การใช้มัลติเพล็กซ์พีซีอาร์เพื่อทำนายการเข้าสร้างปมของไรโซเบียมที่แยกจากปมรากถั่วเหลือง ที่ปลูกในดินจากอำเภอบางระกำ จังหวัดพิษณุโลก

นางสาว ณิชานันท์ กาบเกษร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาจุลชีววิทยาอุตสาหกรรม ภาควิชาจุลชีววิทยา
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2552

USE OF MULTIPLEX PCR TO PREDICT NODULATION OF RHIZOBIA ISOLATED FROM ROOT NODULES OF SOYBEANS GROWN IN BANG RAKAM DISTRICT SOILS, PHITSANULOK PROVINCE

Miss Nichanun Karbkesorn

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Industrial Microbiology
Department of Microbiology
Faculty of Science
Chulalongkorn University
Academic Year 2009

Thesis Title	USE OF MULTIPLEX PCR TO PREDICT NODULATION OF RHIZOBIA ISOLATED FROM ROOT NODULES OF SOYBEANS GROWN IN BANG RAKAM DISTRICT SOILS, PHITSANULOK PROVINCE
By	Miss Nichanun Karbkesorn
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Thesis Advisor	Associate Professor Kanjana Chansa-ngavej, Ph.D.

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ณิชานันท์ กาบเกษร : การใช้มัลติเพล็กซ์พีซีอาร์เพื่อทำนายการเข้าสร้างปมของไรโซเบียมที่แยกจากปมรากถั่วเหลืองที่ปลูกในดินจากอำเภอบางระกำ จังหวัดพิษณุโลก (USE OF MULTIPLEX PCR TO PREDICT NODULATION OF RHIZOBIA ISOLATED FROM ROOT NODULES OF SOYBEANS GROWN IN BANG RAKAM DISTRICT SOILS, PHITSANULOK PROVINCE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รองศาสตราจารย์ ดร.กาญจนา ชาญสง่าเวช, 100 หน้า.

ไรโซเบียมถั่วเหลืองเป็นแบคทีเรียแกรมลบ สามารถเคลื่อนที่ได้ ไม่สร้างสปอร์ เมื่อไรโซเบียมถั่วเหลืองอยู่ในปมรากถั่วเหลือง จะเปลี่ยนไนโตรเจนในอากาศให้เป็นแอมโมเนีย สำหรับถั่วเหลืองใช้ในการเจริญ โดยทั่วไปวิธีการที่ใช้ในการตัดสินหรือบ่งชี้คุณสมบัติการเข้าสร้างปมของไรโซเบียมถั่วเหลืองจะใช้วิธีเติมเชื้อไรโซเบียมถั่วเหลืองแต่ละสายพันธุ์ลงบนเมล็ดถั่วเหลืองงอกรากแล้วในโหลเลียวนาร์ด เลี้ยงถั่วเหลืองในโหลเลียวนาร์ดเป็นเวลา 28 วัน ก่อนตรวจนับจำนวน crown nodules และ total nodules ซึ่งวิธีการที่กล่าวมานี้จะลำบาก อีกทั้งต้องใช้ความพยายามและเวลามาก งานวิจัยนี้จึงมีเป้าหมายเพื่อจะหาสภาวะมัลติเพล็กซ์พีซีอาร์ที่เหมาะสมเพื่อนำมาใช้ทำนายประสิทธิภาพการเข้าสร้างปมของไรโซเบียมถั่วเหลืองประเภทเพิ่มจำนวนช้า จำนวน 33 สายพันธุ์ ที่แยกจากอำเภอบางระกำ จังหวัดพิษณุโลก และงานวิจัยนี้ได้ทำการระบุชนิดของไรโซเบียมถั่วเหลืองประเภทเพิ่มจำนวนช้า จำนวน 5 สายพันธุ์ โดยใช้วิธีอนุกรมวิธานแบบพอลิฟาสิกอีกด้วย ผลที่ได้แสดงให้เห็นว่า สภาวะมัลติเพล็กซ์พีซีอาร์ที่เหมาะสม คือ ใช้ดีเอ็นเอเป้าหมายที่มีความเข้มข้น 200 นาโนกรัม ความเข้มข้นของไพร์เมอร์ *nodD*1F *nodD*1R *nodY*F และ *nodY*R เท่ากับ 8 พิคาโมล 12.5 พิคาโมล 12.5 พิคาโมล และ 8 พิคาโมล ตามลำดับ การแยกผลิตภัณฑ์มัลติเพล็กพีซีอาร์บนอกาโรสเจล พบว่า รูปแบบการเรียงของผลิตภัณฑ์มัลติเพล็กซ์พีซีอาร์แบ่งออกเป็น 2 รูปแบบ คือ รูปแบบที่ 1 ประกอบด้วย ชิ้นส่วนขนาด 317 คู่เบสและ 657 คู่เบส รูปแบบที่ 2 ประกอบด้วยชิ้นส่วนขนาด 340 คู่เบสและ 657 คู่เบส ผลการทดลองไม่พบความสัมพันธ์ระหว่างรูปแบบการจัดเรียงชิ้นส่วนพีซีอาร์และประสิทธิภาพการเข้าสร้างปม ในแง่ที่พิจารณาจากค่าเฉลี่ยของ crown nodules และ total nodules นอกจากนี้ การระบุชนิดของไรโซเบียมถั่วเหลืองประเภทเพิ่มจำนวนช้าจำนวน 5 สายพันธุ์ ที่เลือกมาแบบสุ่ม พบว่าสายพันธุ์ D361 D373 และ D388 จัดเป็น *Bradyrhizobium japonicum* ในขณะที่สายพันธุ์ D416 และ D467 จัดเป็น *B. liaoningense*

ภาควิชา จุลชีววิทยา ลายมือชื่อนิสิต ณิชานันท์ กาบเกษร

สาขาวิชา จุลชีววิทยาทางอุตสาหกรรม ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก

ปีการศึกษา 2552

5072646723 : MAJOR INDUSTRIAL MICROBIOLOGY

KEYWORDS : multiplex PCR, soybean rhizobium , soybean root nodulation

NICHANUN KARBKESORN : USE OF MULTIPLEX PCR TO PREDICT NODULATION OF RHIZOBIA ISOLATED FROM ROOT NODULES OF SOYBEANS GROWN IN BANG RAKAM DISTRICT SOILS, PHITSANULOK PROVINCE. THESIS ADVISOR : ASSOC. PROF. KANJANA CHANSA-NGAVEJ, Ph.D., 100 pp.

Soybean rhizobia are motile, non-spore-forming, Gram negative bacteria. When in soybean root nodules, these rhizobia change atmospheric nitrogen to ammonia for soybeans' use for growth. Traditionally, nodulation property of soybean rhizobia is determined by inoculating each soybean rhizobium strain in Leonard jars containing germinating soybean seeds. Soybeans are grown for 28 days before observing numbers of crown and total number of nodules. This method is labor-intensive and time-consuming. The aims of this research are to find optimal conditions for multiplex PCR reaction for use in the prediction of nodulation efficiency of 33 slow-growing soybean rhizobium strains isolated from Bang Rakam district, Phitsanulok province. In addition, identification of 5 slow-growing soybean rhizobia by polyphasic taxonomy will be carried out in this research. The results obtained indicated that the optimized conditions for the multiplex PCR reaction were 200 ng target DNA and concentrations of primer *nodD1*F, *nodD1*R, *nodY*F, and *nodY*R were 8.0, 12.5, 12.5 and 8.0 pmoles respectively. Separation of the multiplex PCR products by agarose gel electrophoresis showed two multiplex PCR patterns. Pattern 1 consisted of DNA fragments of 317 bp and 657 bp while pattern 2 consisted of DNA fragments of 340 bp and 657 bp. No correlation was found between multiplex PCR patterns and nodulation efficiency in terms of the average numbers of crown and total nodules. Identification of 5 randomly selected slow-growing soybean rhizobia showed strains D361, D373, and D388 were *Bradyrhizobium japonicum* while strains D416 and D467 were *B. liaoningense*.

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Field of Study : Industrial Microbiology Advisor's Signature K. Chansa-ngavej

Academic Year : 2009

Acknowledgements

I wish to express sincere thanks and gratitude to my thesis advisor, Associate Professor Dr Kanjana Chansa-ngavej, for her tireless efforts as well as valuable advice and comments throughout the course of research for this thesis.

I would also like to thank Assistant Professor Dr. Suthep Thaniyavarn for serving as the thesis committee chairperson, Associate Professor Dr. Pairoh Pinphanichakarn, Associate Professor Dr Lerluck Chitradon and Assistant Professor Dr. Wipa Homhaul for serving as thesis committee members and their recommendations for the improvement on the writing of the thesis.

Special thanks are expressed to friends and student members in Laboratory 404, and all staff members of the Department of Microbiology, especially, Mr. Weerasak Chongfuengprinya, for their help and friendship during my study.

The last, but most important, is my sincere and deepest gratitude to my parents and every member in my family for their great love, constant support, understanding and heartfelt encouragement extended throughout my study.

The kind permission to use the Biolog machine at the Center for Agricultural Biotechnology, Kasetsart University, KamphaengSaen Campus, Nakorn Pathom Province, is greatly appreciated.

This research was partially funded by a research grant from the "The 90th Anniversary of Chulalongkorn University Fund" (Ratchadaphiseksomphot Endowment Fund). The author wishes to gratefully acknowledge the grant.

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CHAPTER I

INTRODUCTION

Soybean rhizobia are Gram negative, motile, non-spore forming bacteria which change atmospheric nitrogen to ammonia in soybean root nodules. The enzyme which catalyzes this nitrogen fixation is nitrogenase. There are two types of soybean rhizobia: Fast-growers and slow-growers. The fast-growers consist of *Sinorhizobium fredii* and *S. xinjiangense* while there are 4 species of slow-growers, *Bradyrhizobium elkanii*, *B. japonicum, B. liaoningense*, and *B. yuanmingense* (Appunu et al.,2008, Chen et al., 1988, Jordan, 1982, Kuykendall et al., 1992, Peng et al., 2002, Xu et al., 1995). In 1992, Elkan and Bunn listed differences in several characteristics of fast- and slow-growing soybean rhizobia including the doubling time (less than 6 hours in the former and more than 6 hours in the latter), the number and type of flagella (2-6 peritrichous flagella in the fast-growers and one subpolar flagellum in the slow-growers). In addition, in fast-growing soybean rhizobia, *nifH* which encodes the Fe protein of nitrogenase (Fuhrmann and Hennecke, 1984) and *nifDK* which encode the alpha and beta subunits of the MoFe protein of the enzyme are on the same operon while *nifH* and *nifDK* are on separate operons in slow-growing soybean rhizobia. In 1995, van Rhijn and Vanderleyden reported that slow-growing *B. japonicum* contained *nodY* upstream and on the same operon as *nodABC* while there was no *nodY* in fast-growing rhizobia. Therefore , based on the reported finding that slow-growing soybean rhizobium *B. japonicum* contained *nodY* while fast-growing rhizobia did not, Emampaiwong (2006) designed primers for multiplex PCR to distinguish fast- from slow-growing soybean rhizobia isolated from 3,8, and 4 subdistricts of Chart Trakarn, Bang Rakam, and Phrom Piram districts in Phitsanulok province, respectively. The primers were *nodD1*F, *nodD1*R, *nodY*F, and *nodY*R. It was expected that fast-growing soybean rhizobia yield one multiplex PCR product of 317 bp due to the annealing of only *nodD1*F and *nodD1*R to target DNAs. Slow-growing soybean rhizobia were expected to yield two multiplex PCR products of 317 bp and 340 bp due to the annealing of all the 4 primers. However, using 12.5 pmoles for each of the 4 primer, Emampaiwong (2006) found that out of the

58 slow-growing soybean rhizobia, 2 strains (3.4%) yielded one 317 bp multiplex PCR product, 9 strains (15.5%) yielded one 340 bp multiplex PCR product, 2 strains (3.4%) yielded 317 bp and 340 bp multiplex PCR products, 1 strain (1.7%) yielded 317 bp, 340 bp, and 657 bp multiplex PCR products, 6 strains (10.3%) yielded 317 bp and 657 bp multiplex PCR products, and 38 strains (65.5%) yielded 340 bp and 657 bp multiplex PCR products. Emampaiwong (2006) explained the results as follows: The annealing of only *nodD1*F and *nodD1*R yielded the 317 bp multiplex PCR product. The annealing of only *nodY*F and *nodY*R yielded the 340 bp multiplex PCR product. The annealing of all the 4 primers yielded the 317 bp and 340 bp multiplex PCR products. In addition, the 317 bp, 340 bp, and 657 bp multiplex PCR products were due to the additional extension of a PCR product from the annealing of *nodD1*F and *nodY*R. Similarly, the combinations of the 317 bp, 657 bp and 340 bp, 657 bp multiplex PCR products were also due to the additional extension of a PCR product from the annealing of *nodD1*F and *nodY*R. the additional extension of a PCR product from the annealing of *nodD1*F and *nodY*R. The aims of this research are to vary the 4 primers and target DNA concentrations to find out if it is reproducible for some selected strains to get the annealing of either *nodD1*R or *nodY*F only and not both. If the reproducibility is obtained, it might be possible to utilize the multiplex PCR product patterns to predict nodulation efficiency. Moreover, identification of 5 slow-growing soybean rhizobia by polyphasic taxonomy will be carried out in this research. Since the multiplex PCR employed in this research involves the amplification of genes in the nodulation gene cluster, it is expected that the outcome of the research will pave the way for the utilization of the relatively rapid multiplex PCR method to predict nodulation efficiency or the ability of slow-growing soybean rhizobia to rapidly and effectively nodulate soybeans. The identification of the 5 slow-growing soybean rhizobia will likewise contribute to the pool of knowledge of the diversity of slow-growing soybean rhizobia in Thailand for use in the selection of effective soybean rhizobium strains for the commercial production of soybean rhizobium biofertilizers to increase soybean yields, to increase income of soybean growers, to reduce trade deficit due to the presently imported 85% of the amounts of soybean consumed in Thailand (http://www.feedusers.com/thai/cms/html/Inedible/110.html;http://www.oae.go.th/oae_websi

te/oae_imex.php) and to reduce the extent of water pollution in the form of eutrophication caused by agricultural run-offs of residual chemical fertilizers inadvertently “fertilize” cyanobacteria and microalgae in waters so the water coloration is green due to the color of the chlorophylls and water resources are not used to the maximum benefits.

CHAPTER II

LITERATURE SURVEY

2.1 Slow-growing soybean rhizobium nodulation genetics

In slow-growing soybean rhizobium, *Bradyrhizobium japonicum*, the genes involved in nodulation of soybean roots include *nodD1, nodD2, nodYABCSUIJ*, and *nodVW* (GÖttfert et al., 1990; Stacey, 1995). Figure 2.1 shows positions of the genes for nodulation in *B. japonicum*.

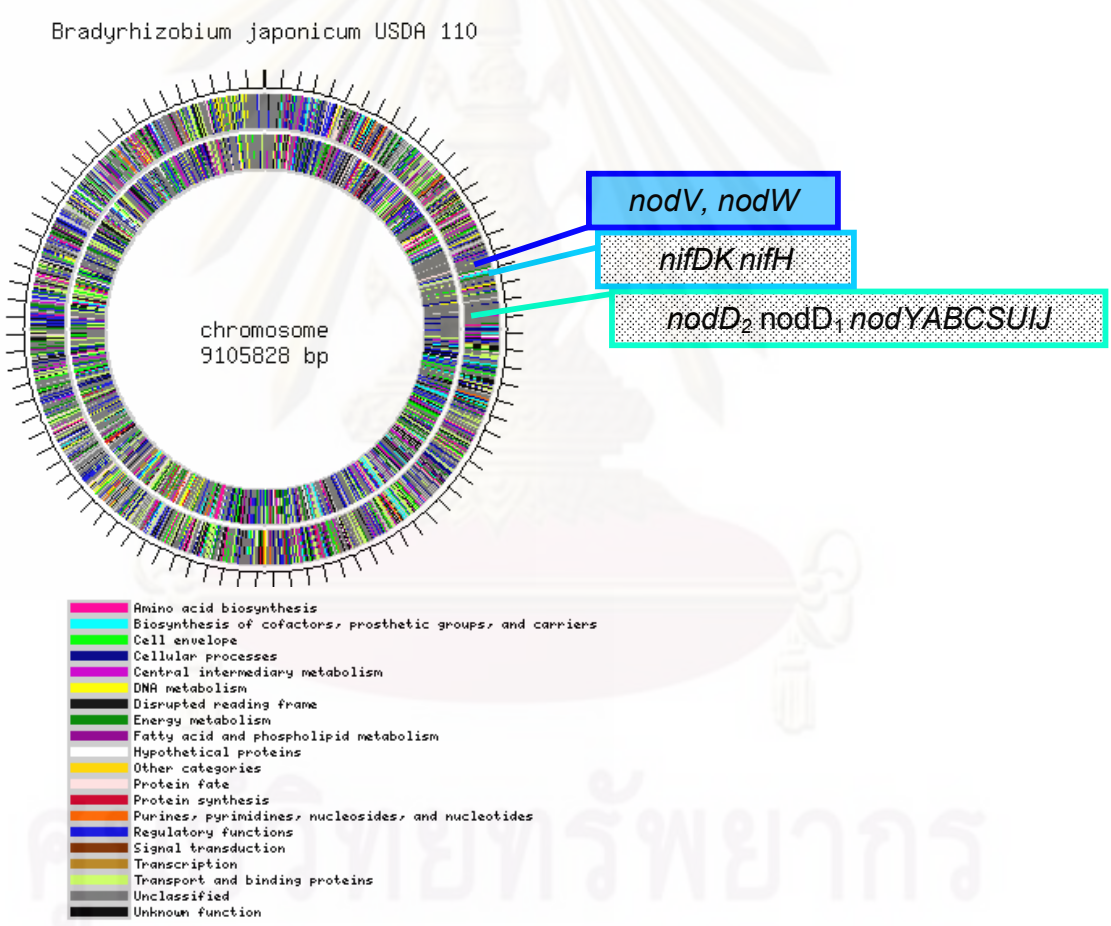


Figure 2.1 Whole genome of *B. japonicum* USDA110. Each interval represents 100,000 bp. All the genes are color-coded according to their functions. Positions of nodulation and nitrogen fixation genes are indicated in the Figure. (http://gib.genes.nig.ac.jp/single/index. php? spid =Bjap_USDA110).

Kosslak et al. (1987) reported that soybean root hair secreted isoflavones such as genistein which diffused into the periplasm of *B. japonicum* to bind to the C terminal of NodD1 to form a complex which bound to the overlapping promoter region of *nodD1* and *nodYABCSUIJ*. Figure 2.2 shows the transcription start site of *nodD1* is in the promoter region of *nodY*, called *nodY*-box, while that of *nodYABC* is in the promoter region of *nodD1*, called *nodD1* box. Each *nod*- box consists of four 9 bp repeats of the following consensus sequence: $A_{74}T_{90}C_{88}G_{85}A_{93}T_{89}T_{71}Y_{74}T_{74}$ (Stacey, 1995; Wang and Stacey, 1991).

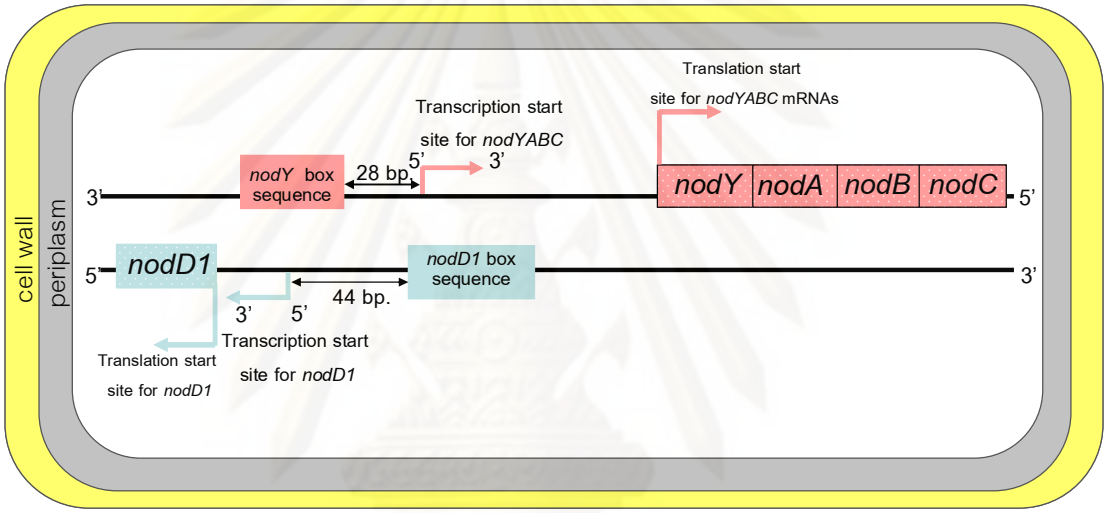
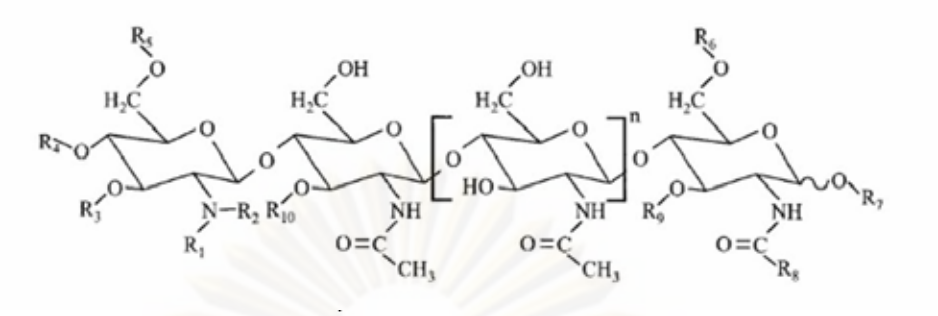


Figure 2.2 Diagrammatic representation of the overlapping promoter region of *nodD1* and *nodY* (drawn from information as reported by Stacey, 1995).

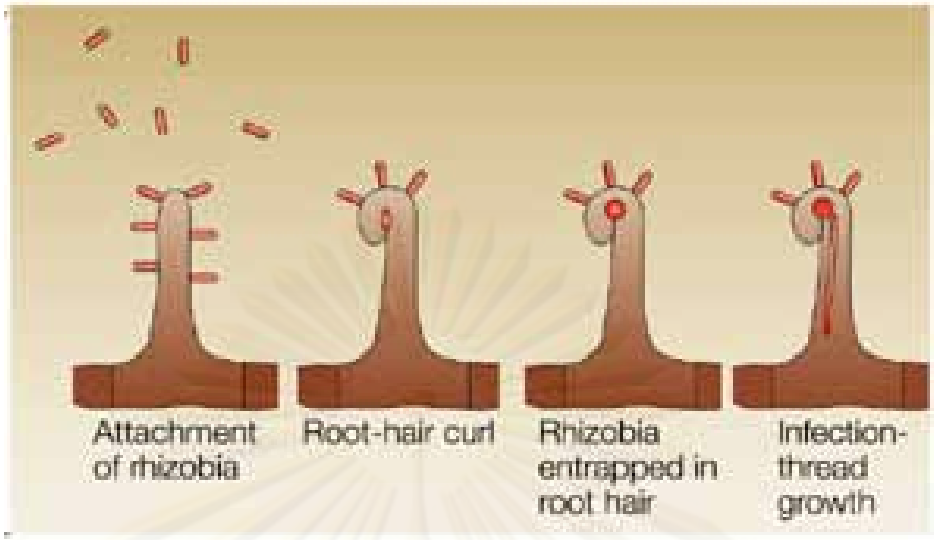
The function of NodY is not yet known (Banfalvi et al., 1988). Proteins NodA, NodB, and NodC are enzymes in the pathway of biosynthesis of Nod factors as follows: NodC is N-acetylglucosaminyl transferase which catalyses the binding of N-acetylglucosaminyl units by Beta 1,4 linkages. NodB is N-deacetylase which catalyzes the removal of N-acetyl group from the N-acetylglucosaminyl unit at the non-reducing end. NodA is N-acyl transferase which catalyzes the addition of an acyl (C18:1) group to the N-acetylglucosaminyl unit at the non-reducing end. Chemical structures of Nod factors of slow-growing soybean rhizobia differ in the number of the N-acetylglucosaminyl units as well as the types of side chains (R_1 to R_{10}) as shown in Figure 2.3.



Rhizobial strain	Nod factor substitutions[ab]										
	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	R_{10}	n[c]
B. elkanii USDA61	C18:1,C16:0	Me,H	Cb,Ac[f] H	Cb,Ac,H	Cb,Ac,H	2-*O*-MeFuc,Fuc	Gro,H	Me	H	H	1, 2
B. japonicum USDA110	C18:1[g]	H	H	H	H	2-*O*-MeFuc	H	Me	H	H	2
B. japonicum USDA135	C18:1,C16:0,C16:1[h]	H	H	H	Ac,H	2-*O*-MeFuc	H	Me	H	H	2

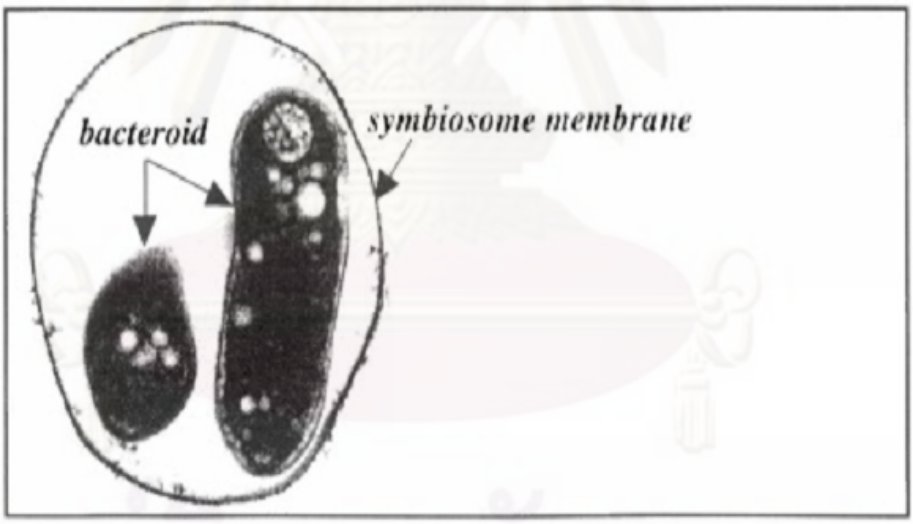
Figure 2.3 Chemical structures of Nod factors of slow-growing soybean rhizobia (D'Haeze and Holsters, 2002).

