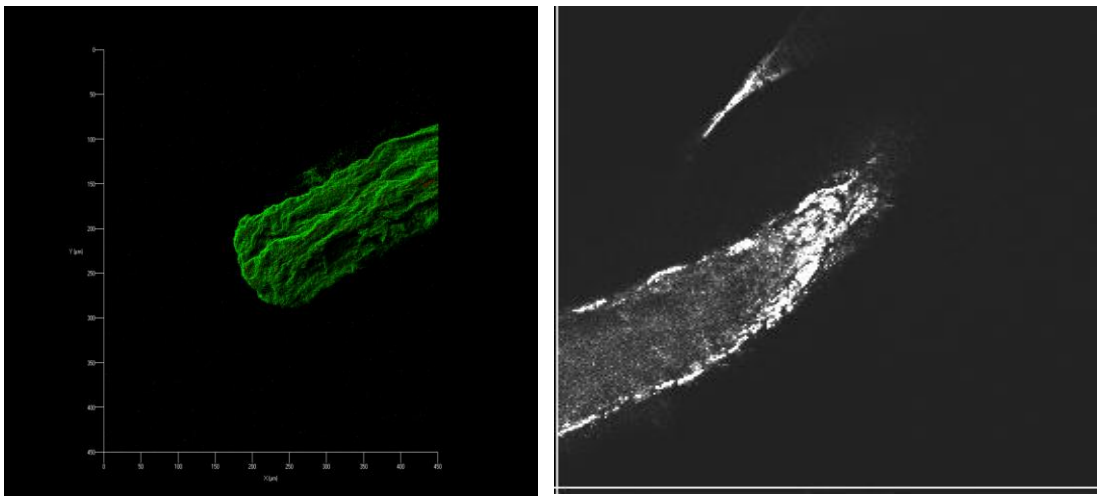


Figure 4.11 The root colonization of *Pseudomonas* sp. SUT19::*gfp* on forage corn and Chinese kale rhizosphere. The root colonization of *Pseudomonas* sp. SUT19 on forage corn rhizosphere, (C) The root colonization of *Pseudomonas* sp. SUT19::*gfp* on Chinese kale rhizosphere and (D) The root colonization of *Pseudomonas* sp. SUT19 on Chinese kale rhizosphere,after 7 days of inoculation

From this study, newly selected PGPR as *Pseudomonas* sp. SUT19, *Brevibacillus* sp. SUT47 for maize and *Bacillus* sp. SUT 1, *Pseudomonas* sp. SUT19 for Chinese kale showed better plant growth promotion than both commercial strains by SUT. Since these PGPR might promote the growth of the plants and increase the root surface area or root architecture, therefore, plants growing better in turn release higher amount of C in root exudates. The release of more C prompts increase in microbial activity, and this process continues in a cycle. The whole process makes more N available from soil pool, influencing N flux into plant roots, and plants are able to uptake more available N. Therefore, inoculants could be used to allow reduction in the current high rates of fertilizers without compromising plant productivity (Adesemoye et al., 2009). However, from this study, it should be noted that no microbial inoculants can be universal for all systems as the effectiveness may be affected by plant species, soil type and some other factors. In addition, the impacts of inoculation of those strains on the microbial community structure of field-grown plants were not negative. A shift in the structure of indigenous microbial community was mainly caused by plant age.

CHAPTER V

CONCLUSION

In conclusion, inoculation of forage corn seeds with *Pseudomonas* sp. SUT 19 and *Brevibacillus* sp. SUT 47 mixed with compost promotes growth and biomass of forage corn better than commercial strains. And in case of Chinese kale cultivation system, the *Bacillus sp.* SUT1 and *Pseudomonas* sp. SUT 19 mixed with compost was able to increase biomass of Chinese kale significantly highest in comparison to uninoculated control, thus they might be applied as inocula. The roles of forage corn and Chinese kale growth promoted by PGPR might come from some other factors as ACC-deaminase, P-solubilization, etc. The impact of all tested PGPR on the indigenous soil microorganisms did not seem to have prominent effect on the structure of microbial population with respect to the control treatments. Recovered and sequenced DGGE bands showed homology with some important eubacterial and fungal groups confirmed that inoculated PGPR did not mainly interfere with other microbes in rhizosphere. However, the plant age mainly caused a shift in the structure of indigenous microbial community. Such mechanisms as plant-microbe and microbe-microbe interaction still remain to be elucidated.

This study demonstrated that no universal strain of PGPR for every plant species, thus selection of PGPR on the basis of host preference is needed in first step of application. This study also recommended that *Pseudomonas* sp. SUT 19 and *Brevibacillus* sp. SUT 47 can be applied as PGPR inoculum for forage corn, and

Bacillus sp. SUT1 and *Pseudomonas* sp. SUT 19 are appropriate for Chinese kale inoculum.

REFERENCES

Adesemoye, A. O., Torbert, H. A., and Kloepper, J. W. (2009): Plant Growth-Promoting Rhizobacteria Allow Reduced Application Rates of Chemical Fertilizers. **Microb. Ecol.** 58, 921-929.

Ahmed, A., Van De Sande, W., Verbrugh, H., Fahal, A., and Van Belkum, A. (2003): *Madurella mycetomatis* strains from mycetoma lesions in Sudanese patients are clonal. **J. Clin. Microbiol.** 41, 4537-4541.

Amann, R. I., Ludwig, W., and Schleifer, K. H. (1995): Phylogenetic identification and in situ detection of individual microbial cells without cultivation. **Microbiol. Mol. Biol. Rev.** 59, 143-169.

Assmus, B., Hutzler, P., Kirchhof, G., Amann, R., Lawrence, J. R., and Hartmann, A. (1995): In situ localization of *Azospirillum brasilense* in the rhizosphere of wheat with fluorescently labeled, rRNA-targeted oligonucleotide probes and scanning confocal laser microscopy. **Appl. Environ. Microbiol.** 61, 1013-1019.

Avrahami, S., and Conrad, R. (2003): Patterns of community change among ammonia oxidizers in meadow soils upon long-term incubation at different temperatures. **Appl. Environ. Microbiol.** 69, 6152-6164.

Bankhead, S. B., Landa, B. B., Lutton, E., Weller, D. M., and Gardener, B. (2004): Minimal changes in rhizobacterial population structure following root colonization by wild type and transgenic biocontrol strains. **FEMS Microbiol. Ecol.** 49, 307-318.

Bashan, Y., and Holguin, G. (1998): Proposal for the division of plant growth-promoting rhizobacteria into two classifications: biocontrol-PGPB (plant growth-promoting bacteria) and PGPB. **Soil Biol. Biochem.** 30, 1225-1228.

Baudoin, E., Benizri, E., and Guckert, A. (2002): Impact of growth stage on the bacterial community structure along maize roots, as determined by metabolic and genetic fingerprinting. **Appl. Soil Ecol.** 19, 135-145.

Baudoin, E., Benizri, E., and Guckert, A. (2003): Impact of artificial root exudates on the bacterial community structure in bulk soil and maize rhizosphere. **Soil Biol. Biochem.** 35, 1183-1192.

Baudoin, E., Nazaret, S., Mougel, C., Ranjard, L., and Mo nne-Loccoz, Y. (2009): Impact of inoculation with the phytostimulatory PGPR *Azospirillum lipoferum* CRT1 on the genetic structure of the rhizobacterial community of field-grown maize. **Soil Biol. Biochem.** 41, 409-413.

Bloemberg, G. V., Wijfjes, A. H. M., Lamers, G. E. M., Stuurman, N., and Lugtenberg, B. J. J. (2000): Simultaneous imaging of *Pseudomonas fluorescens* WCS365 populations expressing three different autofluorescent proteins in the rhizosphere: new perspectives for studying microbial communities. **Mol. Plant Microbe Interact.** 13, 1170-1176.

Boureau, T., Jacques, M. A., Berruyer, R., Dessaux, Y., Dominguez, H., and Morris, C. E. (2004): Comparison of the phenotypes and genotypes of biofilm and solitary epiphytic bacterial populations on broad-leaved endive. **Microb. Ecol.** 47, 87-95.