Functions of Nod factors are unknown but when nanomolar amounts of purified Nod factors were dropped onto soybean root hair, the latter curled (Cohn et al., 1998). Therefore, Nod factors are essential in root hair curling which is the initial step in root nodule formation. While root hairs curl, cell membrane of root hair invaginates to form an infection sac which elongates to become an infection thread. Soybean rhizobia which are attracted to the vicinity of soybean root hair by gradient of the isoflavone, genistein, get into the infection thread which delivers the rhizobia to cortex cells of soybeans. There, the bacteria were cut off the infection thread in the form of root hair-membrane bound structures called symbiosomes containing rhizobia which have by now lost their flagella and become irregularly-shaped cells called bacteroids which fix nitrogen. Cortex cells divide rapidly to form structures known as root nodules. Figures 2.4 and 2.5 show root hair curling and cross-section of a symbiosome as observed under a transmission electron microscope.



http://www.nature.com/nrm/journal/v5/n7/fig_tab/nrm1424_F1.html

Figure 2.4 Diagrammatic depiction of soybean rhizobia (represented by rods) attracted to root hair which curls before an infection thread formation.



http://www.springerlink.com/content/g17v5332404m1x25/

Figure 2.5 Transmission electron micrograph of a symbiosome containing bacteroids.

The functions of *nodS, nodU, nodI* and *nodJ* in soybeans are not known. However, Gottfert et al. (1990) reported that *nodS* conferred host specificity for *Leucaena leucocephala*.

NodD1 acts as a transcriptional activator as previously described. Machado et al.(1998) reported that transformation of plasmid-borne copies of *nodD2* into the fast-growing soybean rhizobium *Sinorhizobium fredii* reduced the level of transcription of *nodD1* to below the detection limit by RNA dot blot hybridization. The functions of NodD1 and NodD2 in *Bradyrhizobium japonicum* were as follows: When *B. japonicum* was grown in minimum medium to more than 10^8 cells.ml^{-1}, cells were found to secrete Bradyoxetin whose structure was determined to be 2-{4-[[4-(3-aminooxetan-2-yl)phenyl](imino)methyl]phenyl}oxetan-3-ylamine (Loh et al., 2002a). Bradyoxetin was found to induce transcription of *nodD2* whose protein product was, in turn, found to inhibit the expression of *nodD1*, probably by the reduction of the content of *nodD1* transcript as found in the fast-growing *Sinorhizobium fredii*. Reduction in the expression of *nodD1* was found to lead to the inhibition of nodulation process via the reduction of expression of nodulation genes *nodABC* which led to a reduction in the amount of the Nod factor. The mechanism of inhibition of nodulation process due to the increased cell density has been termed quorum sensing mechanism (Loh et al., 2002b).

In 2003, Loh and Stacey proposed another regulatory mechanism in the control of nodulation genes in *B. japonicum* which involved the expression of *nodV* and *nodW* as follows: Protein products of *nodVW* formed a two-component regulatory system with NodV as a kinase which autophosphorylated and transferred the phosphate group to NodW. Phosphorylated NodW activated transcription of *nodD1* and *nodYABC* possibly by influencing DNA bending as in the case of the activation mechanism of NodD1-flavonoid complex.

2.2 Methods for the determination of nodulation efficiency

A traditional method for the determination of nodulation efficiency is the time-consuming and labor- intensive growth of soybeans inoculated with each of the test strains of soybean rhizobia in Leonard's jars (Somasegaran and Hoben, 1994). Soybeans are usually grown with nitrogen-free medium in a temperature-controlled greenhouse for 28 days before the determination of the number of crown and total number of nodules. Crown nodules have been designated as those nodules on the tap roots within the length of one centimeter from the crown which is the junction between

the stem and tap root (Spriggs and Dakora, 2007). Distal nodules are those on lateral roots. Soybean rhizobia found in crown nodules are the ones which nodulate tap roots which form prior to the formation of lateral roots. These rhizobia are regarded as the more efficient nodulators compared with the rhizobia in distal nodules which nodulate at a later stage after the formation of lateral roots from the root pericycle layer (Esau, 1977). Research on a novel method for the prediction of nodulation efficiency of soybean rhizobia is in progress (Chansa-ngavej et al., unpublished data). The method is based on a reported suggestion by Vlassak and Vanderleyden (1997) that soybean rhizobia with longer flagella could be better nodulators because they moved faster to the root hair along the concentration gradient of genistein. In addition, different species of slow-growing soybean rhizobia had been reported to exhibit different nodulation and symbiotic properties. Hungria et al. (1998) reported that natural variants of *B. japonicum* strains adapted to the nutrient-poor Cerrados region of Brazil after being used as inoculants from 1966-1978 were better nodulators and nitrogen fixers than the corresponding natural variants of *B. elkanii* . Total number of nodules per plant and nodule dry weight per plant were used as indicators for nodulation efficiency while total N accumulated per plant was used as an indicator for nitrogen-fixing ability (Hungria et al., 1998). In the new method, Chansa-ngavej et. al. (unpublished data) proposed that soybean rhizobia used in the production of biofertilizers should be able to compete with indigenous soybean rhizobia in nodulating soybeans possibly by having longer flagella for faster move to the root hair along the genistein concentration gradient. The hypothesis is as follows: Slow-growing soybean rhizobia with high ratios between length of flagella (f) and length of cell periphery (c) could be good soybean root nodulators. At present, there is a set of preliminary data on the f/c ratios of several strains of slow-growing soybean rhizobia as shown in Table 2.1. Relationship is being found out between high f/c ratios and the ability to increase soybean yield in field experiments. Preliminary results on the f/c ratios obtained so far showed that *B. liaoningense* strain D467 obtained in this thesis has the highest ratio of f/c.

Table 2.1 Ratios of length of flagella and length of cell periphery (f/c) of some strains of slow-growing soybean rhizobia (Chansa-ngavej et al., unpublished data).

Soybean rhizobium strains	Identification by sequences of 16S rDNA	f/c ratios
NM22-8	*Bradyrhizobium japonicum* strain NM22-8	0.71
NM22-30	*Bradyrhizobium japonicum* strain NM22-30	0.60
D373	*Bradyrhizobium japonicum* strain D373	1.00
NM22-11	*Bradyrhizobium elkanii* strain NM22-11	1.00
NM22-15	*Bradyrhizobium elkanii* strain NM22-15	1.00
NA7	*Bradyrhizobium elkanii* strain NA7	1.12
D467	*Bradyrhizobium liaoningense* strain D467	1.28

2.3 Multiplex PCR for the determination of nodulation efficiency

In this thesis, multiplex PCR-amplified fragments of DNA of two slow-growing soybean rhizobium genes in the nodulation gene cluster, namely *nodD1* and *nodY*, are separated by the standard agarose gel electrophoresis to yield multiplex PCR patterns. Correlation will be determined between the multiplex PCR patterns and nodulation efficiency in terms of numbers of crown and total nodules as well as plant dry weight. The patterns will then be used in the prediction of nodulation efficiency for additional slow-growing soybean rhizobial strains. Multiplex PCR with the use of three or more primers have been used in many fields of research in Microbiology. For example, Dyer et al. (2001) designed three primers for use in the determination of the mating type of heterothallic ascomycete cereal pathogenic fungi, *Tapesia yallundae* and *Tapesia acuformis.* The structural genes encoding mating types in both species are *MAT-1* and *MAT-2* were reported to be 3.9 kb and 3.3 kb respectively. Although sequences of both genes were different which were termed “idiomorphs” , those on the flanking regions of both genes had identical sequences of one kilobase. Therefore, Dyer et al. (2001) designed two sets of primers. Each set consisted of three primers with one “common”

primer annealling to the flanking region and each of the remaining two primers was specific to either the *MAT-1* or *MAT-2* structural gene. As indicated in Figure 2.6 , the first set of primers consisted of the "common" primer MT5315 which annealled to the flanking region, with primer MT317 which annealled to the alpha-domain of the *MAT-1* structural gene, and primer MT3311 which annealled to *MAT-2* structural gene. The multiplex PCR products were 812 bp and 418 bp. The second set of primers consisted of the "common" primer MT378, and the two primers, MT511 and MT155 which annealled to the HMG2-box of the structural gene *MAT-2*. Dyer et al. (2001) reported that the second set of multiplex PCR primers did not give reproducible results. Therefore , the first set of primers was used which yielded the expected PCR products as shown in Figure 2.7. The multiplex PCR was successfully used to predict the mating type of 118 isolates of the pathogenic fungi collected from Europe (UK, Belgium, France, and Germany), USA, and New Zealand.

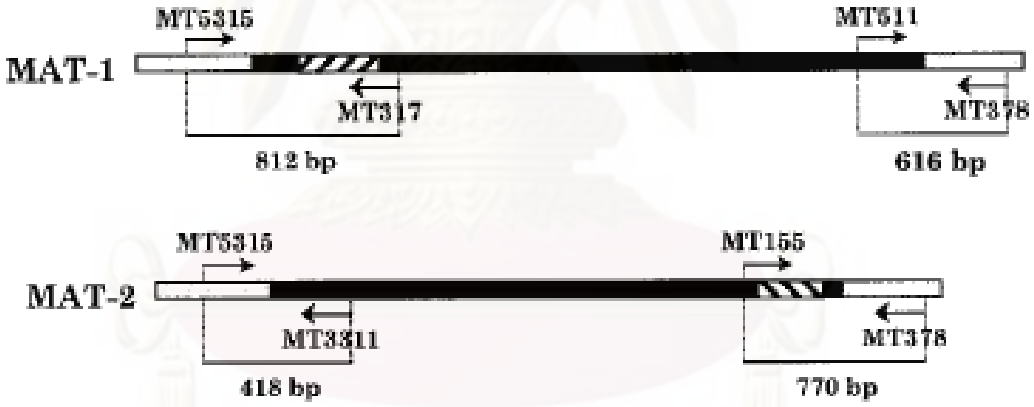


Figure 2.6 Multiplex PCR test for determination of mating type in *Tapesia yallundae* and *T. acuformis.* Drawn to approximate scale to show location of primers (MT prefix) and size of resulting PCR products. Solid boxes represent idiomorphs; lightly shaded boxes represent flanking sequences; forward hatched box represents the alpha-domain gene; reverse hatched box represents the HMG2-box gene. (Dyer et al., 2001)

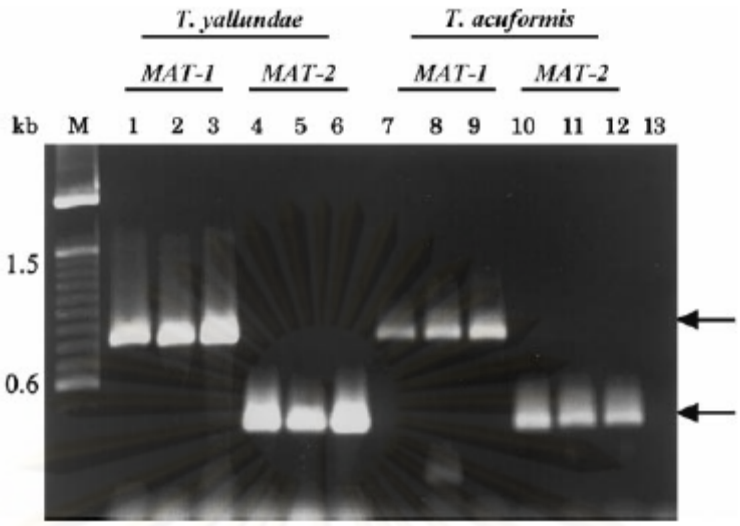


Figure 2.7 Ethidium bromide-stained agarose gel showing representative results of the multiplex PCR test with *Tapesia yallundae* and *T. acuformis.* Species and mating type of isolates are indicated above the appropriate lanes. M, 100-bp molecular weight marker; lanes 1–12, amplicons produced by isolates 22-435, 37-39-3, PB3-2, 22-481, 22-483, 22-495, 22-419, 22-424, 99-6-1, 22-442, 99-9-7, and 99-20-4, respectively; lane 13, water control. Arrows indicate PCR products characteristic of *MAT-1* and *MAT-2* isolates. (Dyer et al., 2001)

Multiplex PCR had also been used in the rapid and specific identification of four *Agrobacterium* species and biovars (Putawska et. al., 2006), and in the quality control of *Epinotia aporema* granulovirus production (Manzan et al., 2008).

2.4 Previous research on soybean rhizobium diversity in soybean exporting countries and in Thailand

Chantapetch (2009) reported in countries which are leading soybean exporters there have been large amounts of research on soybean rhizobium strain selection for inoculant production. (Aguilar et al., 2001; Brutti et al., 1998; Chen et al., 2002; de Jensen et al., 2004; Hungria et al., 2001; Thomas-Oates et al., 2003). In addition, there are many patents on soybean rhizobia and soybean inoculant production in these countries. In Thailand there has been relatively few research on soybean rhizobium (Nuntagij et al., 1997; Shutsrirung et al., 2002; Teaumroong and Boonkerd, 1998; Thompson et al., 1991; Yokoyama et al.,1996). Most of the research conducted in Thailand concerns with the isolation and identification of soybean rhizobia from various

soybean-growing areas (Nuntagij et al., 1997 ; Thompson et al., 1991 ; Yokoyama et al., 1996 ; Ly and Chansa-ngavej, 2006)

Teaumroong & Boonkerd (1998) used primer RAPD (Random Amplified Polymorphic DNA, 5'GGAAGTCGCC3') to obtain fingerprints of 18 *B. japonicum* isolates from root nodules of soybean which the authors did not specify the cultivar. The authors also obtained fingerprints of 4 strains of soybean rhizobia: TAL377, THA7, THA5, and TAL216 and 4 USDA strains (USDA, United States Department of Agriculture) USDA 8-0, USDA 94, USDA 35 and USDA 117.

2.5 Identification of slow-growing soybean rhizobia by polyphasic taxonomy

Polyphasic taxonomy has widely been employed to identify bacteria (Vandamme et al., 1996). In the identification of slow-growing soybean rhizobia, the taxonomy includes use of several morphological characteristics such as colony morphology, type and number of flagella, use of several biochemical and physiological properties such as the ability to use or not use a number of carbon and nitrogen sources, growth rates at different temperatures. Molecular characterization of the rhizobia includes use of PCR-DNA fingerprints and 16S rDNA sequences. At present, there is not much information on polyphasic taxonomy of soybean rhizobia in Thailand. In 2009 Chantapetch employed polyphasic taxonomy to identify 5 strains of slow-growing soybean rhizobia consisting of strain NM22-8 and NM22-30 as *Bradyrhizobium japonicum* USDA 110 with identities of 16S rDNA = 1450/1456 (99%), gaps = 5/1456, strain NM 22-11, NM11-13, and NM11-15 as *Bradyrhizobium elkanii* strain GZ1 with identities of 16S rDNA = 1451/1455 (99%), gaps = 4/1455. The results showed that the relatively conserved sequences of 16S rDNAs yielded only two species of slow-growing soybean rhizobia whereas the PCR-DNA fingerprints using either RPO1 or CRL-7 as the primer showed the 5 strains had different DNA fingerprints. The results agreed with those obtained by Binde et al. (2009) which showed that DNA fingerprints of 54 strains of rhizobia used in the commercial production of inoculants for leguminous plants in Brazil showed more genetic diversity than when sequences of 16S rDNA were used to determine the genetic diversity.

CHAPTER III

MATERIALS AND METHODS

3.1 Bacterial strains

3.1.1 Soybean rhizobia strains used in variations of concentrations of target DNA and primers in multiple PCR reactions

Soybean rhizobium strains used in experiments where concentrations of target DNA and primers were varied, consisted of 23 strains isolated from nodules of soybeans grown in soils from Bang Rakam district, Phitsanulok province, by Emampaiwong (2006). These strains were some of those used in multiplex PCR reactions by Emampaiwong (2006) to distinguish fast- from slow-growing soybean rhizobia. The strains were D286, D291, D293, D306, D314, D316, D337, D345, D361, D366, D373, D388, D404, D412, D416, D459, D464, D467, D481, D490, D494, D509, and D521.

3.1.2 Bacterial isolates used in the prediction of nodulation efficiency by multiplex PCR

Bacterial isolates used in the prediction of nodulation efficiency by multiplex PCR consisted of 57 isolates obtained by Emampaiwong (2006) from Bang Rakam district, Phitsanulok, which were not used by Emampaiwong (2006) in multiplex reactions to distinguish fast- from slow-growing soybean rhizobia. The strains were isolated from the following subdistricts of Bang Rakam district, Phitsanulok province:

Pan Sao subdistrict : D281, D282, D288, D292, D296, D303, D307, D308 D309, D311, D312

Nong Ku-la subdistrict : D355, D367, D368, D378

Bung Kok subdistrict : D320, D322, D323, D324, D326, D329, D330, D331, D333, D334, D340, D342, D343, D344, D346, D347, D349

Kui Muang subdistrict : D460, D465, D466, D469, D470, D471, D472, D473, D479, D480, D482, D483, D484, D487, D488

Bang Rakam subdistrict: D492, D495, D496, D499, D500, D510, D514, D515

Chum Saeng Songkram subdistrict : D395, D411

3.2 DNA fingerprinting of 57 bacterial isolates

3.2.1 Isolation of chromosomal DNA

One loop of each bacterial isolate grown in YMA slant stored at 4°C was activated by streaking onto petri dish containing YM agar with congo red at the final concentration of 25 μg.ml^{-1}. Composition of YM as described by Somasegaran and Hoben (1994) was given in Appendix A. One loop of activated cells was inoculated into 50 ml YM broth in 250 ml Ehrenmeyer flask and grown at 30°C, 200 rpm, until mid-log phase. Cells collected by centrifugation at 8,000 rpm for 5 min were broken by incubating with 100 μl of 2.5 mg.ml^{-1} lysozyme at 37°C for one hour, followed by freezing and thawing at -20°C for 5 min and 80°C for 5 min for 2 cycles. Cell debris was removed by centrifugation at 10,000 rpm. 4°C for 10 min. 250 μl DNAZol was added to the supernatant to hydrolyze RNAs. DNA was precipitated by adding 500 μl ice-cold ethanol, left at -80°C for 15 min. DNA precipitate was collected by centrifugation at 12,000 rpm for 10 min, washed with 70% ethanol. 20 μl high-quality distilled water was added to the precipitate and left for DNA to dissolve overnight in a laminar flow hood. Quality of DNA was checked by ratio of OD260/OD280 and agarose gel electrophoresis by standard method (Sambrook et al., 1989). DNA quantity was determined by OD260 with the estimation that OD260 of 1.00 equal DNA solution of 50 μg. ml^{-1} (Sambrook et al., 1989). DNA solution obtained was used in DNA fingerprinting as described in Section 3.2.2

3.2.2 DNA fingerprinting

DNA fingerprinting was obtained by PCR using either RPO1 or CRL-7 as the primer. RPO1 consisted of 20 conserved nucleotides at the promoter of *nifHDK* of 3 strains of *Rhizobium leguminosarum* bv. *trifoli* (Schofield and Watson, 1985). Sequence of RPO1 was as follows: 5'AATTTTCAAGCGTCGTGCCA3'. CRL-7 is an arbitrary GC rich primer: 5'GCCCGCCGCC3' (Mathis and McMillin, 1996). Composition of PCR reaction was 10X PCR buffer (with 1mM $MgCl_2$) 2.0 μl, 2.5mM dNTPs 2.0 μl, primer

RPO1 or CRL-7 (100 pmole. μl^{-1}) 0.2 µl, target DNA 200 ng, *Taq* polymerase (5 U. μl^{-1}) 0.2 µl, distilled water to 20 . µl. PCR program was as follows: 95°C 15 seconds, 50°C 30 seconds, 72°C 90 seconds (5 cycles), 95°C 15 seconds, 55°C 30 seconds, 72°C 90 seconds (25 cycles), followed by 72°C for 10 min. PCR products were separated by 1.25% agarose gel electrophoresis by standard method (Sambrook et al., 1989). Gels were stained with ethidium bromide solution (0.5 µg ml^{-1}) for 15 min, destained in distilled water for 1 hour and placed on UV transilluminator (Bio-Rad) with Polaroid camera using Fuji FP3000B Polaroid film. Strains with identical DNA fingerprints were assigned to be the same strains.

3.3 Multiplex PCR reactions

Primers used in multiplex PCR reactions were *nodD1*F, *nodD1*R, *nodY*F, and *nodYR* with sequences as described by Emampaiwong (2006) as follows: *nodD1*F (5'AAAATGGCAGCAGYTCGAA3'), *nodD1*R (5'CAACATCAATCTGAGCCAG3'), *nodY*F (5'TGTACGCGGGTAAACC3'), *nodY*R (5'AGCGCAACGAGAAGAT3'). Concentrations of target DNA used were 100 ng and 200 ng. Concentrations of primers were varied according to Table 3.1

Table 3.1 Concentrations of primers used in multiplex PCR reactions.

Conditions (pmole)	*nodD1*F	*nodD1*R	*nodY*F	*nodY*R
1	8.0	8.0	8.0	8.0
2	8.0	12.5	8.0	8.0
3	8.0	8.0	12.5	8.0
4	8.0	12.5	12.5	8.0
5	8.0	25.0	25.0	8.0
6	12.5	12.5	12.5	12.5
7	12.5	25.0	12.5	12.5
8	12.5	12.5	25.0	12.5
9	12.5	25.0	25.0	12.5

Composition of other components in multiplex PCR reactions and program of multiplex PCR were the same as described in Section 3.2.2

3.4 Determination of nodulation efficiency

3.4.1 Germination of soybean seeds cultivar Chiangmai 60

Soybean seeds cultivar Chiangmai 60 used in the determination of root nodulation efficiency were surface-sterilized by swirling in 5% H_2O_2 for 5 min, rinsed 6 times with sterilized deionized water, and aseptically transferred onto petri dished containing 0.7% agar. Seeds were allowed to germinate by incubating agar plates at 25^0C in the dark for 2 days.

3.4.2 Determination of CFU.ml^{-1} of soybean rhizobium strains

One loop of each soybean rhizobium strain stored in YM agar slants was activated by streaking onto YM agar plate containing final concentration of 25 µg.ml^{-1} congo red. One loop of activated cells was inoculated into 50 ml of YM broth in 250 ml Ehrenmeyer flask, incubated at 30°C , 200 rpm. Samples were taken at 12 h intervals for serial dilutions by standard method. Graphs of CFU.ml^{-1} versus incubation were constructed to determine time taken to grow cells to 10^8 CFU.ml^{-1} for use in the inoculation onto germinating Chiangmai 60 soybean seeds in Leonard jars.

3.4.3 Determination of root nodule efficiency

Efficiency of root nodule formation was determined by inoculating 1 ml of equal number of 10^8 CFU.ml^{-1} of each strain in YM broth onto each germination seed of soybean cultivar Chiangmai 60 in Leonard jar. Preparation of Leonard jars was as described by Somasegaran and Hoben (1994) as follows: The lower part of the Leonard jar assembly was a bottle containing nitrogen-free medium. Composition of nitrogen-free medium was given in Appendix A. The upper part of the jar was the inverted top part of a beer bottle with one-third of the bottom cut off. The cut beer bottle contained a cotton wool plug, washed fine sand and a robe as a wick for the supply of nitrogen-free medium to the top layer of the fine sand as shown in Figure 3.1. Each Leonard jar assembly was autoclaved at 121°C for 15 min, left cool before use.

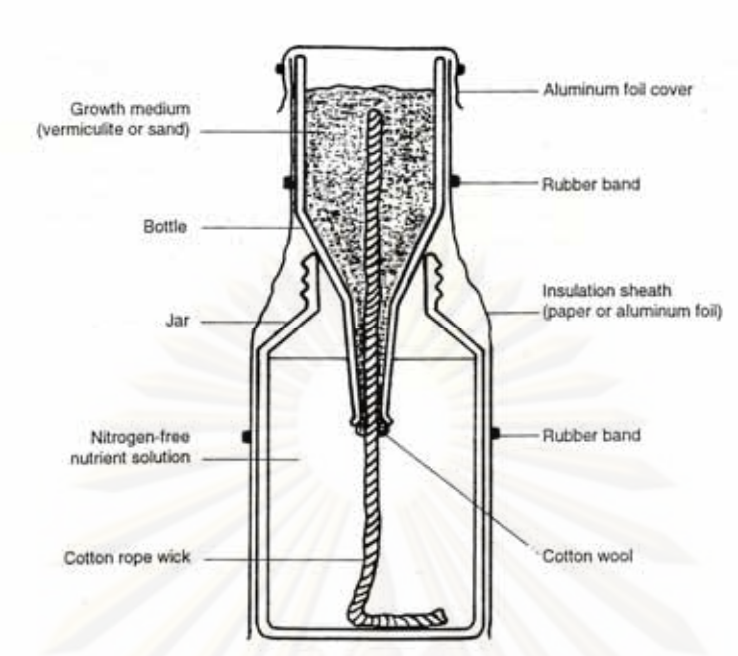


Figure 3.1 Diagram of Leonard jar assembly (Somasegaran and Hoben,1994).