Bremner, J. M. (1996): Nitrogen total, pp. 1085-1122. In D. L. Sparks, A. L. Page, P. A. Helmke, R. H. Loeppert, P. N. Soltanpour, M. A. Tabatabai, C. T.

Johnston, and M. E. Sumner (Eds): *Methods of soil analysis. Part 3 - chemical methods.*, Soil Science Society of America Inc., Madison.

Burdman, S., Okon, Y., and Jurkevitch, E. (2000): Surface characteristics of *Azospirillum brasilense* in relation to cell aggregation and attachment to plant roots. **Crit. Rev. Microbiol.** 26, 91-110.

Carbajo, J. M., Junca, H., Terr n, M. C., Gonz lez, T., Yag e, S., Zapico, E., and Gonz lez, A. E. (2002): Tannic acid induces transcription of laccase gene cglcc1 in the white-rot fungus *Coriolopsis gallica*. **Can. J. Microbiol.** 48, 1041-1047.

Castro-Sowinski, S., Herschkovitz, Y., Okon, Y., and Jurkevitch, E. (2007): Effects of inoculation with plant growth-promoting rhizobacteria on resident rhizosphere microorganisms. **FEMS Microbiol. Lett.** 276, 1-11.

Chen, Y. P., Rekha, P. D., Arun, A. B., Shen, F. T., Lai, W. A., and Young, C. C. (2006): Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. **Appl. Soil. Ecol.** 34, 33-41.

Copenhagen, D. (1997): The Rhizosphere as a Habitat for Soil Microorganisms, pp. 21-45. In E. J. D. Van, J. T. Trevors, and E. M. H. Wellington (Eds): *Modern Soil Microbiology* New York.

Costa, R., G tz, M., Mrotzek, N., Lottmann, J., Berg, G., and Smalla, K. (2005a): Effects of site and plant species on rhizosphere community structure as revealed by molecular analysis of microbial guilds. **FEMS Microbiol. Ecol.** 56, 236-249.

Costa, R., G tz, M., Mrotzek, N., Lottmann, J., Berg, G., and Smalla, K. (2006): Effects of site and plant species on rhizosphere community structure as

revealed by molecular analysis of microbial guilds. **FEMS Microbiol. Ecol.** 56, 236-249.

Costa, R., Gomes, N. C. M., Kr gerrecklenfort, E., Opelt, K., Berg, G., and Smalla, K. (2007): *Pseudomonas* community structure and antagonistic potential in the rhizosphere: insights gained by combining phylogenetic and functional gene-based analyses. **Environ. Microbiol.** 9, 2260-2273.

Costa, R., Götz, M., Mrotzek, N., Lottmann, J., Berg, G., and Smalla, K. (2005b): Effects of site and plant species on rhizosphere community structure as revealed by molecular analysis of microbial guilds. **FEMS Microbiol. Ecol.** 56, 236-249.

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

Da Mota, F. F., Gomes, E. A., and Seldin, L. (2008): Auxin production and detection of the gene coding for the Auxin Efflux Carrier (AEC) protein in *Paenibacillus polymyxa*. **J. Microbiol.** 46, 257-264.

de Kievit, T. R. (2009): Quorum sensing in *Pseudomonas aeruginosa* biofilms. **Environ. Microbiol.** 11, 279-288.

Duncan, D. B. (1955): Multiple range and multiple F tests. **Biometrics** 11, 1-42.

El Zemrany, H., Cortet, J., Peter Lutz, M., Chabert, A., Baudoin, E., Haurat, J., Maughan, N., F lix, D., D fago, G., and Bally, R. (2006): Field survival of the phytostimulator *Azospirillum lipoferum* CRT1 and functional impact on maize crop, biodegradation of crop residues, and soil faunal indicators in a context of decreasing nitrogen fertilisation. **Soil Biol. Biochem.** 38, 1712-1726.

Espinosa-Urgel, M., Kolter, R., and Ramos, J. L. (2002): Root colonization by *Pseudomonas putida*: love at first sight. **Microbiol.** 148, 341-344.

Fallik, E., Sarig, S., and Okon, Y. (1994): Morphology and physiology of plant roots associated with *Azospirillum*, pp. 77–86. In Y. Okon (Ed.): *Azospirillum/plant associations*, Boca Raton.