Two germinating soybean seeds were placed in each Leonard jar followed by inoculation each germinating seed with 1 ml of YM broth containing equal number of 10^8 CFU.ml^{-1} of each soybean rhizobium strain. Leonard jars were placed in a 28°C-32°C temperature-controlled greenhouse for 28 days. Root nodules were counted by assigning nodules on the tap root within the length of 1 cm down from the crown (point where stem ends and root begins) as described by Spriggs and Dakora (2007). Total nodules were also counted. Experiments were repeated one time. Histograms showing average numbers of crown and total nodules obtained for each soybean rhizobium strain were constructed for the determination of nodulation efficiency.

3.4.4 Correlation between multiplex PCR patterns and nodulation efficiency

Multiplex PCR patterns were determined based on results of separation of multiplex PCR products by agarose gel electrophoresis. Correlation was then made between multiplex PCR patterns and average numbers of crown and total nodules.

3.5 Use of multiplex PCR patterns to predict nodulation efficiency

Time taken to reach 10^8 CFU.ml^{-1} for each of the strains obtained from the assignment of 57 root nodule bacterial isolates to the same strains was determined by

construction growth curves (CFU.ml^{-1} versus incubation time) as described in Section 3.4.2. Cells of each strain were grown to 10^8 CFU.ml^{-1} and used to inoculate germinating soybean seeds cultivar Chiangmai 60 and average numbers of crown and total nodules obtained as described in Section 3.4.3. Correlation between multiplex PCR patterns and nodulation efficiency were then obtained to find out the possibility of using multiplex PCR patterns to predict nodulation efficiency.

3.6 Polyphasic taxonomy of 5 selected strains of soybean rhizobia

Five soybean rhizobium strains used in the multiplex PCR reactions were randomly selected for polyphasic taxonomy to identify the strains up to the species level according to the following methods. The 5 strains were D361, D373, D388, D416, and D467.

3.6.1 Colony morphology

One loop of each strain stored in YM agar slant was streaked onto petri dish containing YM agar with 25 µg.ml^{-1} congo red. Plates were incubated at 25°C for 5 days before observation and recording of colony morphology.

3.6.2 Number and type of flagella

One loop of each strain stored in YM agar slant was streaked onto petri dish containing YM agar with 25 µg.ml^{-1} congo red to activate the cells. One loop of activated cells was restreaked onto petri dish containing YM agar with 25 µg.ml^{-1} congo red and incubated at 25°C for 2 days before use in the preparation of cells by negative staining for the observation of number and type of flagella by the transmission electron microscope (JEOL model JEM-2100) at the Scientific and Technological Equipment Center, Chulalongkorn University. Preparation of negatively-stained cells was as follows: One drop of distilled water was placed next to a colony of cells. The petri dish was tilted to allow distilled water to flow through the colony to form cell suspension. A copper grid for the electron microscope was put on top of the cell suspension to pick up cells. The suspension on the grid was allowed to partially dry. Cells were negatively-stained by placing a drop of 1% phosphotungstic acid on top of the cells for 1 min, then dried

completely with a rugged torn of Whatman No. 1 filter paper. The grid was left to dry in a clean petri dish in a desiccator overnight before observation of cells for number and type of flagella under the transmission electron microscope.

3.6.3 Bromthymol blue reactions

The procedure for testing for bromthymol blue reactions was similar to that for the observation of colony morphology as described in Section 3.6.1 except bromthymol blue at the final concentration of 25 $\mu g.ml^{-1}$ was added into YM agar instead of congo red. Plates were incubated at 25°C for 10 days with the observation of bromthymol blue reactions at the end of the 5- and 10- day incubation periods. Bromthymol blue which was green in YM agar with pH 6.8 turned blue when cells secreted alkali products and yellow when acidic products were secreted.

3.6.4 Growth at different temperatures

One loop of activated cells of each strain was inoculated into 50 ml YM broth in a 250 ml Ehrenmeyer flask, incubated at 30°C, 200 rpm for 3 days to be used as seed culture. Five ml of seed culture were inoculated into 45 ml YM broth in a 250 ml Ehrenmeyer flasks and incubated at 25°C, 30°C, 37°C, and 40°C at 200 rpm for 5 days with 100 µl samplings at 1 day intervals. Each sample was serially diluted by standard method and plated onto petri dishes containing YM agar with final concentration of 25 $\mu g.ml^{-1}$ congo red. Plates were incubated at 25°C for 5 days before viable counts of $CFU.ml^{-1}$. Growth curves of cells grown at different temperatures were constructed with $CFU.ml^{-1}$ versus incubation time at different temperatures.

3.6.5 Determination of ability/inability to use carbon and nitrogen sources

Ability/inability of the 5 selected strains of soybean rhizobia to use 95 carbon and nitrogen sources was determined by using the Biolog™ test kit according to the manufacturer 's instruction (Biolog Inc., USA). Cells stored in YM agar slants containing 25 $\mu g.ml^{-1}$ congo red were activated by streaking onto petri dishes containing TY media. Plates were incubated at 25°C for 5 days. Colonies were suspended in inoculation fluid which was supplied with the kit until the percent transmission as measured by the Biolog's

spectrophotometer was 52%. 150 µl of cell suspension was aseptically loaded into each well of a 96-well GN2 MicroPlate which was supplied by the manufacturer. The plates were incubated at 25°C for 24 h. Absorbance reading at 590 nm and 730 nm (DWD, Dual Wavelength Data of OD_{590} and OD_{730} were obtained with the Biolog spectrophoto meter. DWD values were processed by the Biolog processing unit which calculated the values according to the following formula given in the Biolog's instruction manual:

$$DWD = (OD_{590} - OD_{730})\ A\ -\ (OD_{590} - OD_{730})\ A_1 \times 1000$$

A = any of the 95 wells; A_1 = control well , the first well at row A

The Biolog instrument and processing unit which belonged to the the Center for Agricultural Biotechnology, Kasetsart University, Kamphangsaen Campus, Nakorn Pathom Province, were used. Since the Center's Biolog database for the identification of Gram negative bacteria does not contain data for the identification of soybean rhizobia, the data obtained for the utilization/non-utilization of 95 carbon and nitrogen sources are used as supplementary data to aid the identification of soybean rhizobia by polyphasic taxonomy. Data for the utilization/non-utilization of 95 carbon and nitrogen sources will be arbitrarily recorded as follows:

$DWD < A_1 + 25\%A_1 =$ - (non-utilize that sources)

$A_1 + 25\%A_1 < DWD < 0.25 =$ + (utilize that sources)

$DWD > 0.25 < 0.50 =$ ++ (utilize that sources)

$DWD > 0.50 =$ +++ (utilize that sources)

The following reference strains of slow-growing soybean rhizobia were used in the Biolog test. *B. elkanii* NBRC 14791, *B. japonicum* 14783, and *B. liaoningense* NBRC 100396. The reference strains were purchased from NITE Biological Resource Center (NBRC) (NITE = National Institute of Technology and Evaluation), Tokyo, Japan

3.6.6 Identification of slow-growing soybean rhizobia by 16S rDNA sequences

16S rDNA of each of the 5 randomly selected strains were isolated by PCR using 27f and 1492r as the primers. PCR composition was : 10x PCR buffer 2 µl, 2.5mM dNTPs 2 µl, primer 27f (10 pmol•µl $^{-1}$) and primer 1492r (10 pmol•µl $^{-1}$) 0.5 µl, DNA 200 ng, *Taq* polymerase(5 units•µl $^{-1}$) 0.2 µl, distilled water to 20 µl. PCR program was as follows: 95^{o}C 30 seconds, 95^{o}C 60 seconds, 48^{o}C 60 seconds, 72^{o}C 120 seconds (30 cycles) followed by 48^{o}C 60 seconds, 72^{o}C 300 seconds (1 cycle). Sequences of the primers 27f and 1492r were as described by Dorsch and Stackerbrandt (1992) :

27f (9-27)* : 5'GAGTTTGATCCTGGCTCAG3', 1492r (1492-1512)* : 5'ACGGCTACCTTG TTACGACCT3'

* = Position of nucleotides on consensus sequence of 16S rDNA of *E. coli*

PCR product of 1,500 bp was sent to the BioDesign Company, Thailand science Park for sequencing. The following 9 primers with sequences as described by Dorsch and Stackerbrandt (1992) were sent for use in the sequencing of 16S rDNA :

27 f (9-27)* :5'GAGTTTGATCCTGGCTCAG3

1492r (1492-1512)* : 5' ACGGCTACCTTGTTACGACCT3'

343r (343-357)* : 5'CTGCTGCCTCCCGTA3'

519r (519-536)* : 5'GTATTACCGCGGCTGCTC3'

787r (787-803)* : 5'CTACCAGGGTATCTAAT3'

907r (907-926)* : 5'CCGTCAATTCATTTGAGTTT3'

1100r (1100-1115)* : 5'AGGGTTGCGCTCGTTG3'

1385r (1385-1401)* : 5'CGGTGTGTACAAGGCCC3'

1241f (1224-1241)* : 5'TACACACGTGCTACAATG3'

* Position of nucleotides on consensus sequence of 16S rDNA of *E. coli*

The BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) was used to obtain the sequence of sense strand of each 16S rDNA.

Sequences of 16S rDNA obtained were compared with sequences deposited at GenBank database (http://www.ncbi.nlm.nih.gov/) using the Blast program for the identification of microbial strains using similarity of nucleotide sequences.

CHAPTER IV

RESULTS

4.1 PCR-DNA fingerprints of 23 soybean rhizobium strains isolated from root nodules

Figure 4.1 showed PCR-DNA fingerprints of 23 slow-growing soybean rhizobium strains isolated by Emampaiwong (2006) from root nodules of soybeans grown in soils from Bang Rakam district, Phitsanulok province. The results confirmed the 23 strains had different DNA fingerprints. Thus, they were considered as belonging to different strains and were used in the experiments on the optimization of multiplex PCR conditions by varying concentrations of target DNAs and primers.

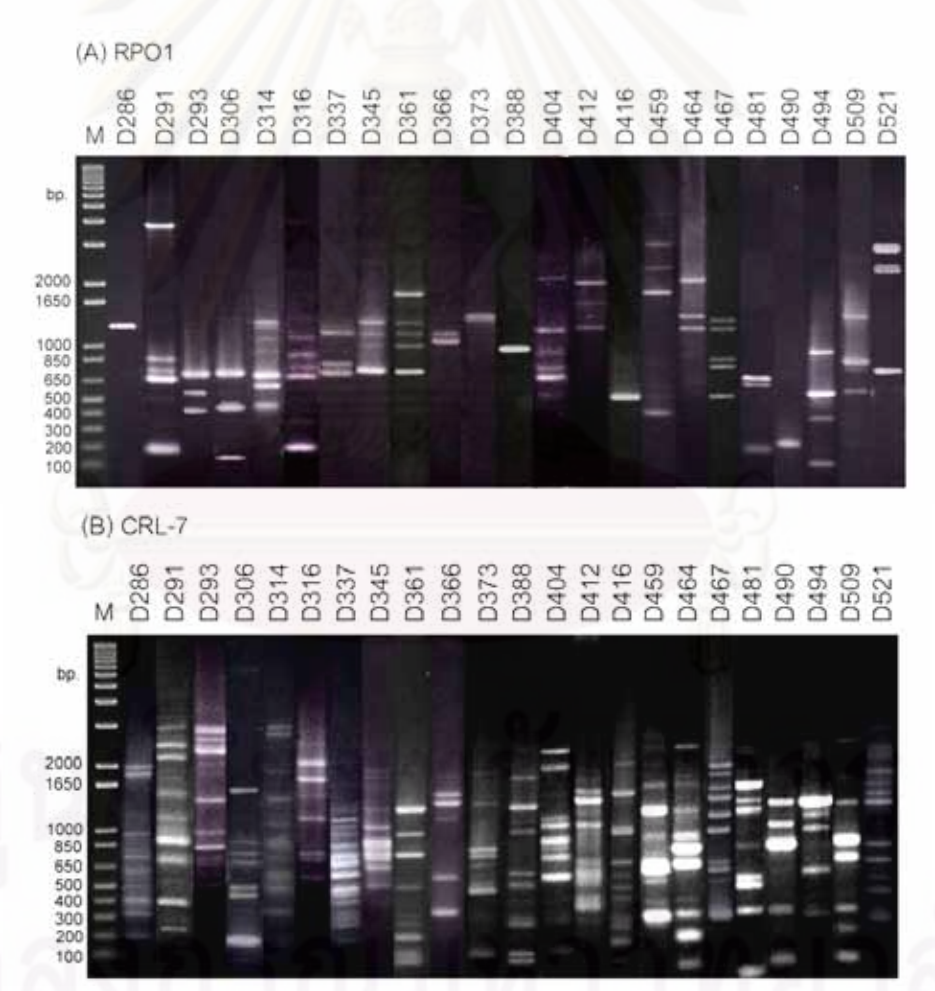
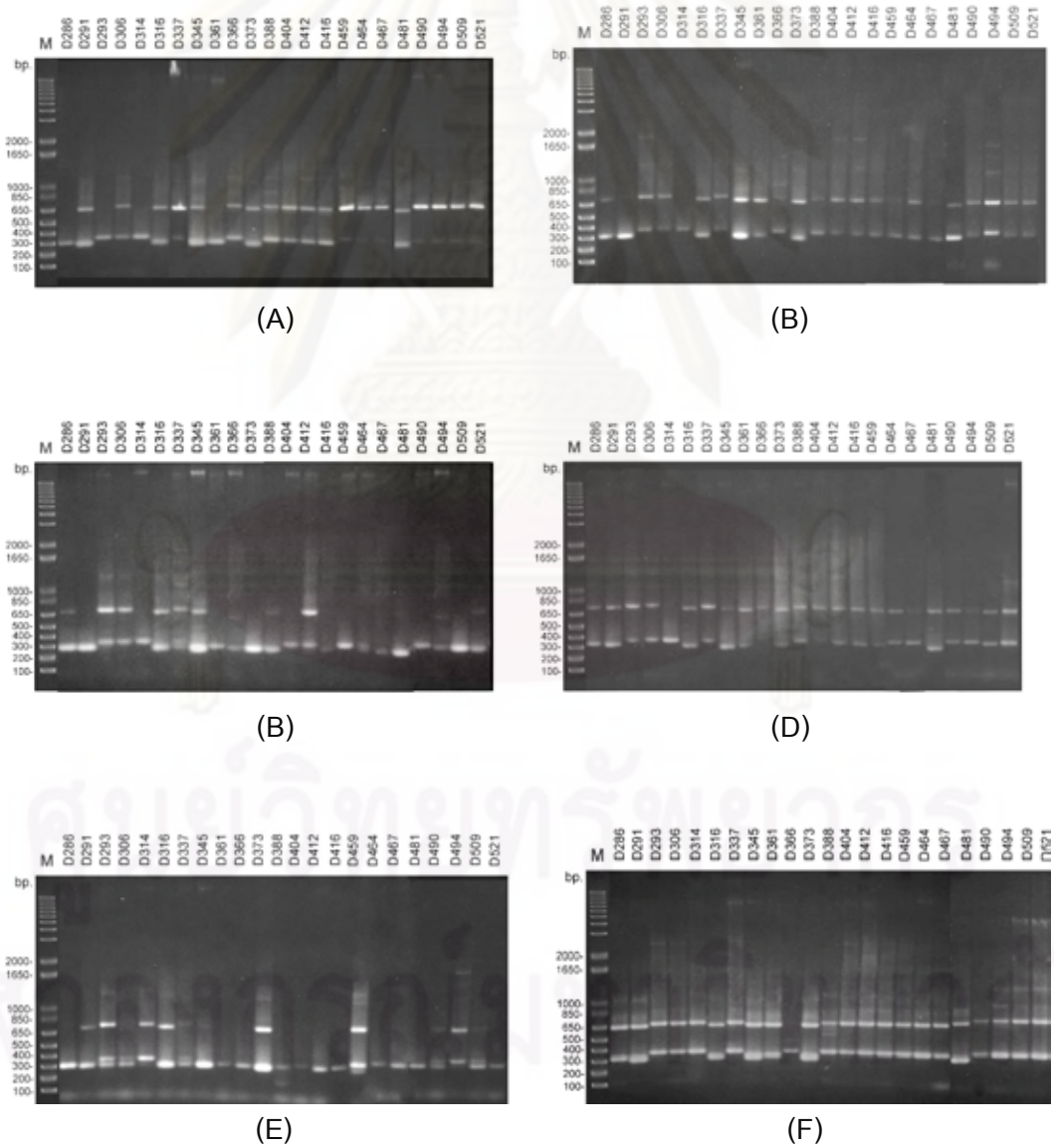


Figure 4.1 PCR-DNA fingerprints of 23 slow-growing rhizobium strains isolated from root nodules of soybeans grown in soils from Bang Rakam district, Phitsanulok Province when (A) RPO1 or (B) CRL-7 was used as the primer.

4.2 Optimization of multiplex PCR conditions

Figure 4.2 (A-I) showed multiplex PCR patterns when 100 ng target DNAs were used with different concentrations of primers as indicated in the Figure. The corresponding results with 200 ng of target DNAs were shown in Figure 4.3 (A-I). The results consistently showed two patterns of multiplex PCR products with pattern 1 consisting of 317 bp and 657 bp and pattern 2 consisting of 340 bp and 657 bp regardless of the different target DNA and primer concentrations used. Therefore, the two multiplex PCR patterns were used in the determination of correlation between multiplex PCR patterns and nodulation efficiency.



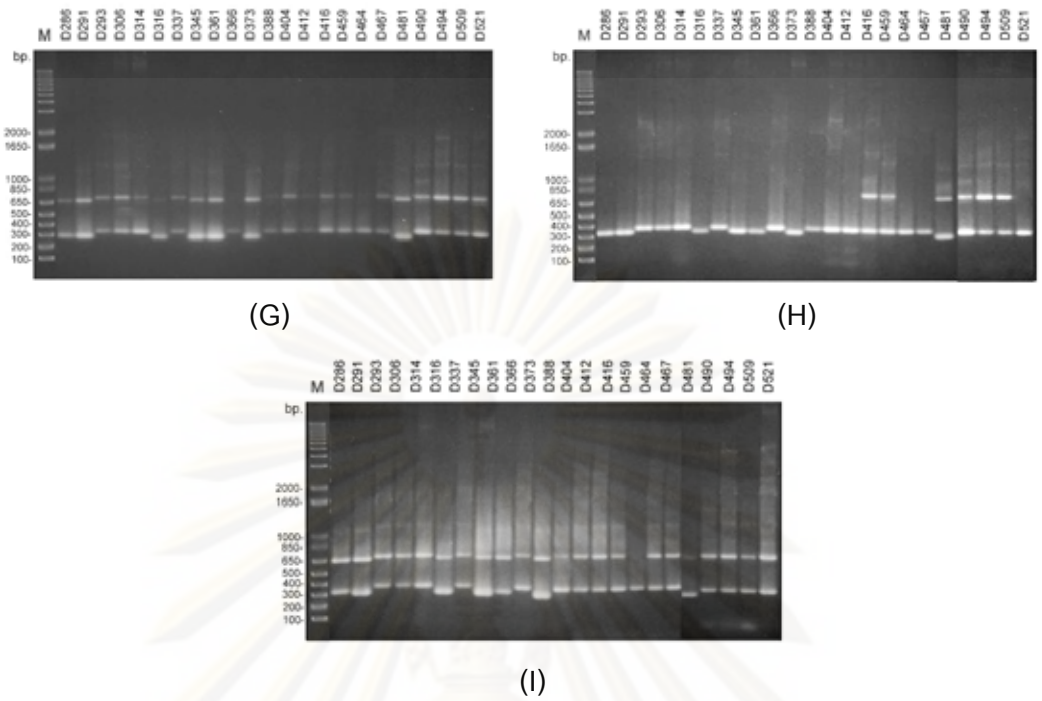


Figure 4.2 (A-I) Multiplex PCR results of 23 soybean rhizobium strains with 100 ng DNA as target DNA and the following combinations of primer concentrations:

A. *nodD1*F 8.0 pmoles *nodD1*R 8.0 pmoles *nodY*F 8.0 pmoles *nodY*R 8.0 pmoles

B. *nodD1*F 8.0 pmoles *nodD1*R 12.5 pmoles *nodY*F 8.0 pmoles *nodY*R 8.0 pmoles

C. *nodD1*F 8.0 pmoles *nodD1*R 8.0 pmoles *nodY*F 12.5 pmoles *nodY*R 8.0 pmoles

D. *nodD1*F 8.0 pmoles *nodD1*R 12.5 pmoles *nodY*F 12.5 pmoles *nodY*R 8.0 pmoles

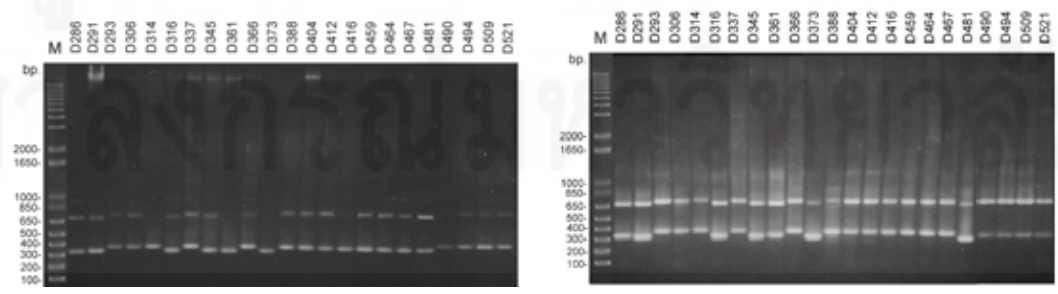
E. *nodD1*F 8.0 pmoles *nodD1*R 25.0 pmoles *nodY*F 25.0 pmoles *nodY*R 8.0 pmoles

F. *nodD1*F 12.5 pmoles *nodD1*R 12.5 pmoles *nodY*F 12.5 pmoles *nodY*R 12.5 pmoles

G. *nodD1*F 12.5 pmoles *nodD1*R 25.0 pmoles *nodY*F 12.5 pmoles *nodY*R 12.5pmoles

H. *nodD1*F 12.5 pmoles *nodD1*R 12.5 pmoles *nodY*F 25.0 pmoles *nodY*R 12.5pmoles

I. *nodD1*F 12.5 pmoles *nodD1*R 25.0 pmoles *nodY*F 25.0 pmoles *nodY*R12.5 pmoles



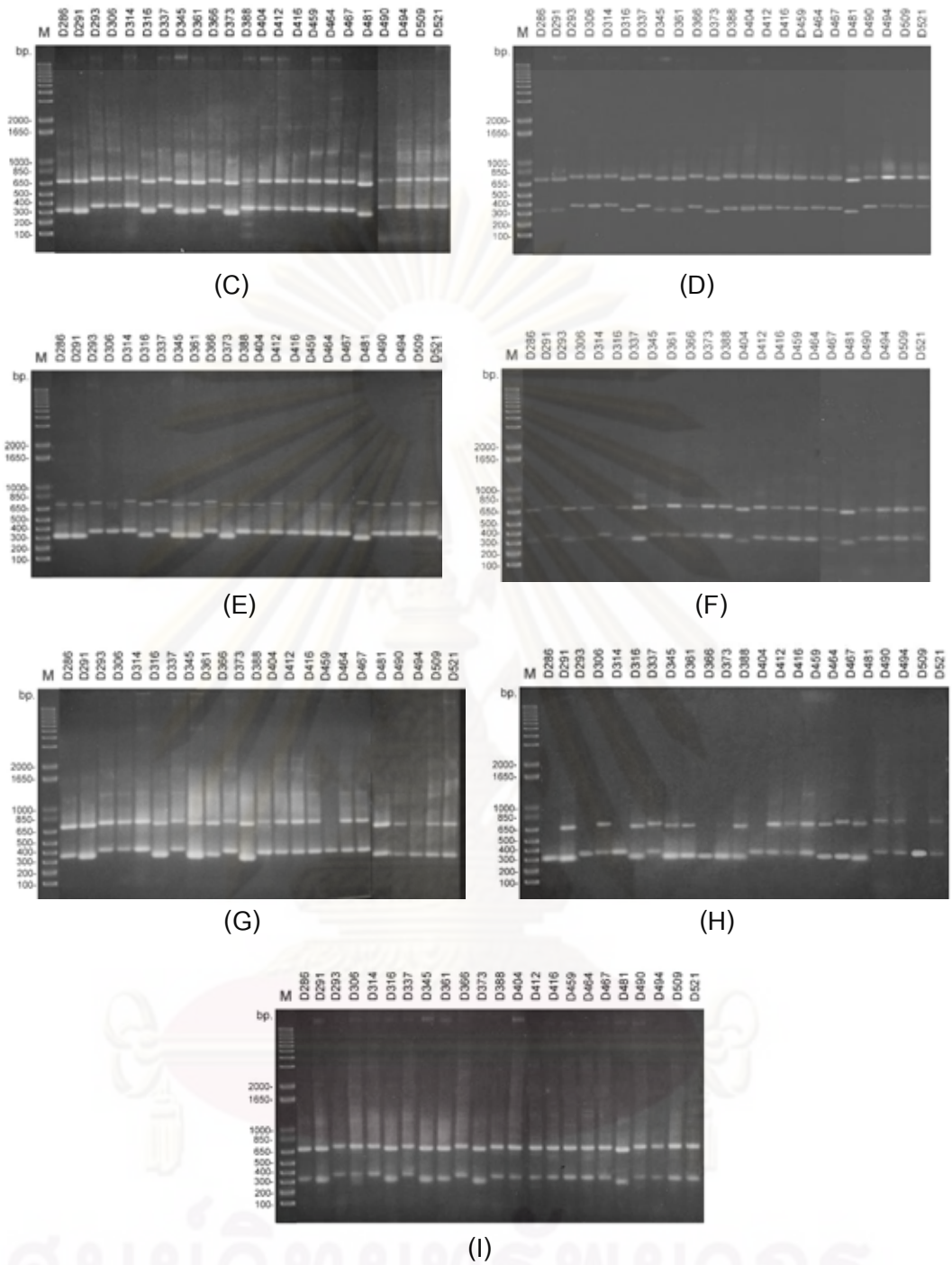


Figure 4.3 (A-I) Multiplex PCR results of 23 soybean rhizobium strains with 200 ng DNA as target DNA and the following combinations of primer concentrations:

A. *nodD1*F 8.0 pmoles *nodD1*R 8.0 pmoles *nodY*F 8.0 pmoles *nodY*R 8.0 pmoles

B. *nodD1*F 8.0 pmoles *nodD1*R 12.5 pmoles *nodY*F 8.0 pmoles *nodY*R 8.0 pmoles

C. *nodD1*F 8.0 pmoles *nodD1*R 8.0 pmoles *nodY*F 12.5 pmoles *nodY*R 8.0 pmoles

D. *nodD1*F 8.0 pmoles *nodD1*R 12.5 pmoles *nodY*F 12.5 pmoles *nodY*R 8.0 pmoles

E. *nodD1*F 8.0 pmoles *nodD1*R 25.0 pmoles *nodY*F 25.0 pmoles *nodY*R 8.0pmoles

F. *nodD1*F 12.5 pmoles *nodD1*R 12.5 pmoles *nodY*F 12.5 pmoles *nodY*R 12.5 pmoles

G. *nodD1*F 12.5 pmoles *nodD1*R 25.0 pmoles *nodY*F 12.5 pmoles *nodY*R 12.5pmoles

H. *nodD1*F 12.5 pmoles *nodD1*R 12.5 pmoles *nodY*F 25.0 pmoles *nodY*R 12.5pmoles

I. *nodD1*F 12.5 pmoles *nodD1*R 25.0 pmoles *nodY*F 25.0 pmoles *nodY*R12.5 pmoles

From the results of the optimization of target DNA and primer concentrations as shown in Figures 4.2 and 4.3, the following set of target DNA and primer concentrations: 200 ng target DNA and concentrations of primers *nodD1*F, *nodD1*R, *nodY*F, and *nodY*R of 8.0, 12.5, 12.5 and 8.0 pmoles, respectively, was chosen as the optimized conditions for use in the use of multiplex PCR patterns to predict nodulation efficiency because the conditions yielded clean multiplex PCR products (Figure 4.3D). The optimized multiplex PCR conditions were used to obtain results as shown in Figure 4.4 where soybean rhizobium strains exhibiting the same multiplex PCR pattern were placed in the same group during agarose electrophoresis. The results showed, out of the 23 strains, the following 7strains: D286, D291, D316, D345, D361, D373, and D481 were in the same group with multiplex PCR pattern 1 containing 317 bp and 657 bp multiplex PCR products. The remaining 16 strains were in the same group of multiplex PCR pattern 2 with 340 bp and 657 bp multiplex PCR products.

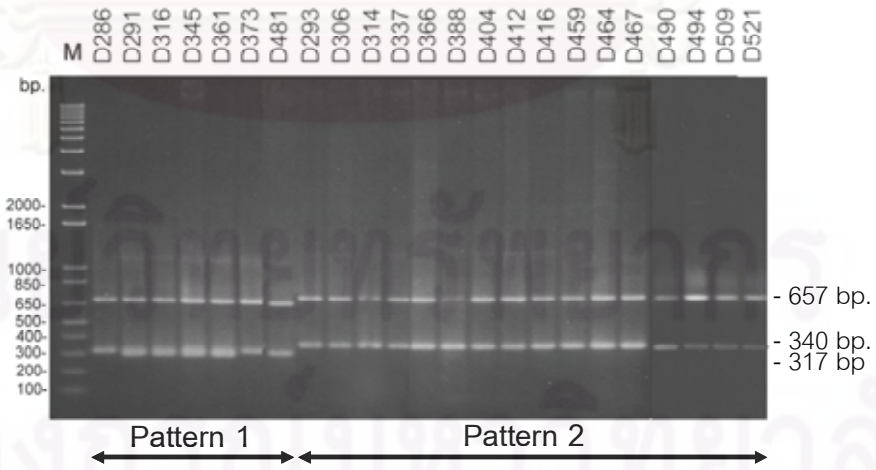


Figure 4.4 Multiplex PCR patterns of 23 soybean rhizobium strains using the optimized conditions of 200 ng target DNA and concentrations of primers *nodD1*F *nodD1*R *nodY*F and *nodY*R were 8.0, 12.5, 12.5 and 8.0 pmoles respectively.

4.3 Determination of nodulation efficiency

4.3.1 Determination of incubation time to grow cells of 23 soybean rhizobium strains to 10^8 CFU/ml at 30°C

Since nodule efficiency might depend on the number of soybean rhizobial cells in the inoculants, growth curves at 30°C of the 23 soybean rhizobium strains were obtained as shown in Figure 4.5 in order to determine the incubation time taken to grow cells of each strain to 10^8 CFU/ml . The results indicated that it was not possible to grow cells of all the 23 soybean strains to 10^8 CFU/ml which were considered to be optimal cell condition based on the cell density dependent inhibition of nodulation genes by the quorum sensing mechanism as reported by Loh et al. (2002) as previously discussed in Chapter II. Therefore, all the 23 strains were grown at 30°C for one day before use as the inoculants with cells from 4 X10^7 to 7 X 10^8 CFU/ml. The results as shown in Figures 4.4 and 4.5 showed no correlation between the multiplex PCR patterns and the extent of growth at 30^0C. For example, strains D373 and D459 had the lowest extent of growth , about 4 x 10^7 CFU/ml after one day incubation at 30^0C, and yet, D373 belonged to the multiplex PCR pattern 1 while D459 was found with the multiplex PCR pattern 2. Similarly, strains D345 and D464 were found to have approximately 8 x 10^8 CFU/ml after one day incubation at 30^0C, but D345 belonged to the multiplex PCR pattern 1 while D464 belonged to pattern 2.

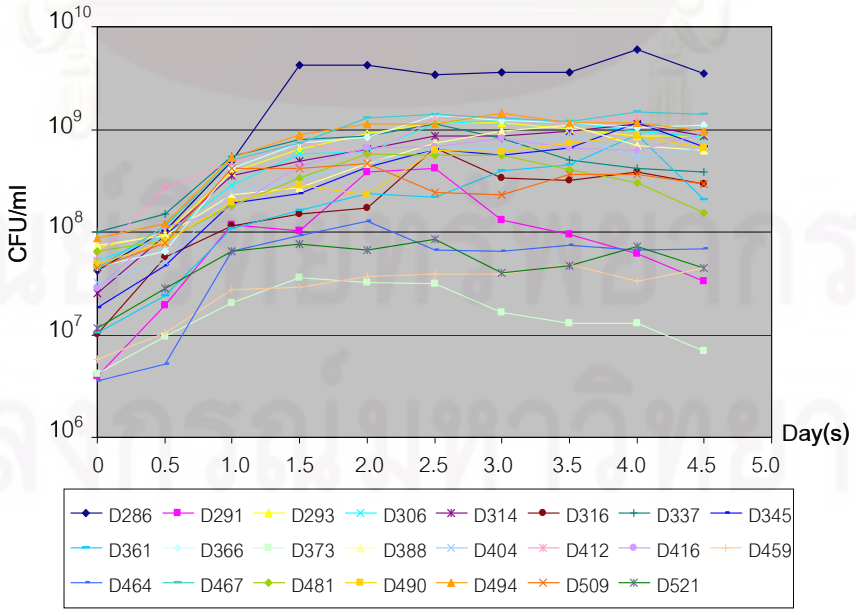
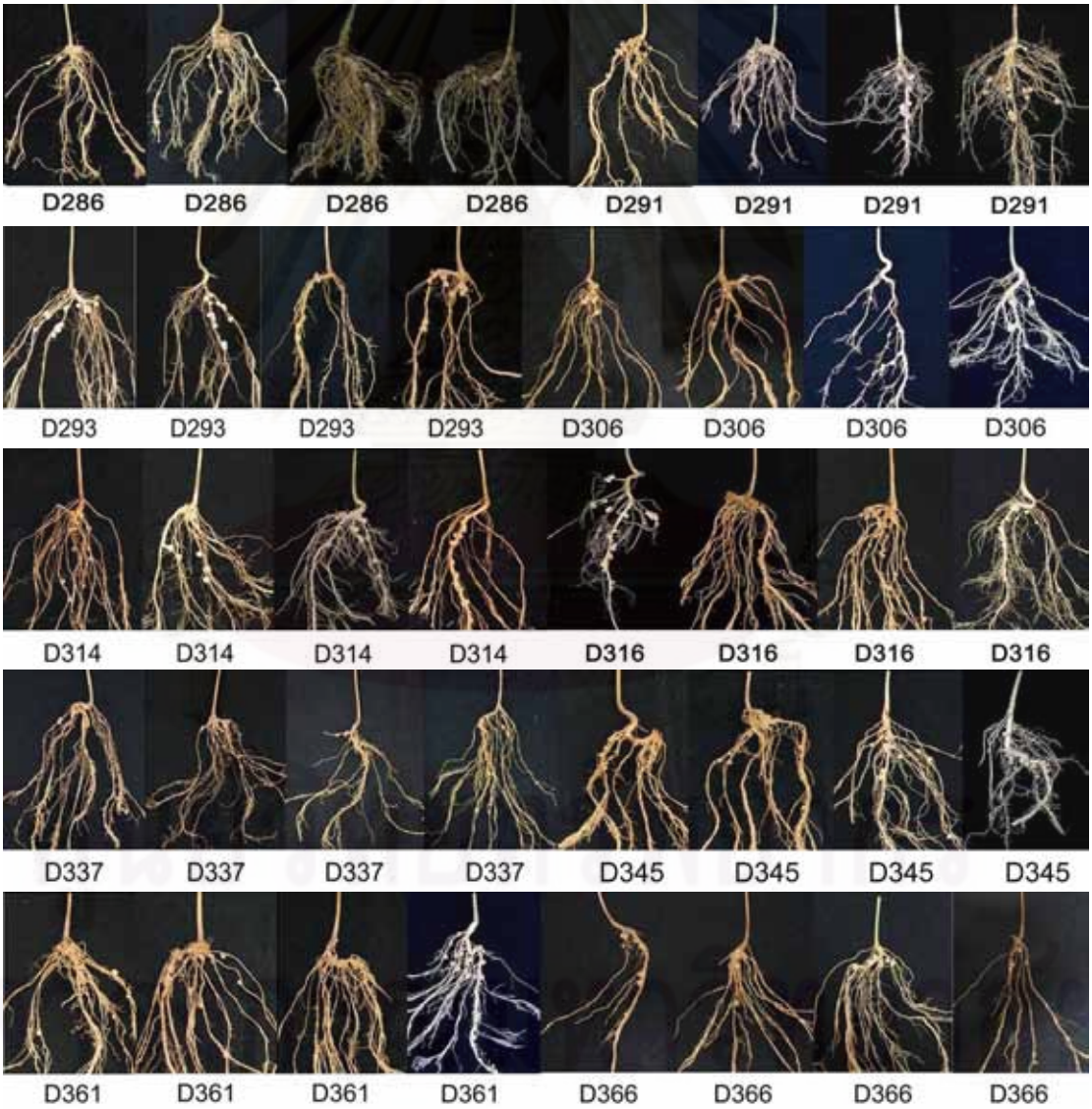
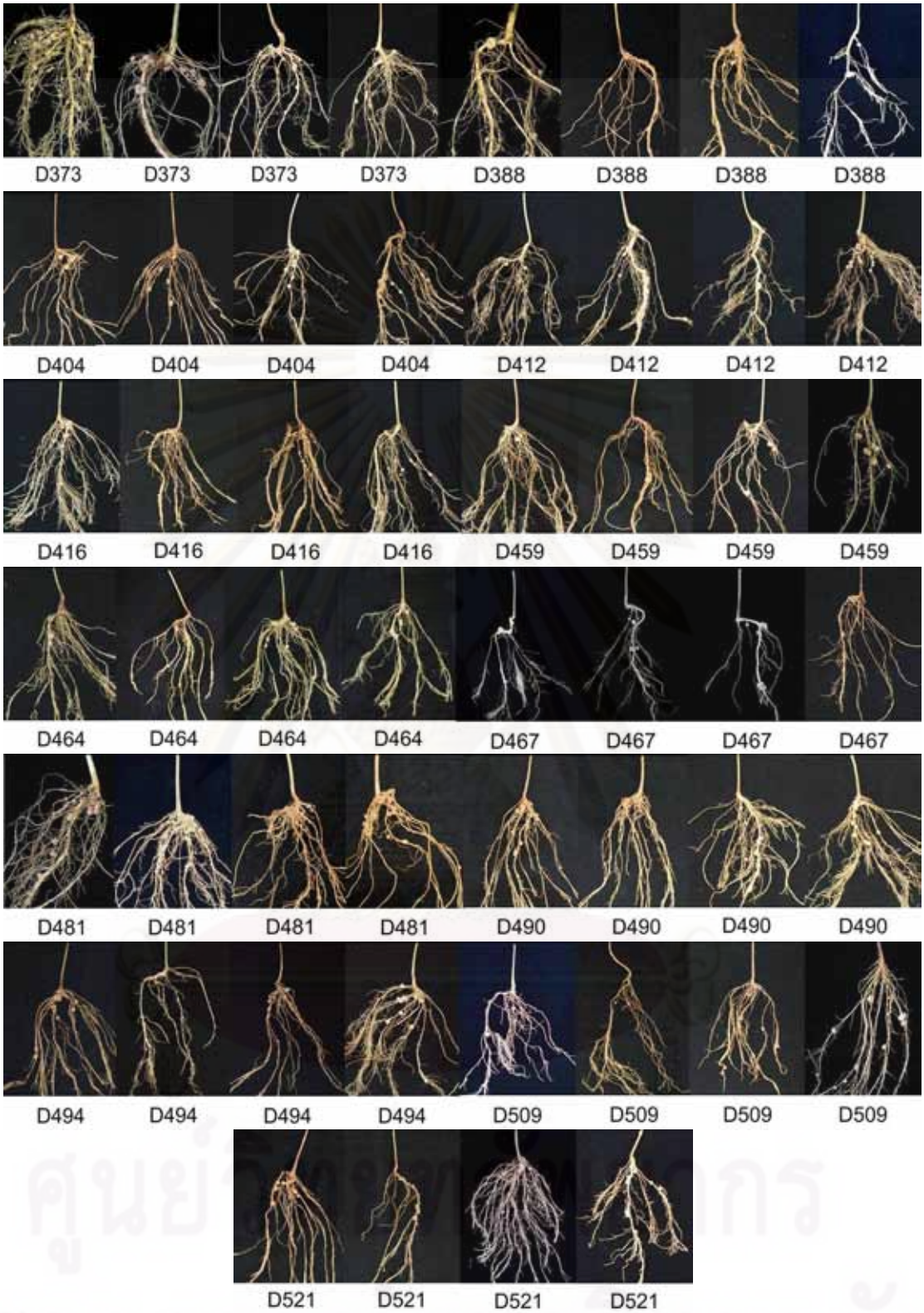


Figure 4.5 Growth of 23 soybean rhizobium strains grown in yeast extract mannitol medium at 200 rpm and 30 °C.

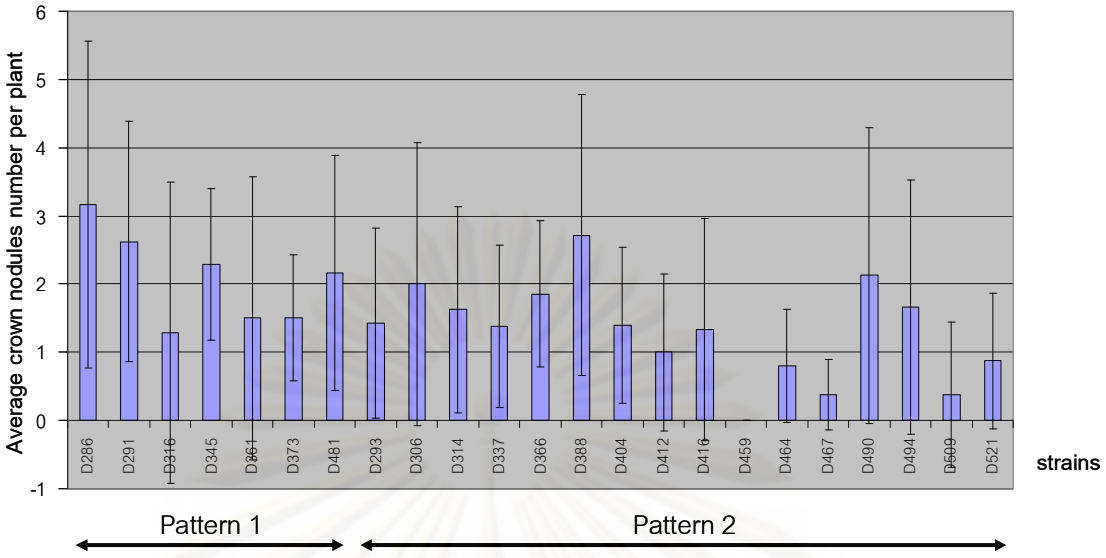
4.3.2 Determination of root nodulation efficiency

Figure 4.6 showed root nodules obtained from four replicates of soybean cultivar Chiangmai 60 inoculated with each of the 23 rhizobium strains and grown in Leonard jars with nitrogen-free medium for 28 days in a 28^0C-32^0C temperature-controlled greenhouse. The number of crown and total nodules were counted and the average numbers were plotted as histograms shown in Figures 4.7.

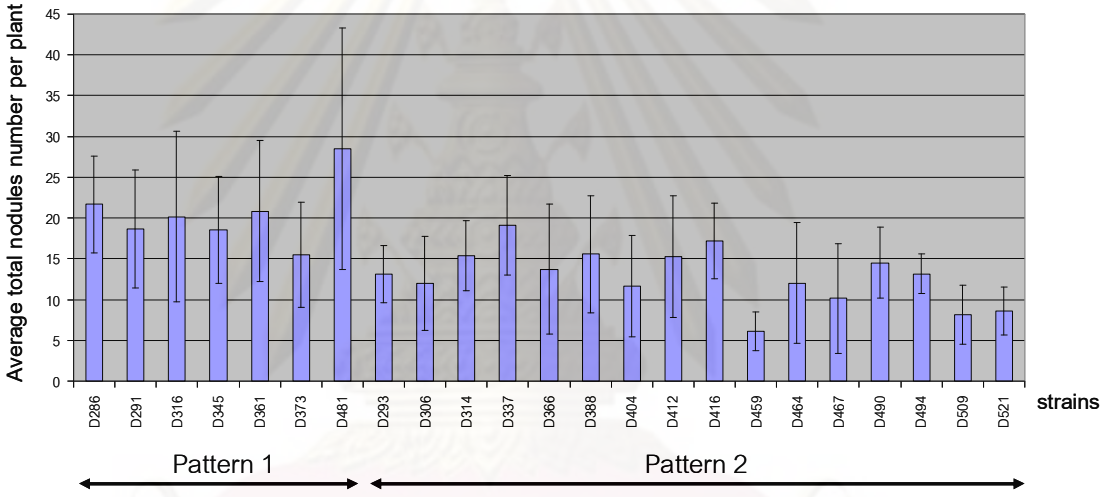




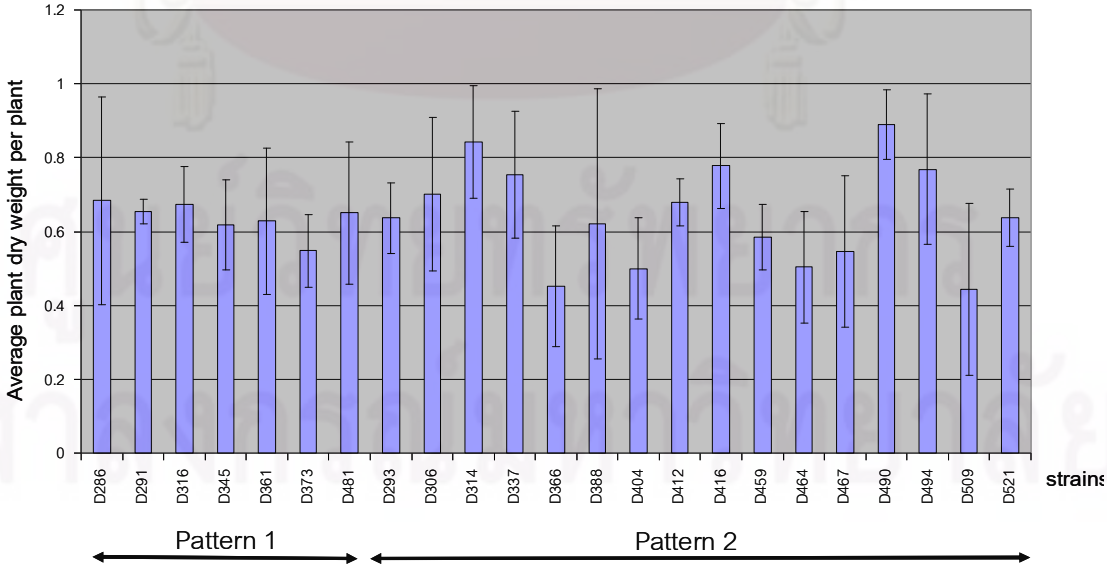
Figures 4.6 : Root nodules of soybean cultivar Chiangmai 60 inoculated with each of the 23 soybean rhizobium strains and grown with nitrogen-free medium in Leonard jars in a 28^{0}C-32^{0}C temperature-controlled greenhouse for 28 days.


Average crown nodules number per plant
6
5
4
3
2
1
0
-1
D286
D291
D316
D345
D361
D373
D481
D293
D306
D314
D337
D366
D388
D404
D412
D416
D459
D464
D467
D490
D494
D509
D521
strains
Pattern 1
Pattern 2

(A)


Average total nodules number per plant
45
40
35
30
25
20
15
10
5
0
D286
D291
D316
D345
D361
D373
D481
D293
D306
D314
D337
D366
D388
D404
D412
D416
D459
D464
D467
D490
D494
D509
D521
strains
Pattern 1
Pattern 2

(B)


Average plant dry weight per plant
1.2
1
0.8
0.6
0.4
0.2
0
D286
D291
D316
D345
D361
D373
D481
D293
D306
D314
D337
D366
D388
D404
D412
D416
D459
D464
D467
D490
D494
D509
D521
strains
Pattern 1
Pattern 2

(C)

Figure 4.7 (A-C) Histogram showing (A) average numbers of crown nodules per plant (B) average numbers of total nodules and (C) average plant dry weight per plant were obtained from the inoculation of 23 soybean rhizobium strains onto soybean cultivar Chiangmai 60 grown in Leonard jars in a 28°C-32°C temperature-controlled greenhouse for 28 days.

The results showed no correlation between multiplex PCR patterns and the average numbers of crown and total nodules. The average number of crown nodules obtained when soybean rhizobia with multiplex PCR pattern 1 were used as inoculants ranged from 1-3 which was the same number as those found when soybean rhizobia with multiplex PCR pattern 2 were used as inoculants in Leonard jars (Figures 4.7 and Appendix C). The average number of total nodules found when soybean rhizobia with multiplex PCR patterns 1 and 2 were used as inoculants were 16-29 and 6-20 respectively (Figures 4.7 and Appendix C). The results showed no correlation between the average number of total nodules and the average plant dry weight. Although soybean rhizobia with multiplex PCR pattern 1 yielded higher average number of total nodules (16-29), the range of the plant dry weight (0.54-0.68 g per plant) was less than the weight obtained when soybean rhizobia of multiplex PCR pattern 2 with lesser average number of total nodules were used (0.44-0.89 g per plant). As a matter of fact, there is no correlation whatsoever between the average plant dry weight and the average numbers of crown and total number of nodules. For example, data in Appendix C showed that strain D286 yielded the most average number of 3 crown nodules and the relatively high average number of total nodules (22 nodules), but the strain did not yield the highest plant dry weight (0.68 g per plant). On the other hand, strain 490 which yielded an average of 2 crown nodules and an average of 15 total nodules resulted in the statistically highest average plant dry weight (0.89 g per plant) as determined by the Duncan's multiple range test with $p < 0.05$. In addition, it was noted that had more soybean rhizobium strains in the multiplex PCR pattern 1 group been used in the experiments, more strains with multiplex PCR pattern 1 with lower average numbers of total nodules could have been detected.

4.9 PCR-DNA fingerprints of additional 57 bacterial isolates from root nodules of soybeans grown in soils from Bang Rakam district

Figure 4.8 showed PCR-DNA fingerprints of 57 additional slow-growing bacteria isolated by Emampaiwong (2006) from root nodules of soybeans grown in soils from Bang Rakam district, Phitsanulok province. The results indicated some isolates had identical DNA fingerprints, therefore, they were assigned to the same strains as shown in Table 4.1 and Appendix D with the total number of 20 strains. However, 10 of the 20 strains had identical fingerprints with 10 of the 23 previously used strains. Therefore, the 10 remaining strains were used to obtain multiplex PCR patterns and to determine if it is possible to use the multiplex PCR patterns to predict nodulation efficiency.