Felici, C., Vettori, L., Giraldi, E., Forino, L. M. C., Toffanin, A., Tagliasacchi, A. M., and Nuti, M. (2008): Single and co-inoculation of *Bacillus subtilis* and *Azospirillum brasilense* on *Lycopersicon esculentum*: Effects on plant growth and rhizosphere microbial community. **Appl. Soil. Ecol.** 40, 260-270.

Fukuhara, H., Minakawa, Y., Akao, S., and Minamisawa, K. (1994): The Involvement of Indole-3-Acetic Acid Produced by *Bradyrhizobium elkanii* in Nodule Formation. **Plant Cell Physiol.** 35, 1261-1265.

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

Gomes, N. C. M., Fagbola, O., Costa, R., Rumjanek, N. G., Buchner, A., Mendona-Hagler, L., and Smalla, K. (2003): Dynamics of fungal communities in bulk and maize rhizosphere soil in the tropics. **Appl. Environ. Microbiol.** 69, 3758-3766.

Gomes, N. C. M., Heuer, H., Schönfeld, J., Costa, R., Mendonca-Hagler, L., and Smalla, K. (2001): Bacterial diversity of the rhizosphere of maize (*Zea mays*) grown in tropical soil studied by temperature gradient gel electrophoresis. **Plant Soil** 232, 167-180.

Guido, B., and Bloemberg, V. (2001): Molecular determinants of rhizosphere colonization by *Pseudomonas*. **Annu. Rev. Phytopathol.** 39, 461-490.

Hardy, R. W. F., Holsten, R. D., Jackson, E. K., and Burns, R. C. (1968): The acetylene-ethylene assay for N2 fixation: laboratory and field evaluation. **Plant Physiol.** 43, 1185-1207.

Herschkovitz, Y., Lerner, A., Davidov, Y., Okon, Y., and Jurkevitch, E. (2005a): *Azospirillum brasilense* does not affect population structure of specific rhizobacterial communities of inoculated maize (*Zea mays*). **Environ. Microbiol.** 7, 1847-1852.

Herschkovitz, Y., Lerner, A., Davidov, Y., Rothballer, M., Hartmann, A., Okon, Y., and Jurkevitch, E. (2005b): Inoculation with the plant-growth-promoting rhizobacterium *Azospirillum brasilense* causes little disturbance in the rhizosphere and rhizoplane of maize (*Zea mays*). **Microb. Ecol.** 50, 277-288.

Hietala, A. M., Korhonen, K., and Sen, R. (2003): An unknown mechanism promotes somatic incompatibility in *Ceratobasidium bicorne*. **Mycologia** 95, 239-250.

Hoagland, D. R., and Arnon, D. I. (1950): The water culture method for growing plants without soil. **Calif. AES. Circular.** 347, 32-39.

Hong, Y., Glick, B. R., and Pasternak, J. J. (1991): Plant-microbial interaction under gnotobiotic conditions: a scanning electron microscope study. **Curr. Microbiol.** 23, 111-114.

Inbar, E., Green, S. J., Hadar, Y., and Minz, D. (2005): Competing factors of compost concentration and proximity to root affect the distribution of streptomycetes. **Microb. Ecol.** 50, 73-81.

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

Julián, M. C., Acero, J., Salazar, O., and Keijer, J. (1999): Mating type-correlated molecular markers and demonstration of heterokaryosis in the phytopathogenic fungus *Thanatephorus cucumeris* (*Rhizoctonia solani*) AG 1-IC by AFLP DNA fingerprinting analysis. **J. Biotechnol.** 67, 49-56.

Justesen, A. F., Yohalem, D., Bay, A., and Nicolaisen, M. (2003): Genetic diversity in potato field populations of *Thanatephorus cucumeris* AG-3, revealed by ITS polymorphism and RAPD markers. **Mycol. Res.** 107, 1323-1331.

Kang, S. M., Hamayun, M., Joo, G. J., Khan, A. L., Kim, Y. H., Kim, S. K., Jeong, H. J., and Lee, I. J. (2010): Effect of *Burkholderia* sp. KCTC 11096BP on some physiochemical attributes of cucumber. **Eur. J. Soil Biol.** 46, 264-268.

Kende, H. (1993): Ethylene biosynthesis. **Annu. Rev. Plant Physiol. Plant Mol. Biol.** 44, 283-307.

Kumar, B., Trivedi, P., and Pandey, A. (2007): *Pseudomonas corrugata*: A suitable bacterial inoculant for maize grown under rainfed conditions of Himalayan region. **Soil Biol. Biochem.** 39, 3093-3100.

Lipman, J. G. (1904): Soil bacteriological studies. Further contributions to the physiology and morphology of the members of the *Azotobacter* group. **New Jersey Agr. Exp. Sta. Ann. Rpt.** 25, 237-289.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951): Protein measurement with the Folin phenol reagent. **J. Biol. Chem.** 193, 265-275.

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

Lynch, J. M. (1990): **The rhizosphere**. Wiley New York. New York.

Lynch, J. M., and Whipps, J. M. (1990): Substrate flow in the rhizosphere. **Plant Soil** 129, 1-10.

Mar Vazquez, M., Cesar, S., Azc n, R., and Barea, J. M. (2000): Interactions between arbuscular mycorrhizal fungi and other microbial inoculants (*Azospirillum*, *Pseudomonas*, *Trichoderma*) and their effects on microbial population and enzyme activities in the rhizosphere of maize plants. **Appl. Soil Ecol.** 15, 261-272.

Marschner, P., and Baumann, K. (2003): Changes in bacterial community structure induced by mycorrhizal colonisation in split-root maize. **Plant Soil** 251, 279-289.

Moeseneder, M. M., Arrieta, J. M., Muyzer, G., Winter, C., and Herndl, G. J. (1999): Optimization of terminal-restriction fragment length polymorphism analysis for complex marine bacterioplankton communities and comparison with denaturing gradient gel electrophoresis. **Appl. Environ. Microbiol.** 65, 3518-3525.

Murray, A. E., Hollibaugh, J. T., and Orrego, C. (1996): Phylogenetic compositions of bacterioplankton from two California estuaries compared by denaturing gradient gel electrophoresis of 16S rDNA fragments. **Appl. Environ. Microbiol.** 62, 2676-2680.

Nakatsu, C. H. (2007): Soil microbial community analysis using denaturing gradient gel electrophoresis. **Soil Sci. Soc. Am. J.** 71, 562-571.

O'Gara, F., and Shanmugam, K. T. (1976): Control of symbiotic nitrogen fixation in rhizobia regulation of NH_4^+ assimilation. **BBA-Gen. Subjects** 451, 342-352.

O'Toole, G. A., and Kolter, R. (1998): Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. **Mol. Microbiol.** 28, 449-461.

Oros-Sichler, M., Gomes, N., Neuber, G., and Smalla, K. (2006): A new semi-nested PCR protocol to amplify large 18S rRNA gene fragments for PCR-DGGE analysis of soil fungal communities. **J Microbiol Methods** 65, 63-75.

Ovreas, L., Forney, L., Daae, F. L., and Torsvik, V. (1997): Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. **Appl. Environ. Microbiol.** 63, 3367-3373.

Owen, A., and Zdor, R. (2001): Effect of cyanogenic rhizobacteria on the growth of velvetleaf (*Abutilon theophrasti*) and corn (*Zea mays*) in autoclaved soil and the influence of supplemental glycine. **Soil Biol. Biochem.** 33, 801-809.

Pascual, C. B., Todab, T., Raymondoc, A. D., and Hyakumachib, M. (2001): Characterization by conventional techniques and PCR of *Rhizoctonia solani* isolates causing banded leaf sheath blight in maize. **Plant Pathol.** 49, 108-118.

Patten, C. L., and Glick, B. R. (1996): Bacterial biosynthesis of indole-3-acetic acid. **Can. J. Microbiol.** 42, 207-220.

Patten, C. L., and Glick, B. R. (2002): Role of *Pseudomonas putida* Indoleacetic Acid in Development of the Host Plant Root System. **Appl. Environ. Microbiol.** 68, 3795-3801.