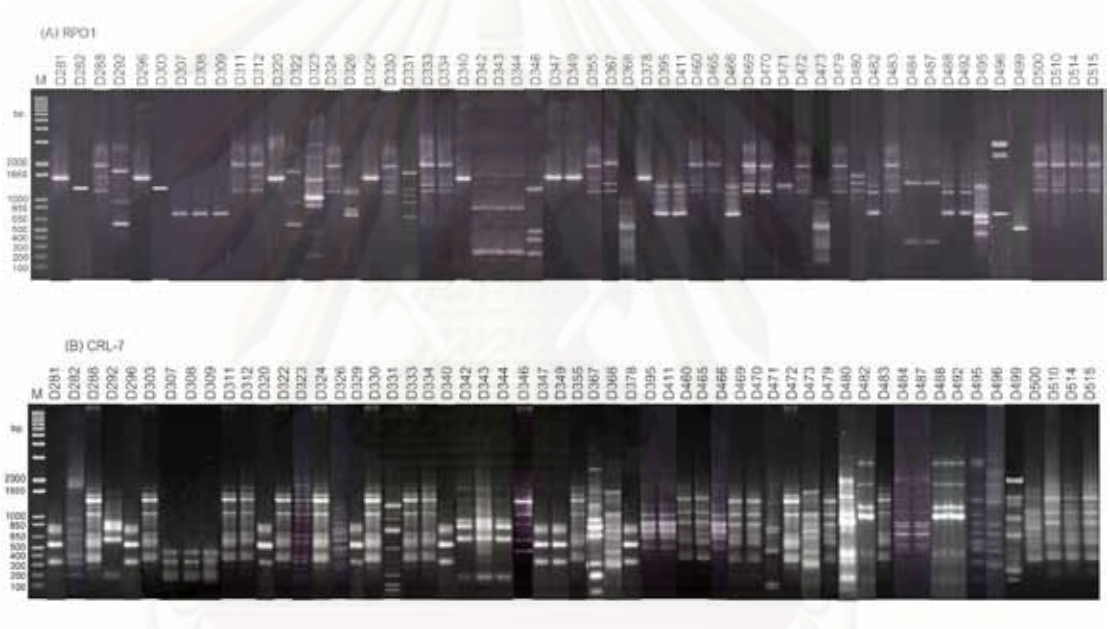


Figure 4.8 PCR-DNA fingerprints of 57 bacterial isolates from root nodules when (A) RPO1 or (B) CRL-7 was used as the primer.

Table 4.1 The 57 bacterial isolates with identical PCR-DNA fingerprints were grouped into 20 different strains.

Strain	Isolates with identical fingerprints	Subdistricts where soil samples were taken	Root nodules of soybean cultivars
D281	D281	Pan Sao	ST1
	D296	Pan Sao	SJ4
	D320	Bung Kok	ST2

Strain	Isolates with identical fingerprints	Subdistricts where soil samples were taken	Root nodules of soybean cultivars
	D329	Bung Kok	ST3
	D340	Bung Kok	SJ5
	D347	Bung Kok	CM60
	D349	Bung Kok	CM60
	D378	Nong Ku-la	CM2
D282	D282	Pan Sao	ST1
D288	D288	Pan Sao	ST2
	D303	Pan Sao	SJ5
	D311	Pan Sao	CM 60
	D312	Pan Sao	CM 60
	D324	Bung Kok	ST2
	D330	Bung Kok	ST3
	D333	Bung Kok	SJ4
	D334	Bung Kok	SJ4
	D355	Nong Ku-la	ST1
	D460	Kui Muang	ST1
	D465	Kui Muang	ST2
	D469	Kui Muang	ST3
	D470	Kui Muang	ST3
	D472	Kui Muang	SJ4
	D479	Kui Muang	SJ5
	D483	Kui Muang	CM2
	D500	Bang Rakam	ST3
	D510	Bang Rakam	ST3
	D514	Bang Rakam	SJ5
	D515	Bang Rakam	SJ5
D292	D292	Pan Sao	ST3
	D322	Bung Kok	ST2
D307	D307	Pan Sao	CM2

Strain	Isolates with identical fingerprints	Subdistricts where soil samples were taken	Root nodules of soybean cultivars
	D308	Pan Sao	CM2
	D309	Pan Sao	CM2
D323	D323	Bung Kok	ST2
D326	D326	Bung Kok	ST3
D331	D331	Bung Kok	SJ4
D342	D342	Bung Kok	CM2
	D343	Bung Kok	CM2
	D344	Bung Kok	CM2
D346	D346	Bung Kok	CM60
D367	D367	Nong Ku-la	SJ4
D368	D368	Nong Ku-la	SJ4
	D473	Kui Muang	SJ4
D395	D395	Chum Saeng Songkram	SJ2
	D411	Chum Saeng Songkram	CM2
	D466	Kui Muang	ST3
D471	D471	Kui Muang	SJ4
D480	D480	Kui Muang	SJ5
D482	D482	Kui Muang	CM2
	D488	Kui Muang	CM60
	D492	Bang Rakam	ST1
D484	D484	Kui Muang	CM2
	D487	Kui Muang	CM60
D495	D495	Bang Rakam	ST1
D496	D496	Bang Rakam	ST2
D499	D499	Bang Rakam	ST2

4.4 Multiplex PCR patterns of the 10 additional soybean rhizobium strains

Figure 4.9 showed multiplex PCR patterns of the 10 additional soybean rhizobium strain

isolated from root nodules of soybeans grown in soils from Bang Rakam district, Phitsanulok province. The results indicated strains D292 and D342 with multiplex PCR pattern 1 and the remaining 8 strains exhibited multiplex PCR pattern 2.

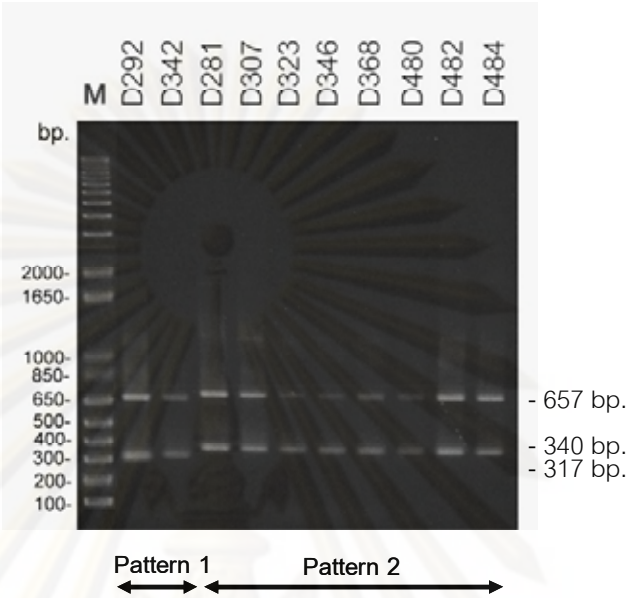


Figure 4.9 Multiplex PCR patterns of 10 additional soybean rhizobium strains. The optimized conditions for multiplex PCR of 200 ng DNA as target DNA and concentrations of primer *nodD1*F, *nodD1*R, *nodY*F, and *nodY*R of 8.0, 12.5, 12.5 and 8.0 pmoles respectively were used.

4.5 Determination of nodulation efficiency of the additional 10 soybean rhizobium strains

Figure 4.10 showed it was not possible to grow cells of each of the 10 additional strains to 10^8 CFU.ml^{-1} . The additional soybean rhizobial strains seemed to be divided into 2 groups with different extent of growth. Therefore, it was decided that cells between 1 x 10^7 and 8 X 10^8 CFU.ml^{-1} were used in the determination of nodulation efficiency in Leonard jars. Figures 4.11 showed pictorial results of root nodules obtained for 4 replicates of the 10 additional soybean rhizobial strains and histograms showing average numbers of crown and total nodules. The results also showed no correlation between the multiplex PCR patterns and nodulation efficiency as previously described in Section 4.3.

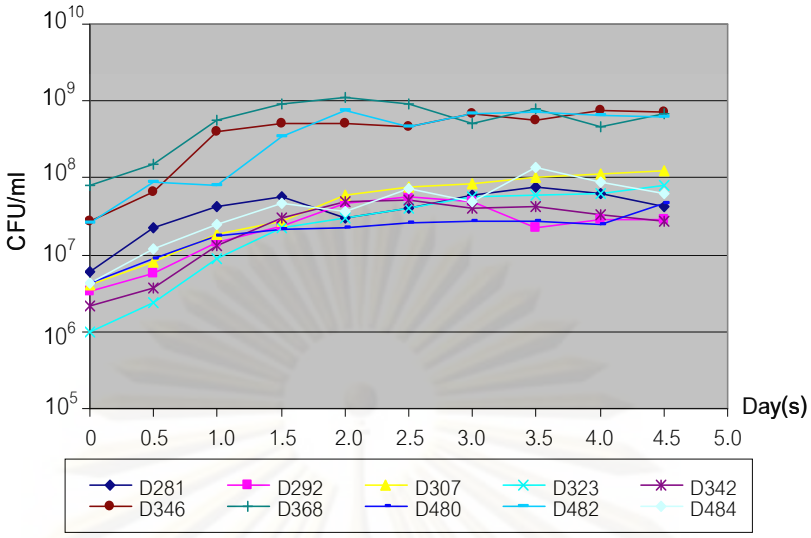


Figure 4.10 Growth of the 10 additional soybean rhizobium strains grown in yeast extract mannitol medium at 200 rpm and 30 °C.

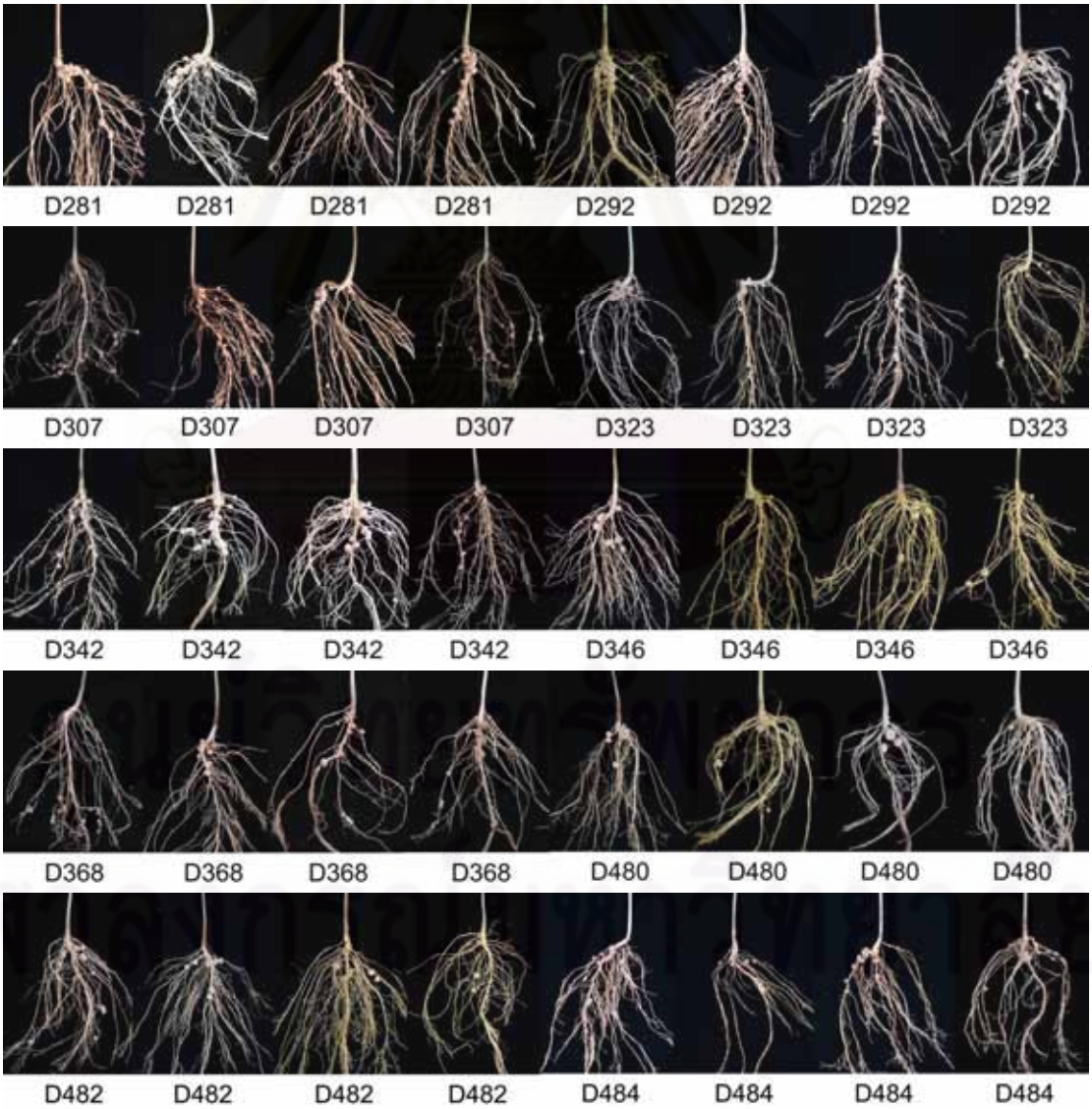
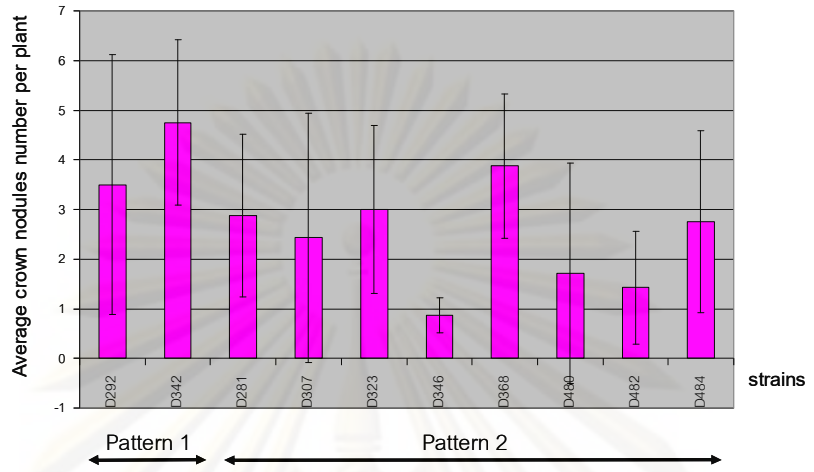
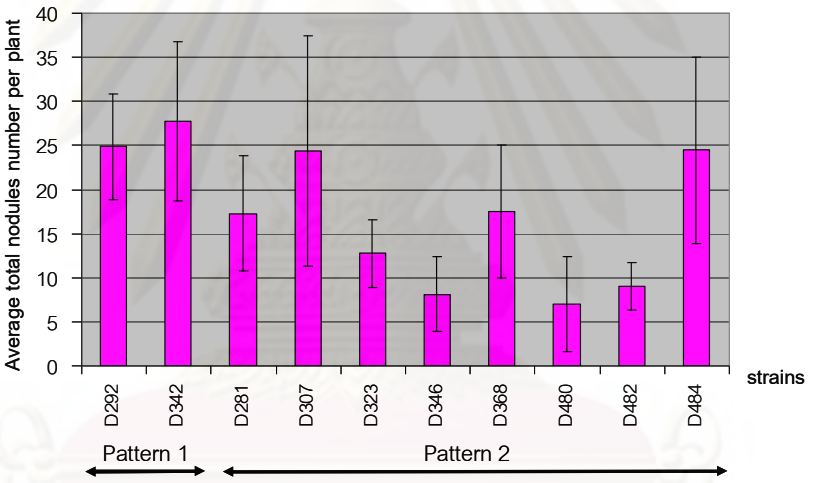


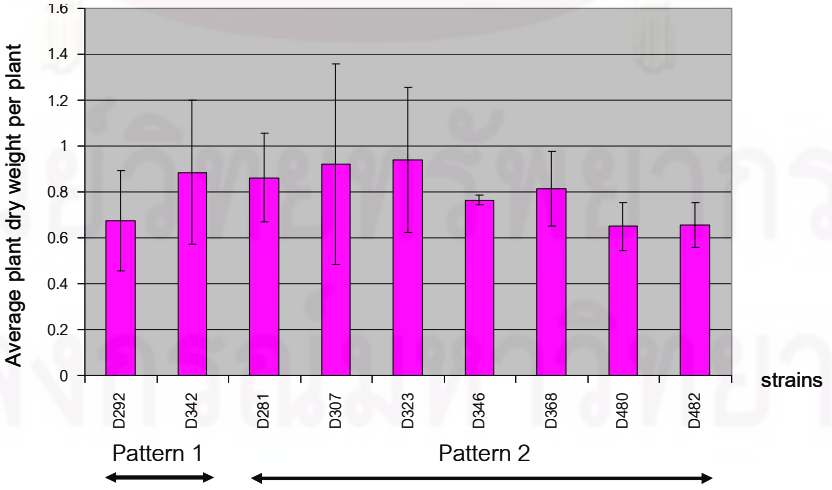
Figure 4.11 Root nodules obtained for 4 replicates of the 10 additional rhizobium strains isolated from root nodules of soybeans grown in soils from Bang Rakam district, Phitsanulok province.



(A)



(B)



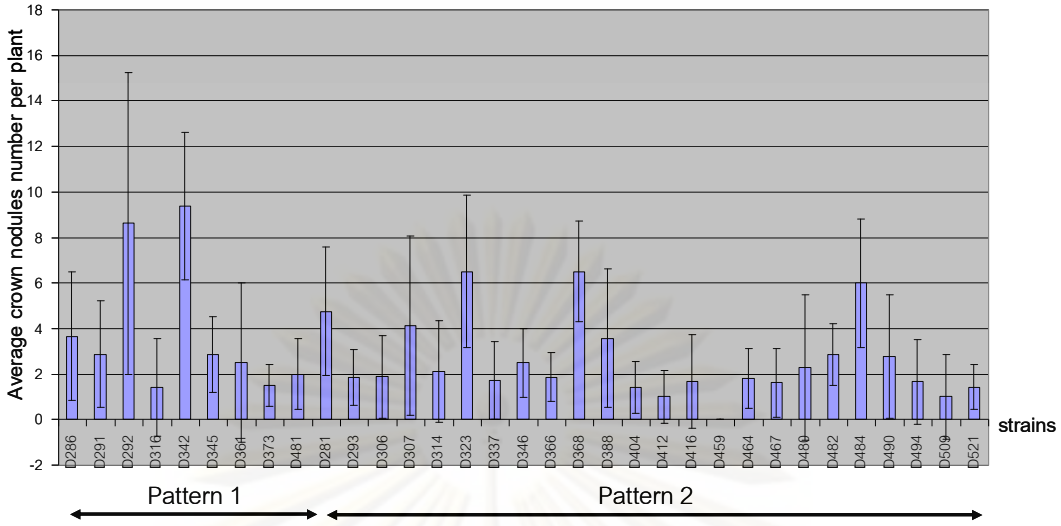
(C)

Figure 4.12 (A-C) Histogram showing (A) average numbers of crown nodules (B) average numbers of total nodules and (C) plant dry weight per plant were obtained when each of the 10 additional soybean rhizobium strains was used as the inoculant for soybean cultivar Chiangmai 60 grown in Leonard jars in a 28°C-32°C temperature-controlled greenhouse for 28 days.

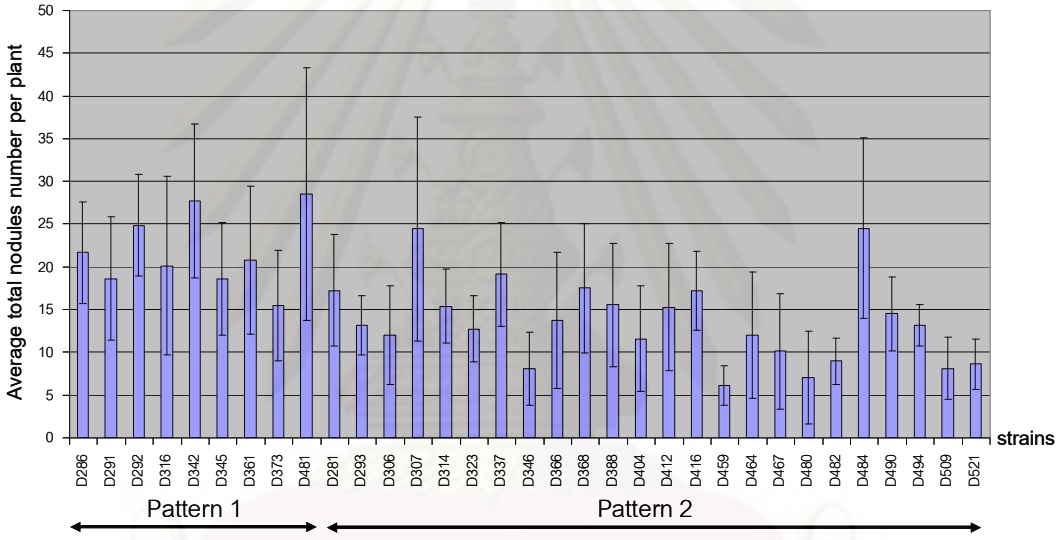
Figures 4.12 (A-B) and Appendix C indicated that the average number of crown nodules obtained when strains D342, and D292 which were found to belong to the multiplex PCR pattern 1 was 3-5 nodules while that obtained from the remaining 8 strains which belonged to the multiplex PCR pattern 2 was 1-4 nodules. The average number of total nodules ranged from 20-28 in the pattern 1 strains and 7-25 in the pattern 2 strains. Here again, figure 4.12 (C), the results showed no correlation between the average numbers of crown and total nodules and the average plant dry weight because all the 10 strains with different numbers of low and high average numbers of both crown and total nodules yielded plant dry weight (0.62-0.94 g per plant) which was not statistically different as determined by the Duncan's multiple range test at $p<0.05$.

4.6 Combining the results obtained for the 23 and the additional 10 slow-growing soybean rhizobium strains

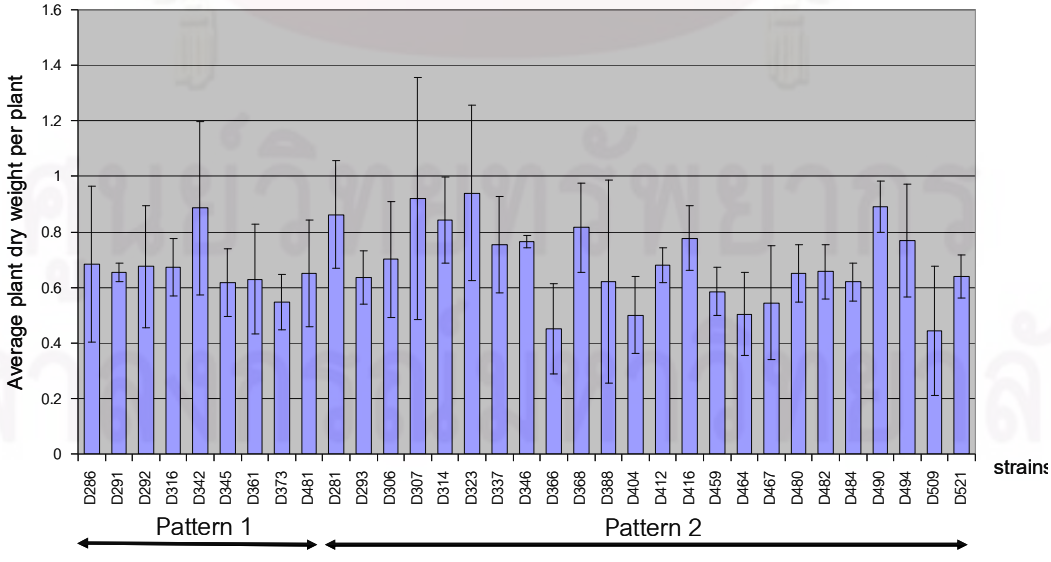
In order to obtain more meaningful data from the experiments, results of the multiplex PCR patterns and the average numbers of crown and total nodules of the 23 strains and the additional 10 strains were combined as results from a total of 33 slow-growing soybean rhizobium strains. Figures 4.15 and 4.16 showed histograms of the average numbers of crown nodules and those of the total nodules obtained from the use of the 33 slow-growing soybean rhizobium strains as the inoculants in Leonard jars.


Average crown nodules number per plant
18
16
14
12
10
8
6
4
2
0
-2
D286 D291 D292 D316 D342 D345 D361 D373 D481 D281 D293 D306 D307 D314 D323 D337 D346 D366 D368 D388 D404 D412 D416 D459 D464 D467 D480 D482 D484 D490 D494 D509 D521
strains
Pattern 1
Pattern 2

(A)


Average total nodules number per plant
50
45
40
35
30
25
20
15
10
5
0
D286 D291 D292 D316 D342 D345 D361 D373 D481 D281 D293 D306 D307 D314 D323 D337 D346 D366 D368 D388 D404 D412 D416 D459 D464 D467 D480 D482 D484 D490 D494 D509 D521
strains
Pattern 1
Pattern 2

(B)


Average plant dry weight per plant
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
D286 D291 D292 D316 D342 D345 D361 D373 D481 D281 D293 D306 D307 D314 D323 D337 D346 D366 D368 D388 D404 D412 D416 D459 D464 D467 D480 D482 D484 D490 D494 D509 D521
strains
Pattern 1
Pattern 2

(C)

Figure 4.13 (A-B) Histogram showing (A) combined results of the average numbers of crown nodules (B) combined result of the average numbers of total nodules and (C) combined results of the average plant dry weight per plant were obtained when each of the 33 slow-growing soybean rhizobium strains was used as an inoculant for growing soybean cultivar Chiangmai 60 in Leonard jars.

Combining data of the average numbers of crown and total nodules obtained when each of the 33 slow-growing soybean rhizobium strains was used as an inoculant for growing soybean cultivar Chiangmai 60 in Leonard jars as shown in Figures 4.13 (A-B) still showed no correlation between the multiplex PCR patterns and the average numbers of crown and total nodules. Strains belonging to either multiplex PCR pattern 1 or 2 were found to yield both high and low average numbers of crown and total nodules as previously described in detail in Sections 4.3 and 4.5.

4.7 Polyphasic taxonomy of 5 randomly selected strains of slow-growing soybean rhizobia

4.7.1 Colony morphology

Figure 4.14 showed colony morphology of 5 randomly selected soybean rhizobium strains consisting of strains D361, D373, D388, D416, and D467 grown on YMA plus congo red plates. All colonies were round, pearly, less than 1mm in diameter and did not absorb congo red.

Figure 4.14 Colony morphology of 5 randomly selected soybean rhizobium strains grown on YMA plus congo red plates at 25^0C for 10 days.

4.7.2 Number and type of flagella

Figure 4.15 showed negative staining results for the 5 randomly selected soybean rhizobium strains. Each strain was found to have one subpolar flagellum as expected (Elkan and Bunn, 1992).