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

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

Rohlf, F. J. (2000): **NTSYS-pc: Numerical taxonomy and multivariate analysis system, version 2.2 User Guide**. Applied Biostatistics, Inc. New York.

Shaharoona, B., Arshad, M., Zahir, Z. A., and Khalid, A. (2006): Performance of *Pseudomonas* spp. containing ACC-deaminase for improving growth and yield of maize (*Zea mays* L.) in the presence of nitrogenous fertilizer. **Soil Biol. Biochem.** 38, 2971-2975.

Sheng, X., Chen, X., and He, L. (2008): Characteristics of an endophytic pyrene-degrading bacterium of *Enterobacter* sp. 12J1 from *Allium macrostemon* Bunge. **Int. Biodeterioration Biodegrad.** 62, 88-95.

Sllgiyama, J., and Ogawa, H. (2004): Plectomycetes: Biotechnological Importance and Systematics, pp. 429: *Handbook of fungal biotechnology*.

Smalla, K. (2004): Culture-independent microbiology, pp. 88–99. In A. Bull (Ed.): *Microbial Diversity and Bioprospecting*, ASM, Washington.

Steenhoudt, O., and Vanderleyden, J. (2006): *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. **FEMS Microbiol. Rev.** 24, 487-506.

Stell, R. G. D., Torrie, J. H., and Dickey, D. A. (1980): **Principles and procedures of statistics: a biometrical approach**. McGraw-Hill. New York.

Tarun, A. S., Lee, J. S., and Theologis, A. (1998): Random mutagenesis of 1-aminocyclopropane-1-carboxylate synthase: a key enzyme in ethylene biosynthesis. **Proc. Natl. Acad. Sci. USA.** 95, 9796-9801.

Teaumroong, N., Wanapu, C., Chankum, Y., Arjharn, W., Sang-Arthit, S., Teaimthaisong, K., and Boonkerd, N. (2010): Production and application of bioorganic fertilizers for organic farming systems in Thailand: A Case Study, pp. 293-312. In H. Insam, I. Franke-Whittle, and M. Goberna (Eds): *Microbes at Work*, Springer, Berlin Heidelberg.

Va utov, M. (2008): Taxonomic studies on Psathyrella sect. Spadiceae. **Czech. Mycol.** 60, 131-171.

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

Walker, T. S., Bais, H. P., Grotewold, E., and Vivanco, J. M. (2003): Root Exudation and Rhizosphere Biology. **Plant physiol.** 132, 44-51.

Walkley, A., and Black, I. A. (1934): An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. **Soil sci.** 37, 29-38.

Watanabe, T., Kimura, M., and Asakawa, S. (2006): Community structure of methanogenic archaea in paddy field soil under double cropping (rice-wheat). **Soil Biol. Biochem.** 38, 1264-1274.

Winding, A., Binnerup, S. J., and Pritchard, H. (2004): Non-target effects of bacterial biological control agents suppressing root pathogenic fungi. **FEMS Microbiol. Ecol.** 47, 129-141.

Yang, C. H., and Crowley, D. E. (2000): Rhizosphere microbial community structure in relation to root location and plant iron nutritional status. **Appl. Environ. Microbiol.** 66, 345-351.

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

BIOGRAPHY

Mr. Pongdet Piromyou was born on September 2, 1983 at Nakhonpathom, Thailand. He received his Bachelor degree of Science in Public Health, Suranaree University of Technology, 2006. Then, he had continued his Master degree in 2006 at School of Biotechnology, Suranaree University of Technology, Nakhon Ratchasima. During his study, he had experience on his thesis work in investigation the effects of Plant Growth Promoting Rhizobacteria (PGPR) inoculum on indigenous microbial community structure under cropping system. He received two scholarships as Kagoshima University on August 10-September 14, 2009 and Tsukuba University on April 5 to July 4, 2010. Some past of this work also presented in poster session in the meeting of Kagochima University on August 20, 2009. He will present research work in 1st Asian Conference on Plant-Microbe Symbiosis and Nitrogen Fixation in the topic of Effects of Plant Growth Promoting Rhizobacteria (PGPR) inoculum on indigenous microbial community structure (oral and poster presentation), September 20-24, 2010. Miyazaki, Japan.