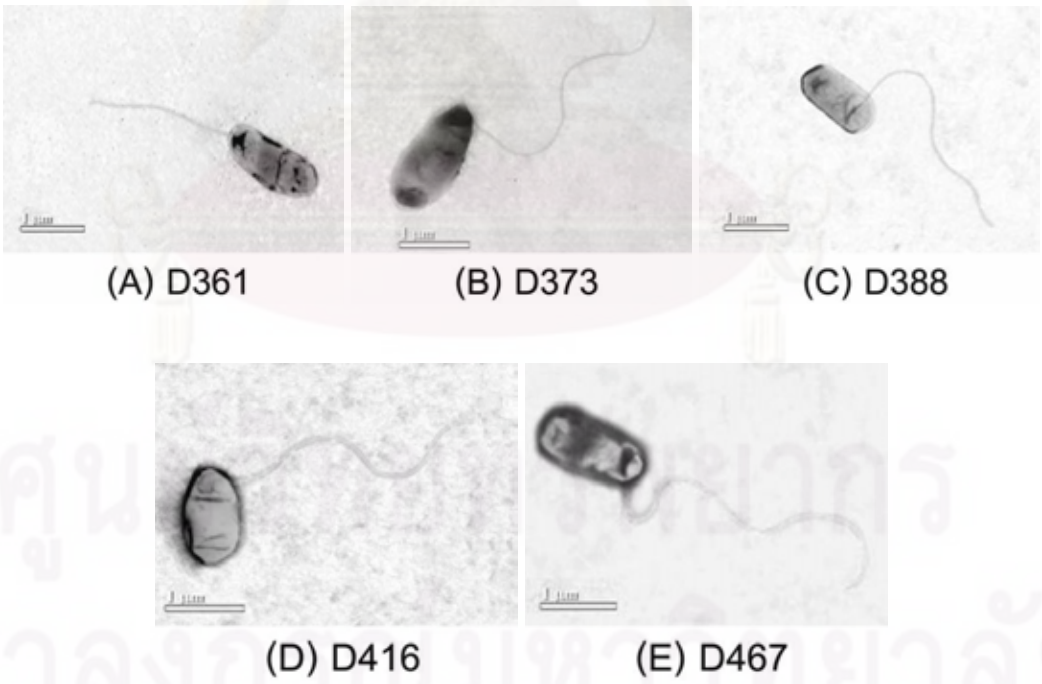


Figure 4.15 Negative staining results for the 5 selected strains of slow-growing soybean rhizobia.

4.7.3 Bromthymol blue reactions

Figure 4.16 showed Bromthymol blue reactions of the 5 selected soybean rhizobium strains grown on YMA plus Bromthymol blue plates. The results showed all strains strongly secreted acidic products after 10 days incubation at 25^0C.

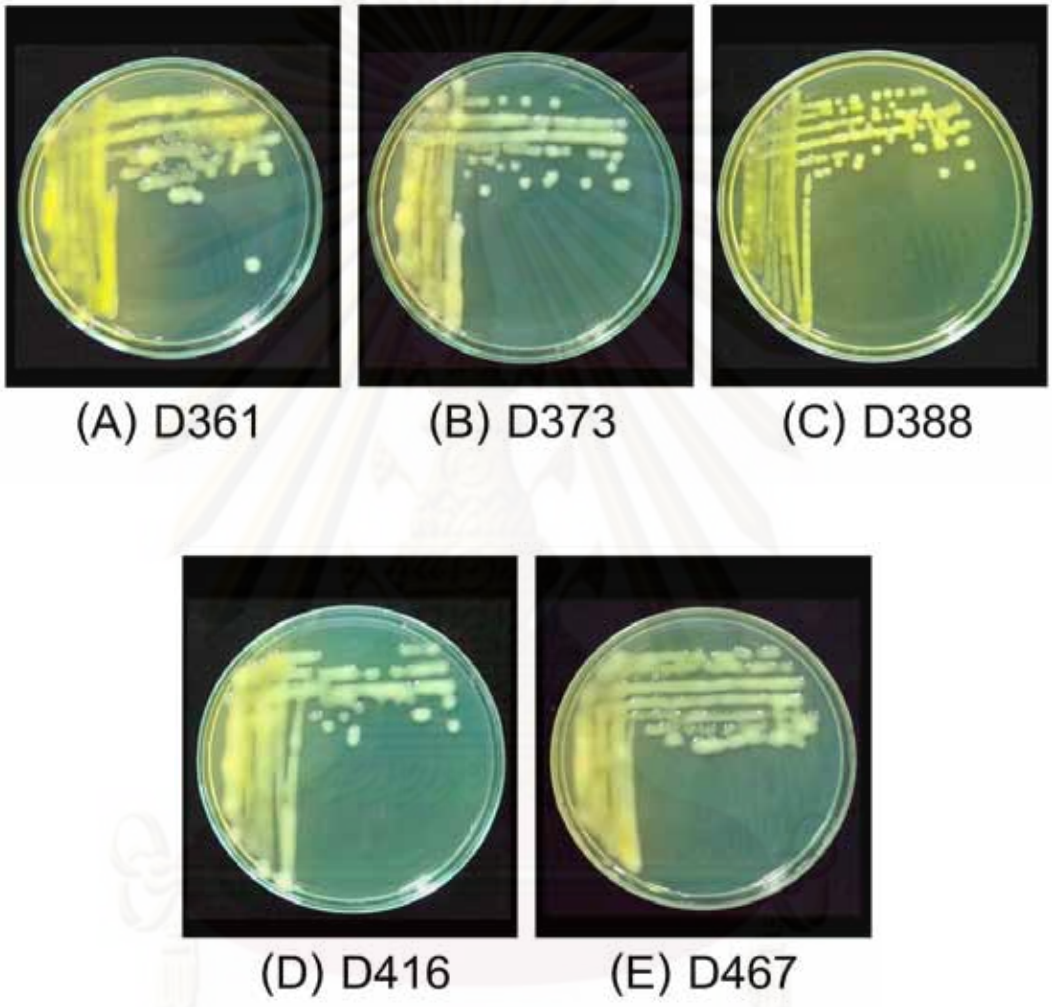
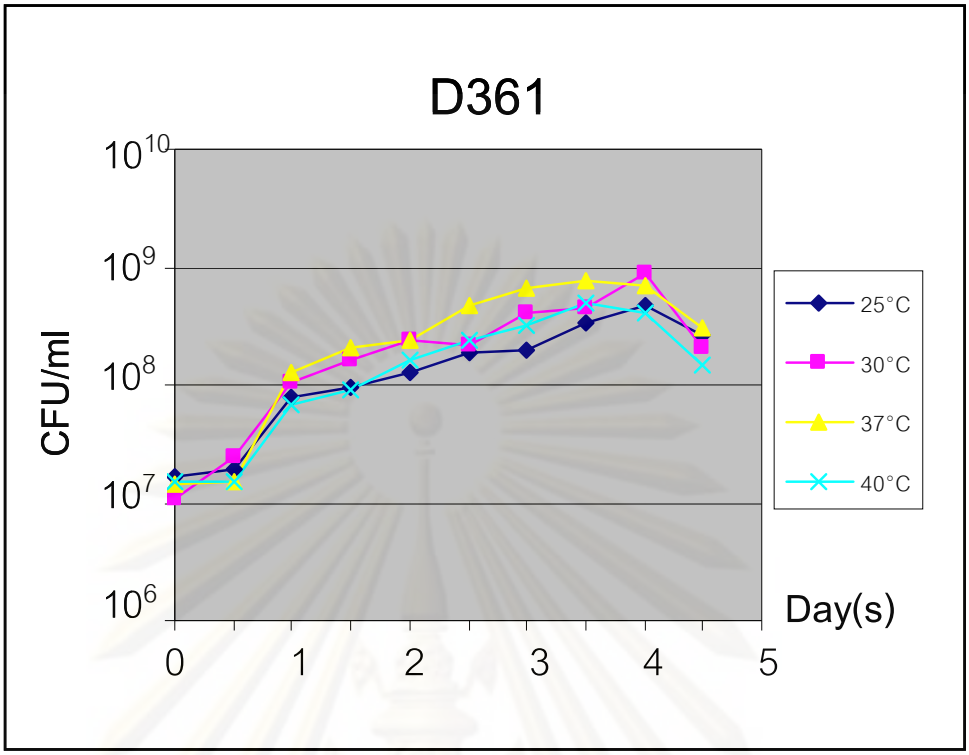


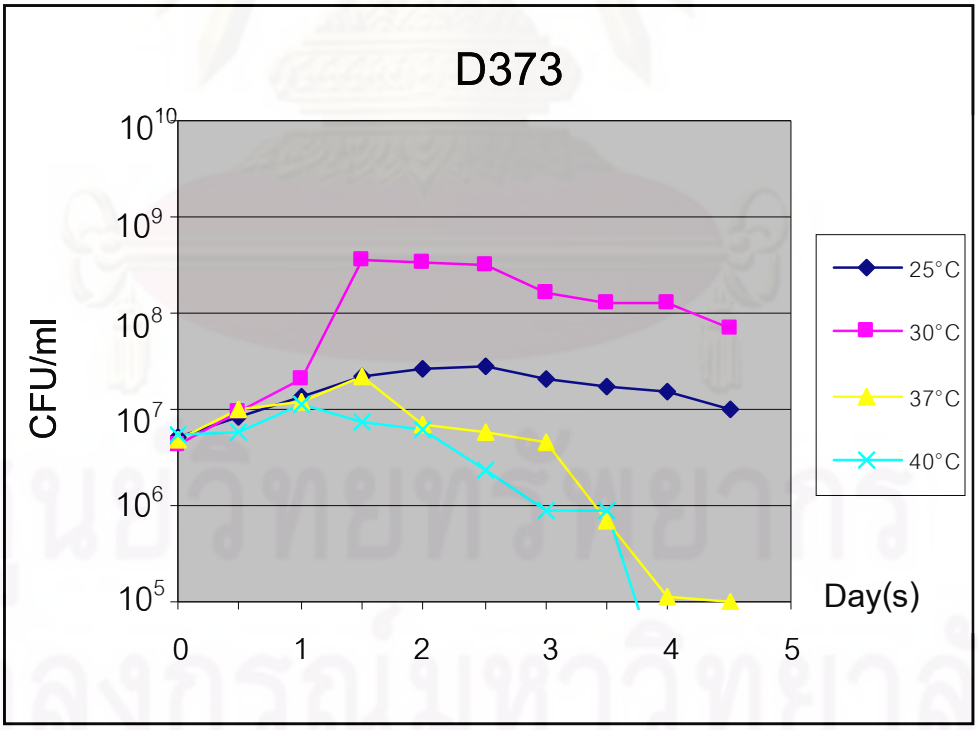
Figure 4.16 Bromthymol blue reactions of the 5 soybean rhizobium strains grown on YMA plus Bromthymol blue plates at 25^0C for 10 days.

4.7.4 Growth at different temperatures

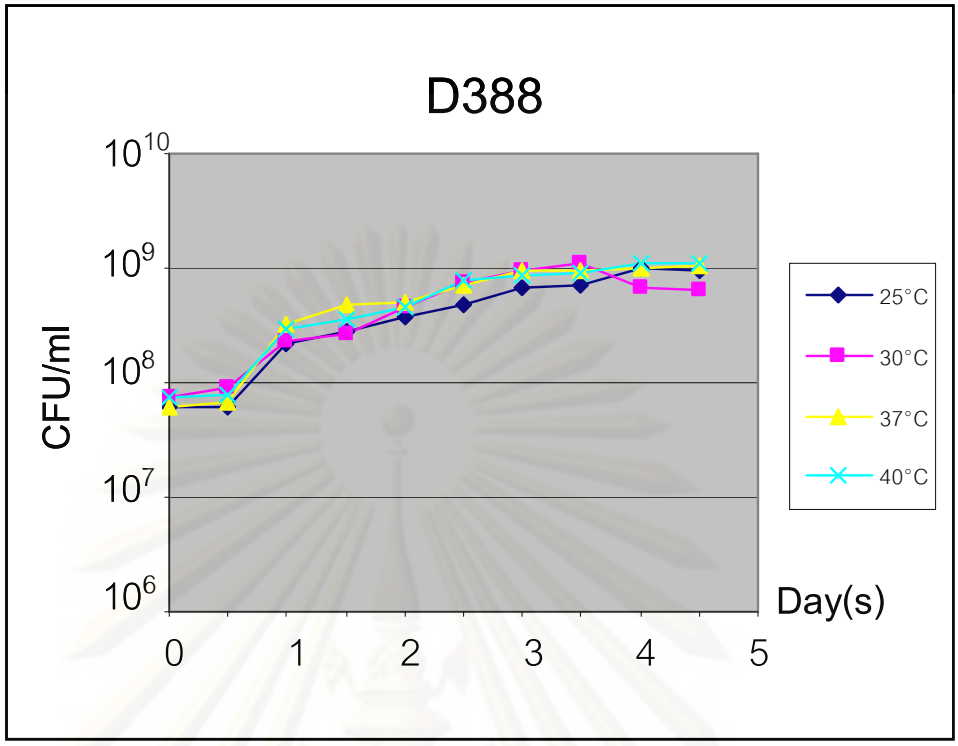
Figure 4.17 showed growth curves of the 5 selected strains at different temperatures. The results as well as the calculated specific growth rates shown in Table 4.2 showed the selected 5 strains had different growth rates at all the incubation temperatures used in the experiments.


D361
CFU/ml
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
0
1
2
3
4
5
Day(s)
25°C
30°C
37°C
40°C

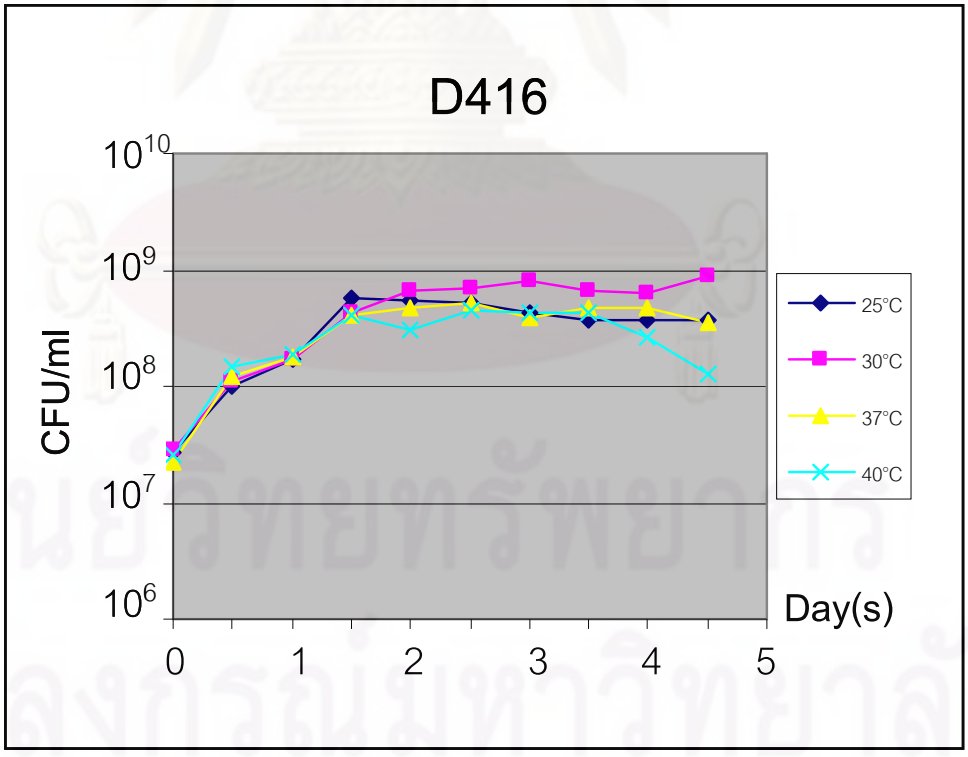
(A)


D373
CFU/ml
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
0
1
2
3
4
5
Day(s)
25°C
30°C
37°C
40°C

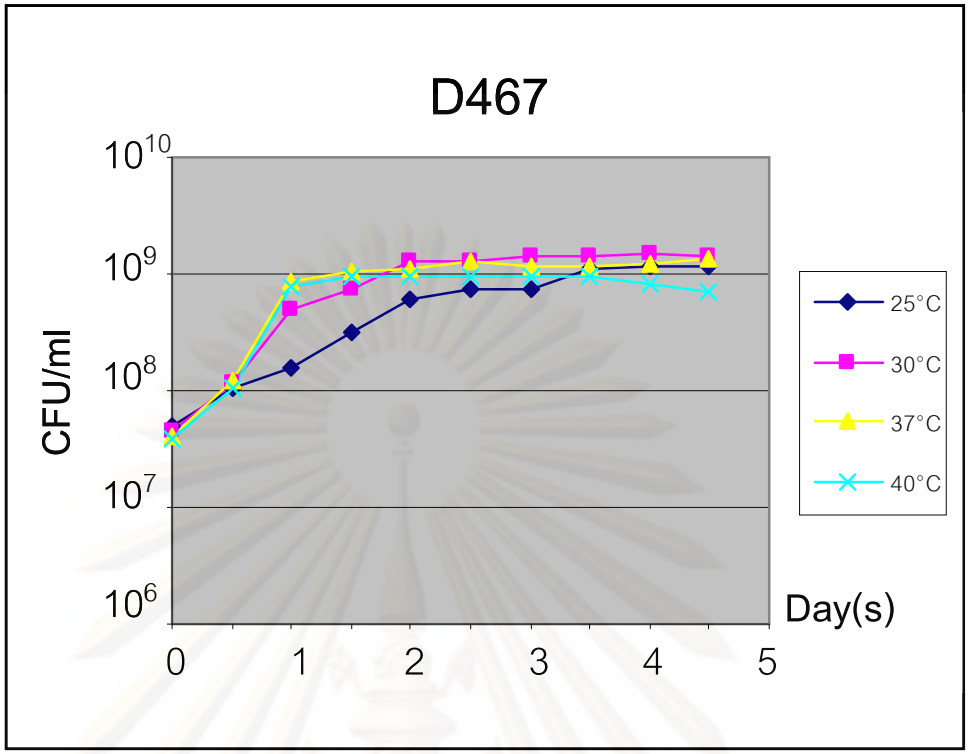
(B)


D388
CFU/ml
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
0
1
2
3
4
5
Day(s)
25°C
30°C
37°C
40°C

(C)


D416
CFU/ml
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
0
1
2
3
4
5
Day(s)
25°C
30°C
37°C
40°C

(D)



(E)

Figure 4.17 Growth of the 5 selected soybean rhizobium strains grown in yeast extract mannitol medium at different temperatures.

Table 4.2 Specific growth rates (μ) of the 5 selected strains of soybean rhizobia at 30 °C

strain	μ (day^{-1})
D361	2.32
D373	2.97
D388	1.82
D416	2.74
D467	2.43

Figure 4.17 showed the slow-growing soybean rhizobium strain D373 was not heat-tolerant. Cell number declined upon incubation at 37°C and 40°C. The other 4 strains grew relatively equally well at all the temperatures tested with strain D467 showing the high specific growth rate at 30°C as shown in Table 4.2.

4.7.5 PCR-DNA fingerprints of the 5 selected slow-growing soybean rhizobium

Figure 4.18 showed PCR-DNA fingerprints of the 5 randomly selected slow-growing soybean rhizobium strains. The DNA fingerprints indicated the 5 selected soybean rhizobium strains had different fingerprints. The results agreed with the growth experiments that the 5 selected strains were different strains.

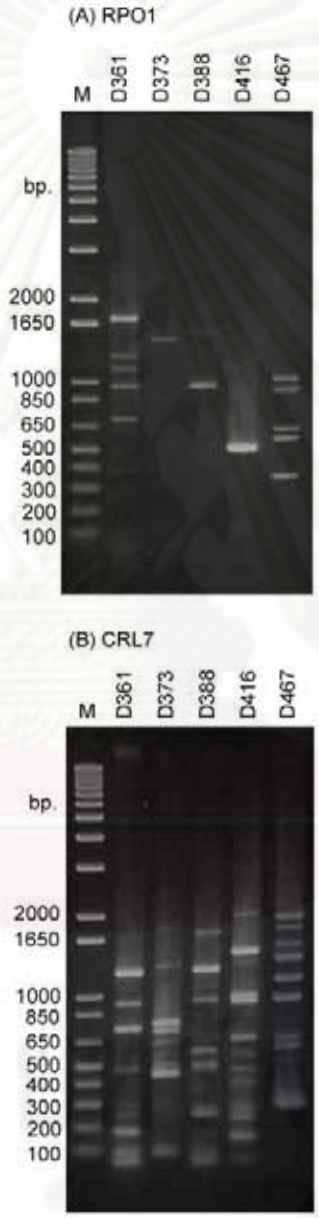


Figure 4.18 DNA fingerprints of the 5 selected soybean rhizobium strains when (A)RPO1 or (B) CRL-7 was used as the primer. Lane (M) was the molecular size standard.

4.7.6 Determination of ability / inability to use carbon and nitrogen sources

Table 4.3 showed the percentage differences in the Biolog tests for an ability/inability to use/not use 95 carbon and nitrogen sources. Raw data were given in Appendix E. The results indicated tentative identification of the selected 5 strains of soybean rhizobia as shown in Table 4.3.

Table 4.3 Tentative identification of the 5 selected strains of soybean rhizobia by using Biolog test results on the utilization/non-utilization of 95 carbon and nitrogen sources.

Strain	*B. elkanii* NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	Tentative identification
D361	33 (34.74%)	22 (23.16%)	22 (23.16%)	*B. japonicum* *B. liaoningense*
D373	27 (28.42%)	20 (21.05%)	23 (24.21%)	*B. japonicum*
D388	26 (27.40%)	24 (25.26%)	21 (22.11%)	*B. liaoningense*
D416	26 (27.40%)	24 (25.26%)	17 (17.89%)	*B. liaoningense*
D467	26 (27.40%)	26 (27.40%)	21 (22.11%)	*B. liaoningense*

4.7.7 Identification by 16S rDNA sequences of the 5 selected soybean rhizobium strains

Figure 4.19 showed 16S rDNA nucleotide sequence of soybean rhizobium strain D361. Comparisons of 16S rDNA sequence of strain D361 (Length=1457 bp) with corresponding sequences deposited at GenBank indicated the strain could be *Bradyrhizobium japonicum* USDA 110 with identities= 1452/1452 (100%), gaps = 0/1452 (0%), or *Bradyrhizobium japonicum* strain USDA 62 with identities = 1452/1452

(99%), gaps = 0/1452(0%). Therefore, D361 could be identified as *Bradyrhizobium japonicum.*

Figure 4.19 16S rDNA sequence of soybean rhizobium strain D361. Nucleotide sequences of sequencing primers were shown in boxes.

Figure 4.20 showed 16S rDNA nucleotide sequence of soybean rhizobium strain D373. Comparisons of 16S rDNA sequence of strain D373 (Length=1456 bp) with corresponding sequences deposited at GenBank indicated the strain could be *Bradyrhizobium japonicum* USDA 110 with identities= 1445/1453 (99%), gaps = 0/1453 (0%), or *Bradyrhizobium japonicum* strain USDA 62 with identities = 1445/1453 (99%), gaps = 0/1453(0%). Therefore, D373 could be identified as *Bradyrhizobium japonicum.*

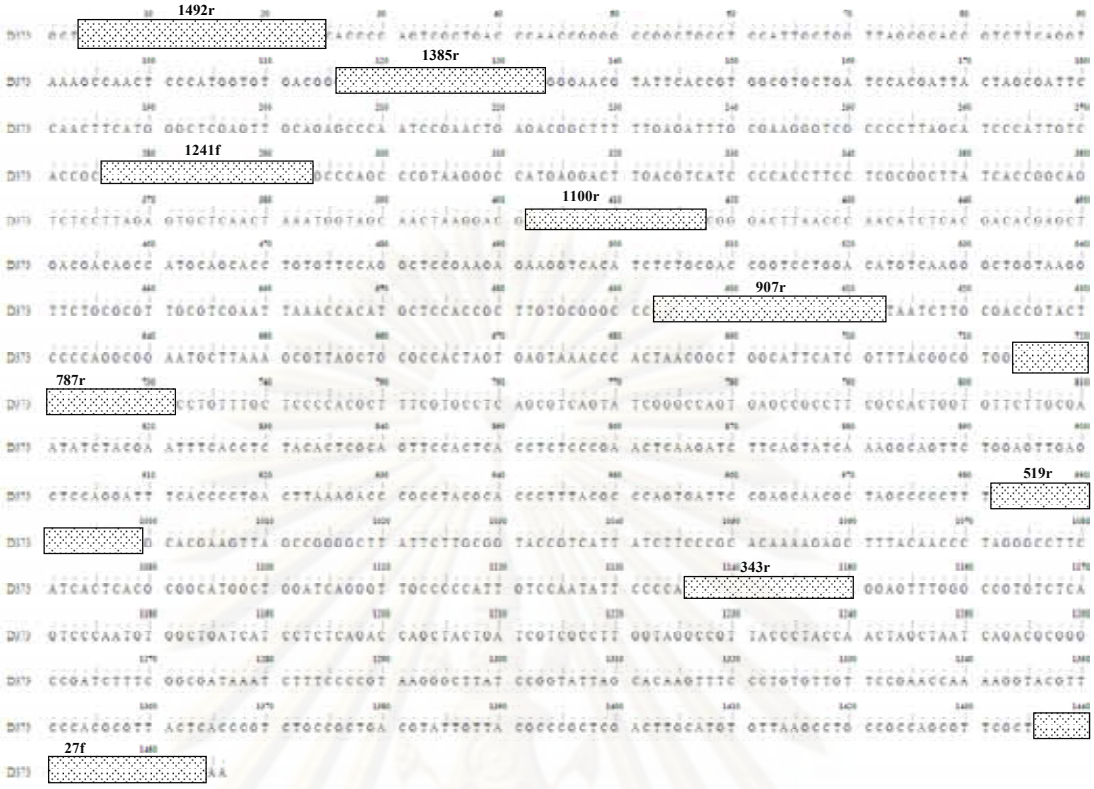


Figure 4.20 16S rDNA sequence of soybean rhizobium strain D373. Nucleotide sequences of sequencing primers were shown in boxes.

Figure 4.21 showed 16S rDNA nucleotide sequence of soybean rhizobium strain D388. Comparisons of 16S rDNA sequence of strain D388 (Length=1456 bp) with corresponding sequences deposited at GenBank indicated the strain could be *Bradyrhizobium japonicum* HMS-02 with identities= 1449/1453 (99%), gaps = 1/1453.

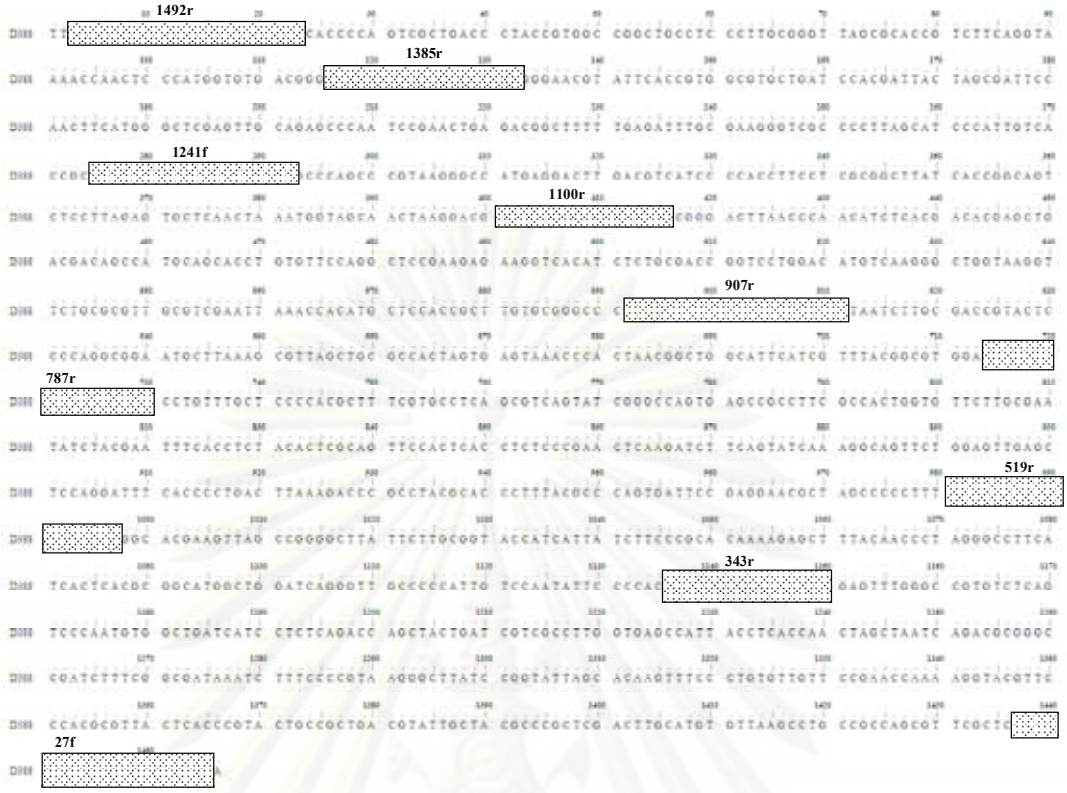


Figure 4.21 16S rDNA sequence of soybean rhizobium strain D388. Nucleotide sequences of sequencing primers were shown in boxes.

Figure 4.22 showed 16S rDNA nucleotide sequence of soybean rhizobium strain D416. Comparisons of 16S rDNA sequence of strain D416 (Length=1458 bp) with corresponding sequences deposited at GenBank indicated the strain could be *Bradyrhizobium liaoningense* LYG2 with identities= 1452/1454 (99%), gaps = 2/1454

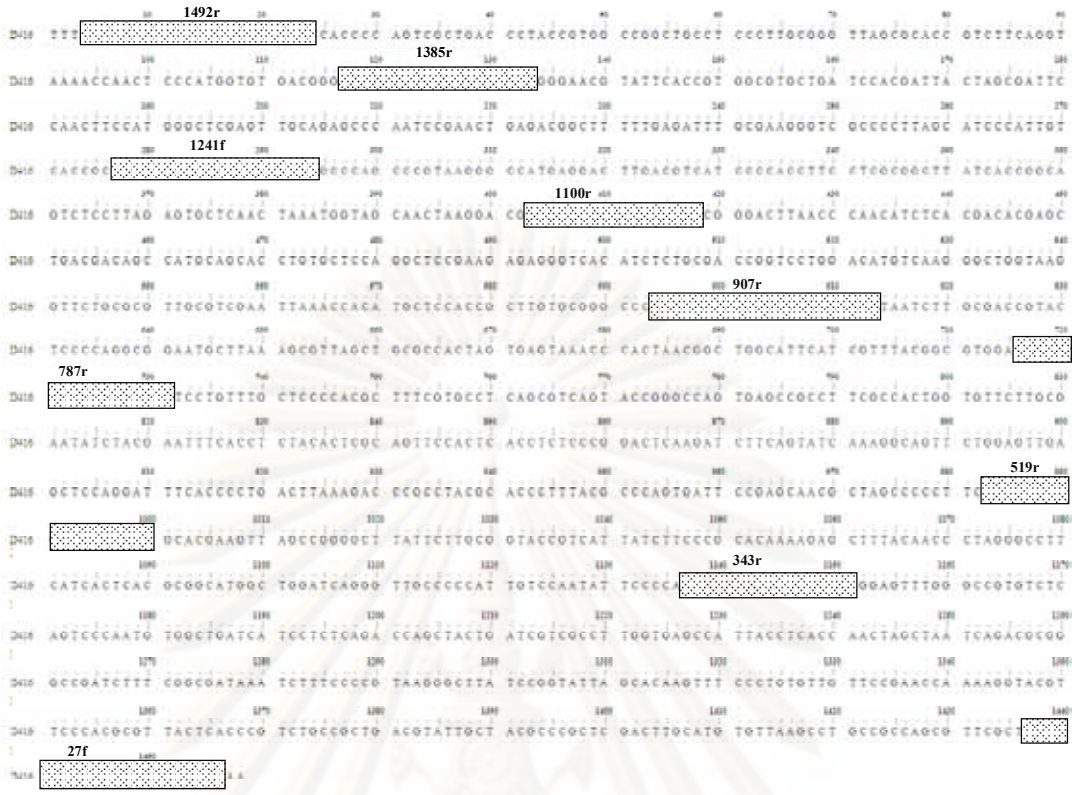


Figure 4.22 16S rDNA sequence of soybean rhizobium strain D416. Nucleotide sequences of sequencing primers were shown in boxes.

Figure 4.23 showed 16S rDNA nucleotide sequence of soybean rhizobium strain D467. Comparisons of 16S rDNA sequence of strain D467 (Length=1456 bp) with corresponding sequences deposited at GenBank indicated the strain could be *Bradyrhizobium liaoningense* LYG2 with identities= 1450/1454 (99%), gaps = 4/1454 .

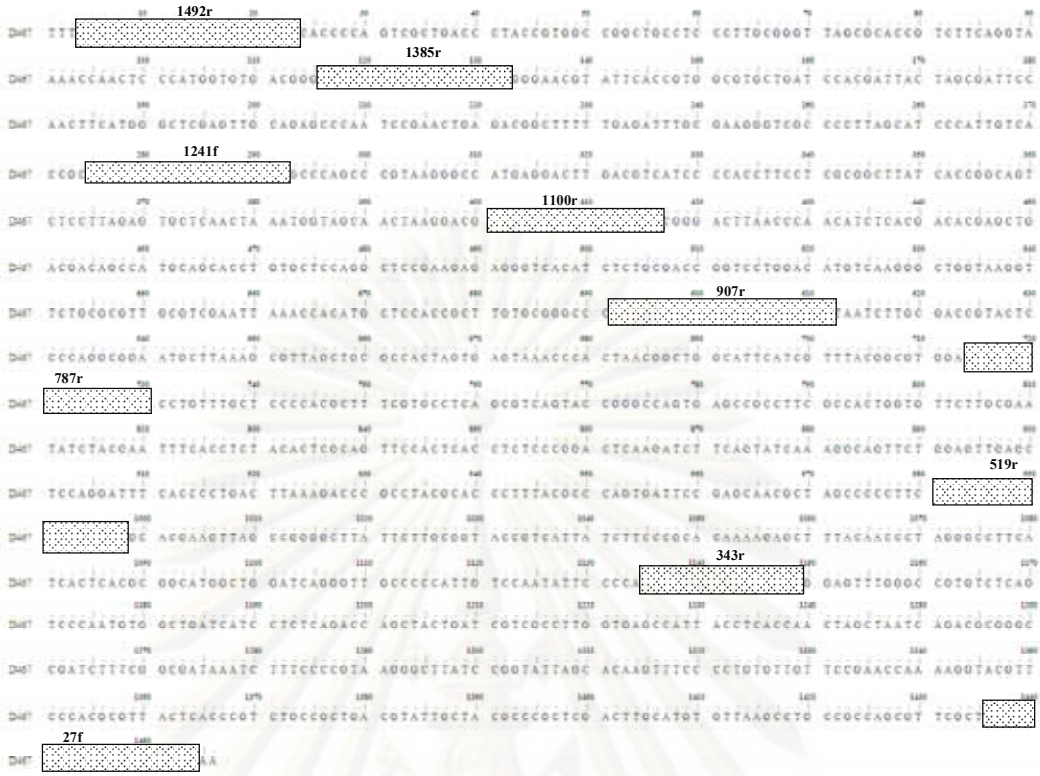


Figure 4.23 16S rDNA sequence of soybean rhizobium strain D467. Nucleotide sequences of sequencing primers were shown in boxes.

Table 4.4 Summary of the identification of the 5 selected soybean rhizobium strains by 16S rDNA sequences.

Strain	length (bp)	bp used in the comparisons with the Blast program	Percent of differences	Identification
D361	1457	1452/1452	0.00	*Bradyrhizobium japonicum* USDA 110
D373	1456	1445/1453	0.55	*Bradyrhizobium japonicum* USDA 110
D388	1456	1449/1453	0.28	*Bradyrhizobium japonicum* strain HMS-02
D416	1458	1452/1454	0.14	*Bradyrhizobium liaoningense* strain LYG2
D467	1456	1450/1454	0.28	*Bradyrhizobium liaoningense* strain LYG2

CHAPTER V

DISCUSSION

5.1 PCR-DNA fingerprinting

In this research , identical PCR-DNA fingerprints were used to initially group bacteria isolated from root nodules into the same strains. Primer RPO1 was chosen for use in the DNA fingerprinting because it annealled to the 20 conserved nucleotide sequence in the promoter of *nifH* of the fast-growing *Rhizobium trifolii* strains Rt 329, Rt RS1 and *R. meliloti* RmP1 (Schofield and Watson,1985). In addition, Richardson et al. (1995) reported that the primer RPO1 could be used in PCR-fingerprinting to differentiate among different strains of fast-growing *Rhizobium* spp. Primer RPO1 was chosen for use in this research because it was expected that the presence of a PCR product due to the extension of a DNA fragment after the annealling of the primer would be a confirmation for the presence of *nifH* which encodes the Fe protein subunit of the enzyme nitrogenase. However, DNA fingerprints of the 23 slow-growing soybean rhizobium strains and the additional 57 bacterial isolates from root nodules of soybeans grown in soils from Bang Rakam district, Phitsanulok province, as shown in Figures 4.1 and 4.9 showed more than one PCR product were obtained for some soybean rhizobium strains and some bacterial isolates. In this research, RPO1-PCR fingerprinting was obtained at least twice. The PCR fingerprints were not always reproducible. Sometimes one PCR product band was obtained, other times more than one PCR product band were obtained for the same strains (results not shown). One reason might be a long extension time at 72^0C of 90 seconds for 5 cycles (annealling temperature 55^0C) and 25 cycles (annealling temperature 60^0C) followed by a final extension of 72^0C at 10 min. It is expected that if the extension time is shortened, only one PCR product band might be obtained for all the test strains. However, identical PCR fingerprints for the same strains were confirmed by the use of another primer, CRL-7, in the fingerprinting. CRL-7 with the sequence 5'GCCCGCCGCC3, is an arbitrarily GC rich primer (Mathis and McMillin, 1996). This primer was chosen for use because it was reasoned that more PCR bands in the fingerprints implied more GC rich regions in the target DNAs which might render

soybean rhizobia more heat-tolerant due to more heat-stable DNA molecules because nucleotide G binds to nucleotide C with three hydrogen bonds as compared with nucleotide A which binds to nucleotide T with two hydrogen bonds. However, Ly and Chansa-ngavej (2006) reported no correlation between the number of CRL-7 PCR products and growth at 40^0C of 34 strains of slow-growing soybean rhizobia isolated from each of 7 soybean cultivars (ST1, ST2, ST3, SJ4, SJ5, Chiangmai 2, and Chiangmai 60) grown in soils from soybean cultivation areas in Nam Moub, Lai Nan, and Tan Choom subdistricts, Wiangsa district, Nan province. Therefore, heat tolerance in slow-growing soybean rhizobia might not be due to the presence of more GC rich regions on DNA molecules but might be due to the increased expression of heat shock protein genes in the genome. Heat shock protein genes in slow-growing soybean rhizobia which include *dnaK, dnaJ, , groESLs*, and several small heat shock proteins including *hspA, hspBC, hspD, and hspEF* have been well-documented (Munchbach et al., 1999a,b).

Apart from the primer RPO1, several other primers have been used to obtain PCR-DNA fingerprints of slow-growing soybean rhizobia. These primers include the consensus REP and ERIC primers with sequences as shown in Figure 5.1

Figure 5.1 Nucleotide sequence of the REP and ERIC primers. (A) REP consensus sequence and nucleotide sequence of the two REP primers (REP1R-I and REP2-I), positioned relative to the REP consensus sequence. The I's denote inosines. (B) ERIC consensus sequence and nucleotide sequence of the two ERIC primers (ERICIR and ERIC2), positioned relative to the ERIC consensus sequence. The arrows denote the direction of *Taq* polymerase extension (de Bruijn, 1992).

De Bruijn (1992) reported that enteric bacteria such as *E. coli* and *Salmonella typhimurium* and several other Gram negative bacteria including *Bradyrhizobium* spp. contained several short intergenic repeated sequences with highly conserved central inverted repeats known as the repetitive extragenic palindromic elements (REPs) and the enterobacterial repetitive intergenic consensus (ERIC) sequences. When all the available REP and ERIC sequences were aligned, the REP and ERIC consensus sequences as shown in Figure 5.1 were obtained. These sequences have been used in PCR-DNA fingerprinting of several soybean rhizobia including the rhizobium strains used in the commercial production of inoculants in Spain (Binde et al. 2009). But for the purpose of grouping soybean rhizobia with identical fingerprints into the same strains which is part of the topic for this thesis, the use of RPO1 or CRL-7 as the primer in the PCR-DNA fingerprinting is satisfactory.

5.2 Optimization of multiplex PCR conditions

The results of the optimization of the multiplex PCR conditions as shown in Figures 4.2-4.4 indicated that it was not possible, despite the various amounts of target DNAs and the primer concentrations, to get both *nodD1*R and *nodY*F primers to anneal to the target DNAs at the same time. If conditions where both primers annealled to the target DNAs at the same time during the multiplex PCR reaction were found, two multiplex PCR products of 317 bp and 340 bp would be obtained and it would not be possible to differentiate the multiplex PCR products into the two patterns 1 and 2. Thus, it was very fortunate to have found optimized conditions which yielded reproducible results of the two multiplex PCR patterns with pattern 1 consisting of 317 bp and 657 bp and pattern 2 consisting of 340 and 657 bp. Previously, Emampaiwong (2006) had reported that the sequence of the 657 bp of strain D345 consisted of sequence of *nodD1* upstream of *nodY.* Hence, the 657 products were obtained by DNA extension from the annealling of *nodD1*F and *nodY*R.

One reason it was not possible to get the annealling of both primers *nodD1*R and *nodY*F at the same time during the multiplex PCR reaction might be because this region of the DNA contained the overlapping promoters of *nodD1* and of *nodYABC* as indicated in Figure 2.2. Therefore, the region might be so tightly packed as to require

extra time, more than 15 seconds at 95^0C , for the denaturation step in the multiplex PCR reaction. In this thesis, the number of base pairs between the translation start sites for *nodD1* and for *nodYABC* was determined from the sequence as reported by Stacey (1995) to be 51.5% which was relatively GC rich and might require longer time in the denaturation step of the mutiplex PCR in order to yield single-stranded DNA for the simultaneous annealling of both primers *nodD1*R and *nodY*F. In any case, the optimized conditions obtained for the multiplex PCR reaction in this thesis are satisfactory because two reproducible multiplex PCR patterns as previously discussed were obtained for the determination of the use of the multiplex PCR patterns to predict root nodulation efficiency.

5.3 Correlation between multiplex PCR patterns and root nodulation efficiency

The results obtained from the experiments as shown in the pictorial presentation of root nodules and the histograms in Figures 4.6, 4.12, 4.15, and 4.16 indicated that it was not possible to use multiplex PCR patterns obtained from the optimized conditions of multiplex PCR (200 ng target DNA with 8, 12.5, 12.5, and 8 pmoles of primers *nodD1*F, *nodD1*R, *nodY*F, and *nodY*R respectively) to predict root nodulation efficiency. There are several reasons contributing to the weakness of obtaining mutiplex PCR and root nodulation results for the determination of a possible correlation. First, the number of 33 soybean rhizobium strains used in the experiments was not sufficient to confidently conclude that strains belonging to multiplex PCR pattern 1 would yield higher average numbers of crown and total nodules. Second. it was impossible to control the number of 10^8 CFU.ml^{-1} for use in the determination of nodulation efficiency. In hindsight, more than 10^8 CFU.ml^{-1}used in the determination in the case of some slow-growing soybean rhizobium strains used in the experiments might have contributed to the low average numbers of crown and total nodules by the quorum sensing mechanism as described by Loh et al. (2002). Furthermore, the multiplex PCR should have been used to amplify a longer stretch of nodulation genes and not to amplify only the two nodulation genes: *nodD1* and *nodY*.

5.4 Identification of 5 slow-growing soybean rhizobium strains by polyphasic taxonomy

16S rDNA sequences yielded satisfactory identification results. The identification of strains D316 and D373 as *Bradyrhizobium japonicum* and strains D416 and D467 as *B. liaoningense* was partially confirmed by the Biolog results of the utilization/non-utilization of the 95 carbon and nitrogen compounds as shown in Tables 4.3 and 4.4. The identification obtained contributes to more information on the species of slow-growing soybean rhizobia in Phitsanulok province.

CHAPTER VI

CONCLUSION

In this thesis, the optimized conditions for a multiplex PCR reaction were obtained for use in the reaction of target DNAs of a total of 33 slow-growing soybean rhizobium strains isolated from Bang Rakam district, Phitsanulok province. The optimized conditions were 200 ng target DNA and concentrations of primer *nodD1*F, *nodD1*R, *nodY*F, and *nodY*R were 8.0, 12.5, 12.5 and 8.0 pmoles respectively. Separation of the multiplex PCR products by agarose gel electrophoresis showed two multiplex PCR patterns. Pattern 1 consisted of 317 bp and 657 bp while pattern 2 consisted of 340 bp and 657 bp. No correlation was found between multiplex PCR patterns and nodulation efficiency in terms of the average numbers of crown and total nodules. Identification of 5 randomly selected slow-growing soybean rhizobia showed strains D361, D373, and D388 were *Bradyrhizobium japonicum* while strains D416 and D467 were *B. liaoningense.*

REFERENCES

Aguilar, D.M., Lopez, M.V. and Riccillo, P.M. 2001. The diversity of rhizobia nodulating beans in Northwest Argentina as a source of more efficient inoculant strains. ***J. Biotechno*** 91: 181-188.

Appunu, C., N' Zoue, A. and Laguerre, G. 2008. Genetic diversity of native bradyrhizobia isolated from soybeans (*Glycine max* L.) in different agricultural-ecological-climatic regions of India. ***Appl. Environ. Microbio*** 74(19): 5991-5996.

Binde, D. R., Menna, P., Bangel, E.V., Barcellos, F.G. and Hungria, M. 2009. rep-PCR fingerprinting and taxonomy based on the sequencing of the 16S rRNA gene of 54 elite commercial rhizobial strains. ***Appl. Microbiol. Biotechnol*** 83: 897-908.

Brutti, L., Rivero, E., Basurco, J. C. P., Nicolas, M., Iriarte, L., Abbiati, N., Ljunggren, H. and Martensson. A. 1998. Persistence of *Bradyrhizobium japonicum* in arable soils of Argentina. ***Appl. Soil Ecology*** 10 : 87-94.

Chantapetch, T. 2009. DNA fingerprinting of soybean rhizobia isolated from nodules of soybeans inoculated with biofertilizer NA7 in Nam Moub subdistrict, Nan Province. MSc thesis. Industrial Microbiology program. Chulalongkorn University.

Chen, L.S., Figueredo, A., Pedrosa, F.O. and Hungria, M. 2002. Genetic characterization of soybean rhizobia in Paraguay. ***Appl. Environ. Microbiol*** 66: 5099-5103.

Chen, W.X., Yan, G.H. and Li, I. L. 1988. Numerical taxonomic study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen.nov. ***Int. J. Syst. Bacteriol*** 38 :392-397.

Cohn, J., Day, R.B., and Stacey, G. 1998. Legume nodule organogenesis. ***Trends in Plant Sci*** 3(3): 105-110.

de Bruijn, F.J. 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. ***Appl. Environ. Microbiol*** 58(7): 2180-2187.

de Jensen, C.E., Kurle, J.E. and Percich, J.A. 2004. Integrated management of edaphic and biotic factors limiting yield of irrigated soybean and dry bean in Minnesota. ***Field Crops Res*** 86: 211-224.

D'Haeze, W., and Holsters, M. 2002. Minireview: Nod factor structures, responses, and perception during initiation of nodule development. ***Glycobiology*** 12(6): 79R-105R.

Dorsch. M. and Stackerbrandt, E. 1992. Some modifications in the procedure of direct sequencing of PCR amplified 16S rDNA. ***J. Microbiol. Meth*** 16 : 271-279.

Dyer, P.S., Furneaux, P.A., Douhen, G., and Murray, T.D. 2001. A multiplex PCR test for determination of mating type applied to the plant pathogens *Tapesia yallundae* ane *Tapesia acuformis*. ***Fungal Genetics Biol*** 33: 173-180.

Elkan, G.H. and Bunn, C.R. 1992. The Rhizobia. In Balows, A., Truper, H.G., Dworkin, M., Harder, W., Schleifer, K-H (eds). ***The Prokaryotes. 2nd Edition. Chapter 107*** New York : Springer Verlag.

Emampaiwong, D. 2006. Use of multiplex PCR to distinguish fast- from slow-growing soybean rhizobia. MSc thesis. Industrial Microbiology program. Chulalongkorn University.

Esau, K. 1977. Anatomy of Seed plants. 2nd Edition. John Wiley & Sons.

Fuhrmann M. and Hennecke H. 1984. *Rhizobium japonicum* nitrogenase Fe protein gene (*nifH*). ***J. Bacteriol*** 158 : 1005-1011.

GÖttfert, M., Hitz, S., and Hennecke, H. 1990. Identification of *nodS* and *nodU*, two inducible genes inserted between *Bradyrhizobium japonicum nodYABC* and *nodIJ* genes. ***Mol. Plant Microbe Interact*** 3(5): 308-316.

Hungria, M., Boddey, L.H., Santos, M.A., and Vargas, M.A.T. 1998. Nitrogen fixation capacity and nodule occupancy by *Bradyrhizobium japonicum* and *B. elkanii* strains. ***Biol. Fertil. Soils*** 27: 393-399.

Hungria, M., Campo, R.J., Chueire, L.M.O., Grange, L. and Megias, M. 2001. Symbiotic effectiveness of fast-growing rhizobial strains isolated from soybean nodules in Brazil. ***Biol. Fertil. Soils*** 33: 387-394.

Jordan, D.C. 1982. Transfer of *Rhizoium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a genus of slow-growing, root nodule bacteria from leguminous plants. ***Int. J. Syst. Bacteriol*** 32: 136-139.

Kosslak, R. M., Bookland, R., Barkei, J., Paaren, H. E. and Applebaum, E. R. 1987. Induction of *Bradyrhizobium japonicum* common *nod* genes by isoflavones isolated from *Glycine max*. ***Proc. Natl. Acad. Sci. USA*** 84: 7428-7432.

Kuykendall, L.D., Saxena, B., Devine, T.E. and Udell, S.E. 1992. Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp. nov. ***Can. J. Microbiol*** 38 : 501-505.

Loh, J., Yuen-Tsai, J.P.Y., Stacey, M.G., Lohar, D., Welborn, A. and Stacey, G. 2001. Population density-dependent regulation of the *Bradyrhizobium japonicum* nodulation genes. ***Mol. Microbiol*** 42(1) : 37-66.

Loh, J., Carlson, R.W., York, W.S. and Stacey, G. 2002a. Bradyoxetin, a unique chemical signal involved in symbiotic gene regulation. ***Proc. Natl. Acad. Sci. USA*** 99(22): 14446-14451.

Loh, J., Pierson, E.A., Pierson, L.S., Stacey, G. and Chatterjee, A. 2002b. Quorum sensing in plant-associated bacteria. ***Curr. Opin. Plant Biol*** 5 : 285-290.

Loh, J. and Stacey, G. 2003. Nodulation gene regulation in *Bradyrhizobium japonicum* : a unique integration of global regulatory circuits. ***Appl. Environ. Microbiol*** 69(1) : 10-17.

Ly K. P. and Chansa-ngavej, K. 2006a. Correlation between RAPD-PCR fingerprints, heat responses, and nitrogen fixing potential of soybean rhizobia. ***Ann. Rep. ICBiotech*** 28 : 931-941.

Ly K. P. and Chansa-ngavej, K. 2006b. Isolation and RAPD-PCR fingerprints of soybean rhizobia from 3 soybean cultivation subdistricts in Wiangsa district, Nan Province, Thailand. ***Ann. Rep. ICBiotech*** 28 : 1093-1096.

Machado, D., Pueppke, S.G., Vinardel, G.S., Ruiz-Sainz, J.E., and Krishnan, H.B. 1998. Expression of *nodD1* and *nodD2* in *Sinorhizobium fredii*, a nitrogen-fixing symbiont of soybean and other legumes. ***Mol. Plant Microbe Interact*** 11: 375-382.

Manzan, M.A., Aljinovic, E.M., Biedma,M.E., Sciocco-Cap, A., Ghiringhelli, P.D., and Romanowski, V. 2008. Multiplex PCR and quality control of *Epinotia aporema* granulovirus production. ***Virus Genes*** 37: 203-211.

Mathis, J.N. and McMillin, D.E. 1996. Detection of genetic variation in *Bradyrhizobium japonicum* USDA 110 variants using DNA fingerprints generated with GC rich arbitrary PCR primers. ***Plant and Soil*** 186 : 81-85.

Munchbach, M., Dainese, P., Staudenmann, W., Narberhaus, F., Janes, P. 1999a. Proteome analysis of heat shock protein expression in *Bradyrhizobium japonicum*. ***Eur. J. Biochem*** 263 : 39-48.

Munchbach, M., Nocker, A., Narberhaus, F. 1999b. Mutiple small heat shock proteins in rhizobia. ***J. Bacteriol*** 181(1): 83-90.

Nuntagij, A., Abe, M., Uchiumi, T., Seki, Y., Boonkerd, N. and Higashi, S. 1997. Characterization of 187 *Bradyrhizobium* strains isolated from soybean cultivation in Thailand. ***J. Gen. Appl. Microbiol*** 43: 183-187.

Peng, G.X., Tan, Z.Y., Wang, E.T., Reinhold-Hurek, B., Chan, W.F. and Chen, W.X. 2002. Identification of isolates from soybean nodules in Xinjiang region as *Sinorhizobium xinjiangense* and genetic differentiation of *S. xinjiangense* from *Sinorhizobium fredii*. ***Int. J. Syst. Evol. Microbiol*** 52 : 457-462.

Pulawska, J., Willems, A., and Sobiczewski, P. 2006. Rapid and specific identification of four *Agrobacterium* species and biovars using multiplex PCR. ***Syst. Appl. Microbiol*** 29: 470-479.

Richardson, A.E., Viccars, L.A., Watson, J.M. and Gibson, A.H. 1995. Differentiation of *Rhizobium* strains using the polymerase chain reaction. ***Soil Biol.Biochem*** 27: 515 – 524.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular Cloning : A Laboratory Manual, Book 1. ***Cold Spring Harbor Laboratory Press***. New York. p. A3

Schofield, P.R. and Watson, J.M. 1985. Conservation of *nif* - and species-specific domains within repeated promoter sequences from fast-growing *Rhizobium* species. ***Nucleic Acid Res*** 13(10): 3407-3418

Shutsrirung, A., Pengnoo, A., Bhromsiri,A., Senoo, K., Tajima, S. and Hisamatsu, M. 2002a. Symbiotic efficiency and compatibility of native rhizobia in northern Thailand with different soybean cultivars. III. Laboratory experiment using native

isolates from upland rainfed soybean-growing areas. ***Soil Sci. Plant Nutri*** 48(4): 511-520.

Shutsrirung, A., Sutigoolabud, P., Santasup, C., Senoo, K., Tajima, S., Hisamatsu. M. and Bromsiri, A. 2002b. Symbiotic efficiency and compatibility of native rhizobia in northern Thailand with different soybean cultivars. I. Field experiment in irrigated traditional soybean-growing areas. ***Soil Sci. Plant Nutri*** 48(4): 491-499.

Shutsrirung, A., Thamapibool, S., Bromsiri, A., Senoo, K., Tajima, S. and Hisamatsu. M. 2002c. Symbiotic efficiency and compatibility of native rhizobia in northern Thailand with different soybean cultivars. II. Laboratory experiment using native isolates from irrigated soybean-growing areas. ***Soil Sci. Plant Nutri*** 48(4): 501-510.

Somasegaran, P. and Hoben, H.J. 1994. Handbook for Rhizobia: Methods in Legume-*Rhizobium* Technology. New York. Springer Verlag pp. 340, 370-1.

Spriggs, A.C. and Dakora, F.D. 2007. Competitive ability of selected *Cyclopia* Vent. rhizobia under glasshouse and field conditions. ***Soil Biol. & Biochem*** 39: 58-67.

Stacey, G. 1995. *Bradyrhizobium japonicum* nodulation genetics. ***FEMS Microbiol. Lett.*** 127: 1-9.

Teaumroong, N. and Boonkerd, N. 1998. Detection of *Bradyrhizobium* spp. and *B. japonicum* in Thailand by primer-based technology and direct DNA extraction. ***Plant and Soil*** 204: 127-134.

Thomas-Oates, J. et al. (36 co-authors). 2003. A catalogue of molecular, physiological and symbiotic properties of soybean-nodulating rhizobial strains from different soybean cropping areas of China. ***Syst. Appl. Microbiol*** 26: 453-465.

Thompson, J.A., Bhromsiri, A., Shutsrirung, A. and Lillakan, S. 1991. Native root-nodule bacteria of traditional soybean growing areas of northern Thailand. ***Plant and Soil*** 135: 53-65.

Vandamme, P., Pot, B., Gillis, M., De Vos, P., Kersters, K., and Swings, J. 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. ***Microbiol. Rev*** 60(2): 407-438.

van Rhijn, P. and Vanderleyden, J. 1995. The *Rhizobium* - plant symbiosis. ***Microbiol. Rev*** 59(1): 124-142.

Vlassak, K.M. and Vanderleyden, J. 1997. Factors influencing nodule occupancy by inoculant rhizobia. ***Crit. Rev. Plant Sci*** 16: 163-229.

Wang, S.-P. and Stacey, G. 1991. Studies of the *Bradyrhizobium japonicum nodD1* gene promoter: A repeated structure for the *nod* box. ***J. Bacteriol*** 173: 3356-3365.

Xu, L.M., Ge, C., Cui, Z. Li, J. and Fan, H. 1995. *Bradyrhizobium liaoningense* sp. nov. isolated from the root nodules of soybeans. ***Int. J. Syst. Bacteriol*** 45 (4): 706-711.

Yokoyama, T., Ando, S., Murakami, T. and Imai, H. 1996. Genetic variability of the common *nod* gene in soybean bradyrhizobia isolated in Thailand and Japan. ***Can. J. Microbiol*** 42: 1209-1218.

APPENDICES

APPENDIX A

BACTERIAL GROWTH MEDIA AND PLANT NUTRIENT SOLUTIONS

Preparation of all bacterial growth media and plant nutrient solutions are as described by Somasegaran and Hoben (1994) unless otherwise stated.

Yeast Extract Mannitol Broth (YMB)

Mannitol	10.0 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g
Yeast extract	0.5 g
Deionized water	1.0 liter

pH of medium was adjusted to 6.8 with 0.1 N NaOH. The medium was autoclaved at 121°C for 15 min.

Yeast Extract Mannitol Agar (YMA)

YMB	1 liter
Agar	15 g

Agar was added to 1 liter of YMB. The solution was shaken to suspend the agar then autoclaved at 121°C for 15 min. After autoclaving, the medium was shaken to ensure even mixing of melted agar with medium before pouring onto petri dishes and left to solidify.

YMA with Congo Red

Congo Red stock solution: 250 mg of Congo Red dissolved in 100 ml of deionized water. 10 ml of Congo Red stock solution were added to 1 liter of YMA. The final Congo Red concentration was 25 $\mu g.ml^{-1}$. The medium was autoclaved at 121°C for 15 min.

YMA with Bromthymol Blue (BTB YMA)

Bromthymol Blue stock solution: 0.5 g of Bromthymol Blue were dissolved in 100 ml of ethanol. 5 ml of Bromthymol Blue stock solution were added to 1 liter of YMA. The final Bromthymol Blue concentration was 25 $\mu g.ml^{-1}$. The medium was autoclaved at 121°C for 15 min.

Tryptone Yeast Agar (TY)

Tryptone	5.0 g.
Yeast extract	3.0 g.
$CaCl_2.H_2O$	0.87 g.
Deionized water	1.0 liter

pH of medium was adjusted to 6.8 with 0.1 N NaOH. Agar was added to 1 liter of TY. The solution was shaken to suspend the agar then autoclaved at 121°C for 15 min. After autoclaving, the medium was shaken to ensure even mixing of melted agar with medium before pouring onto petri dishes and left to solidify.

N-free Nutrient Solutions

Stock Solutions	Chemicals	g/liter
1	$CaCl_2.2H_2O$	294.1
2	KH_2PO_4	136.1
3	$FeC_6H_5O_7.3H_20$	6.7
	$MgSO_4.7H_2O$	123.3
	K_2SO_4	87.0
	$MnSO_4.H_2O$	0.338
4	H_3BO_3	0.247
	$ZnSO_4.7H_2O$	0.288
	$CuSO_4.5H_2O$	0.100
	$CoSO_4.7H_2O$	0.056
	$Na_2MoO_2.7H_2O$	0.048

Warm water was used to prepare stock solutions to get the ferric-citrate into solution. Ten liters of full-strength plant culture solution were prepared as follows:

- To 5 liters of water, add 5 ml of each stock solution and mix,
- Adjust pH to 6.8 with 1 N HCl
- Dilute to 10 liters by adding water
- For nutrient solution, 0.05% KNO_3 was added to give final N concentration of 70 ppm.

APPENDIX B

CHEMICALS AND SOLUTIONS

1. Solutions for DNA extraction

Saline-EDTA solution

15 mM NaCl, 10 mM EDTA, pH 8.0

0.9 g NaCl, 0.29 g EDTA

were added to distilled water. The final volume was made to 100 ml. 0.1 N NaOH was used to adjust pH to 8.0 before autoclaving at 121°C for 15 min.

DNAzol

DNAzol solution (Molecular Research Lab, MRL) was used according to the manufacturer's instruction.

2. Electrophoresis Buffer

50X Tris Acetate Buffer (TAE buffer)

Tris base	242	g.
glacial acetic acid	57.1	ml
0.5 M EDTA pH 8.0	100	ml

were added to double distilled water. 6 N HCl was used to adjust pH to 8.0. The final volume was added to 1000 ml.

APPENDIX C

DETERMINATION OF NODULATION

strain	X bar ± SD No. of nodules per plant		Plant dry wt per plant	nodulation pattern
	crown	total		
D286	3.1667 [a]	21.6667 [ab]	0.6833 [a-d]	1
D291	2.6250 [ab]	18.6250 [b-d]	0.6537 [a-d]	1
D293	1.4286 [a-d]	13.1429 [b-g]	0.6363 [a-d]	2
D306	2.0000 [a-d]	12.0000 [c-g]	0.7013 [a-d]	2
D314	1.6250 [a-d]	15.3750 [b-f]	0.8425 [ab]	2
D316	1.2857 [a-d]	20.1429 [bc]	0.6725 [a-d]	1
D337	1.3750 [a-d]	19.1250 [bc]	0.7537 [a-d]	2
D345	2.2857 [a-c]	18.5714 [b-d]	0.6175 [a-d]	1
D361	1.5000 [a-d]	20.8333 [b]	0.6283 [a-d]	1
D366	1.8571 [a-d]	13.7143 [b-g]	0.4513 [d]	2
D373	1.5000 [a-d]	15.5000 [b-f]	0.5475 [b-d]	1
D388	2.7143 [ab]	15.5714 [b-f]	0.6213 [a-d]	2
D404	1.4000 [a-d]	11.6000 [c-g]	0.5000 [cd]	2
D412	1.0000 [bd]	15.2500 [b-f]	0.6800 [a-d]	2
D416	1.3333 [a-d]	17.1667 [b-e]	0.7775 [a-c]	2
D459	0.0000 [d]	6.1250 [g]	0.5850 [a-d]	2
D464	0.8000 [bd]	12.0000 [c-g]	0.5033 [cd]	2
D467	0.3750 [cd]	10.1250 [d-g]	0.5450 [b-d]	2
D481	2.1667 [a-c]	28.5000 [a]	0.6500 [a-d]	1
D490	2.1250 [a-c]	14.5000 [b-g]	0.8900 [a]	2
D494	1.6667 [a-d]	13.1667 [b-g]	0.7683 [a-c]	2
D509	0.3750 [cd]	8.1250 [fg]	0.4425 [d]	2
D521	0.8750 [b-d]	8.6250 [e-g]	0.6387 [a-d]	2

strain	X bar ± SD No. of nodules per plant		Plant dry wt per plant	nodulation pattern
	crown	total		
D281	2.8750 [a-d]	17.2500 [bc]	0.8628 [a]	2

D292	3.5000 [a-c]	24.8750 [ab]	0.6750 [a]	1
D307	2.4286 [b-d]	24.4286 [ab]	0.9212 [a]	2
D323	3.0000 [a-c]	12.7500 [cd]	0.9400 [a]	2
D342	4.7500 [a]	27.7500 [a]	0.8860 [a]	1
D346	0.8750 [d]	8.1250 [d]	0.7650 [a]	2
D368	3.8750 [ab]	17.5000 [bc]	0.8150 [a]	2
D480	1.7143 [cd]	7.0000 [d]	0.6500 [a]	2
D482	1.4286 [cd]	9.0000 [d]	0.6560 [a]	2
D484	2.7500 [a-d]	24.5000 [ab]	0.6200 [a]	2

Table of raw data and average total nodules number per plant

of 23 soybean rhizobia strains

strain	pattern	1st	2nd	3rd	4th	5th	6th	7th	8th	average total nodules (per plant)
D481	1	25	20	11	44	22	49			28.50
D286	1	23	28	17	29	15	18			21.67
D361	1	8	26	21	27	13	30			20.83
D316	1	13	11	7	22	24	37	27		20.14
D291	1	9	21	25	26	16	8	18	26	18.63
D345	1	18	11	16	20	13	21	31		18.57
D373	1	6	12	11	17	15	19	28	16	15.50
D337	2	16	7	19	18	23	24	19	27	19.13
D416	2	12	15	13	20	19	24			17.17
D388	2	3	11	19	19	15	16	26		15.57
D314	2	15	18	12	10	18	11	23	16	15.38
D412	2	12	9	26	14					15.25
D490	2	22	15	14	15	16	13	6	15	14.50
D366	2	8	12	28	19	16	8	5		13.71
D494	2	11	11	17	13	15	12			13.17
D293	2	18	12	13	10	18	10	11		13.14
D306	2	16	17	9	10	6	13	21	4	12.00
D464	2	7	8	11	9	25				12.00

strain	pattern	1st	2nd	3rd	4th	5th	6th	7th	8th	average total nodules (per plant)
D404	2	22	6	10	12	8				11.60
D467	2	15	19	13	4	10	3	1	16	10.13
D521	2	8	9	6	8	7	10	15	6	8.63
D509	2	9	3	12	7	3	11	8	12	8.13
D459	2	6	6	3	6	6	7	4	11	6.13

Table of raw data and average crown nodules number per plant
of 23 soybean rhizobia strains

strain	pattern	1st	2nd	3rd	4th	5th	6th	7th	8th	average crown nodules (per plant)
D286	1	2	6	6	3	0	2			3.17
D291	1	1	6	4	1	3	3	2	1	2.63
D345	1	3	3	3	0	2	2	3		2.29
D481	1	3	0	4	1	1	4			2.17
D361	1	0	0	0	5	1	3			1.50
D373	1	0	1	1	1	2	3	2	2	1.50
D316	1	0	0	5	0	4	0	0		1.29
D388	2	4	4	0	0	5	2	4		2.71
D490	2	5	0	3	1	0	0	3	5	2.13
D306	2	4	0	0	4	1	2	5	0	2.00
D366	2	2	2	2	3	3	0	1		1.86
D494	2	1	1	4	0	4	0			1.67
D314	2	3	0	3	3	0	3	0	1	1.63
D293	2	0	0	0	2	3	2	3		1.43
D404	2	0	1	3	2	1				1.40
D337	2	0	3	1	3	1	2	1	0	1.38
D416	2	2	2	4	0	0	0			1.33
D412	2	0	2	0	2					1.00
D521	2	2	2	2	1	0	0	0	0	0.88
D464	2	1	2	0	1	0				0.8

strain	pattern	1st	2nd	3rd	4th	5th	6th	7th	8th	average crown nodules (per plant)
D467	2	0	0	0	0	0	1	1	1	0.38
D509	2	0	0	3	0	0	0	0	0	0.38
D459	2	0	0	0	0	0	0	0	0	0.00

Table raw data and average total nodules number per plant of 10 bacterial isolates from root nodules

strain	pattern	1st	2nd	3rd	4th	5th	6th	7th	8th	average total nodules (per plant)
D342	1	26	20	16	23	41	40	25	31	27.75
D292	1	26	23	25	17	20	22	36	30	24.88
D484	2	9	19	14	32	36	39	23	24	24.50
D307	2	9	30	13	34	14	26	45	0	24.43
D368	2	9	11	12	16	32	23	21	16	17.50
D281	2	17	13	15	23	22	28	10	10	17.25
D323	2	18	11	14	18	12	13	8	8	12.75
D482	2	8	9	14	8	6	7	11	0	9.00
D346	2	8	14	14	10	4	5	7	3	8.13
D480	2	14	9	14	1	5	4	2	0	7.00

Table of raw data and average crown nodules number of per plant of 10 bacterial isolates from root nodules

strain	pattern	1st	2nd	3rd	4th	5th	6th	7th	8th	average crown nodules (per plant)
D342	1	2	5	5	3	4	6	6	7	4.75
D292	1	0	2	3	3	3	9	3	5	3.50
D368	2	3	4	5	1	5	5	5	3	3.88
D323	2	3	6	4	1	4	1	2	3	3.00
D281	2	0	4	1	3	3	5	4	3	2.88

strain	pattern	1st	2nd	3rd	4th	5th	6th	7th	8th	average crown nodules (per plant)
D484	2	0	4	1	4	4	3	1	5	2.75
D307	2	4	1	0	0	1	6	5		2.43
D480	2	3	5	4	0	0	0	0		1.71
D482	2	0	0	3	2	1	2	2		1.43
D346	2	1	1	1	1	1	0	1	1	0.88

Table of raw data and average plant dry weight per plant

of 23 soybean rhizobia strains

strain	pattern	1st	2nd	3rd	4th	average plaht dry weight (per plant)
D286	1	0.83	0.86	0.36		0.68
D316	1	0.69	0.59	0.6	0.81	0.67
D291	1	0.665	0.65	0.69	0.61	0.65
D481	1	0.475	0.855	0.62		0.65
D361	1	0.425	0.64	0.82		0.63
D345	1	0.715	0.45	0.7	0.605	0.62
D373	1	0.515	0.525	0.69	0.46	0.55
D490	2	0.805	0.985	0.955	0.815	0.89
D314	2	0.755	1.02	0.915	0.68	0.84
D416	2	0.86	0.62	0.76	0.87	0.78
D494	2	0.79	0.555	0.96		0.77
D337	2	0.565	0.67	0.96	0.82	0.75
D306	2	0.95	0.465	0.615	0.775	0.70
D412	2	0.635	0.725			0.68
D521	2	0.605	0.755	0.6	0.595	0.64
D293	2	0.72	0.615	0.51	0.7	0.64
D388	2	0.95	0.11	0.625	0.8	0.62
D459	2	0.665	0.46	0.6	0.615	0.59
D467	2	0.68	0.63	0.24	0.63	0.55
D464	2	0.585	0.595	0.33		0.50

strain	pattern	1st	2nd	3rd	4th	average plaht dry weight (per plant)
D404	2	0.65	0.47	0.38		0.50
D366	2	0.525	0.26	0.635	0.385	0.45
D509	2	0.375	0.475	0.18	0.74	0.44

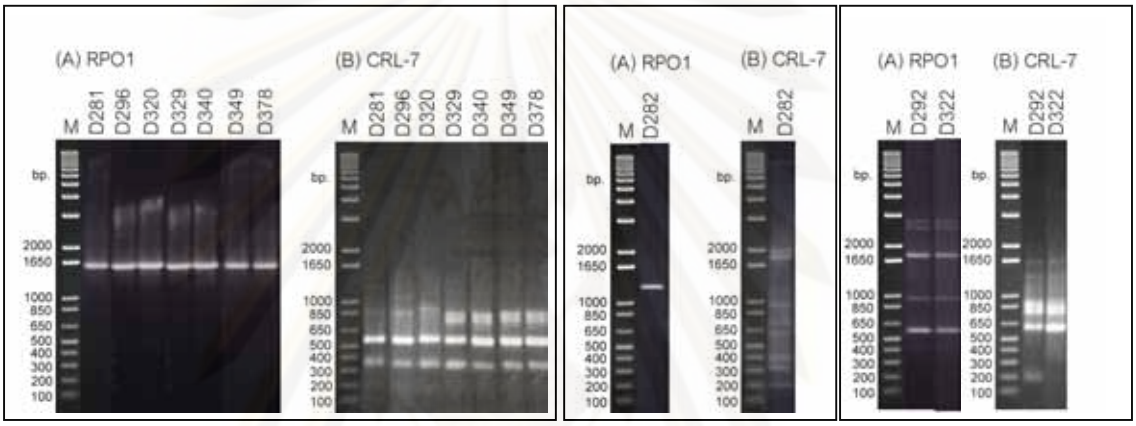
Table raw data and average plant dry weight per plant of 10 bacterial isolates from root nodules

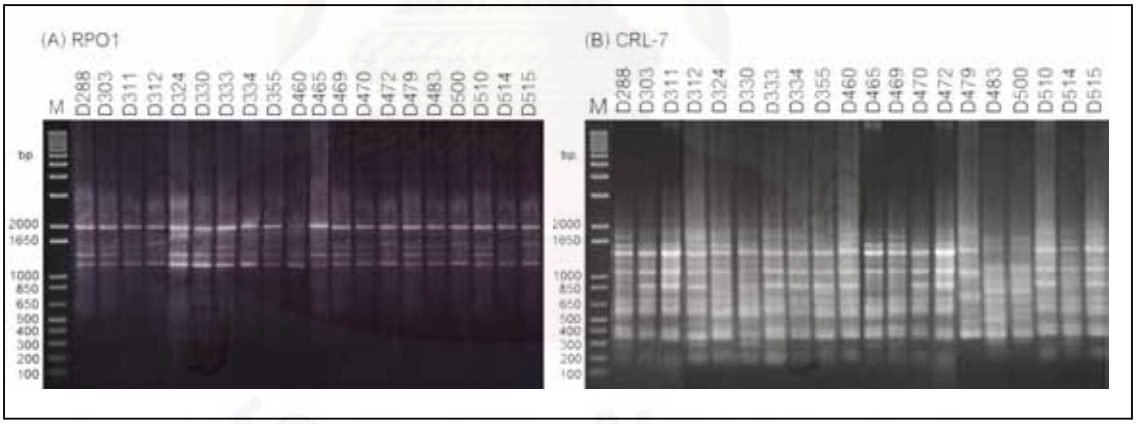
strain	pattern	1st	2nd	3rd	4th	average plant dry weight (per plant)
D342	1	0.69	0.714	1.35	0.79	0.89
D292	1	0.5	0.86	0.47	0.87	0.68
D323	2	0.96	0.56	0.91	1.33	0.94
D307	2	0.76	0.725	0.63	1.57	0.92
D281	2	0.87	0.641	0.83	1.11	0.86
D368	2	0.61	0.76	0.94	0.95	0.82
D346	2	0.74	0.76	0.79	0.77	0.77
D482	2	0.76	0.524	0.68	0.66	0.66
D480	2	0.54	0.65	0.79	0.62	0.65
D484	2	0.54	0.66	0.59	0.69	0.62

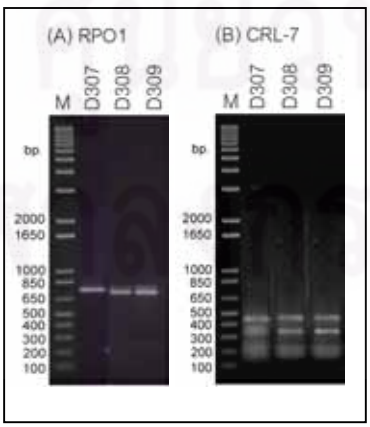
APPENDIX D

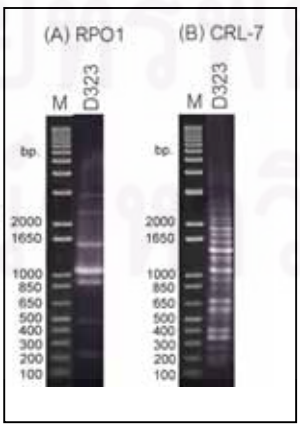
PCR - DNA FINGERPRINTS OF ISOLATES WITH IDENTICAL FINGERPRINTS

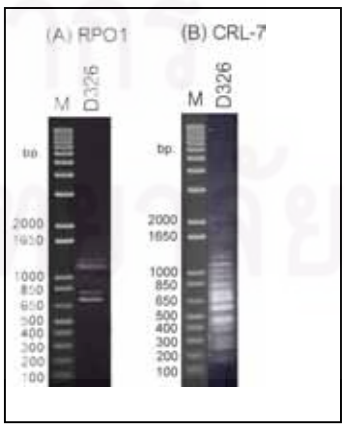
57 soybean root nodule bacterial isolates from Bang Rakam district, Phitsanulok Province were group into 20 strains.

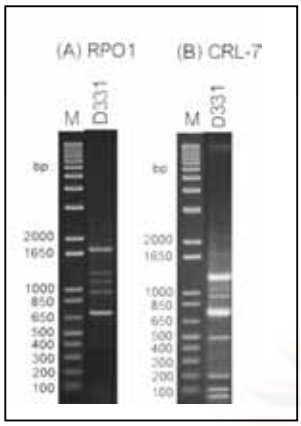


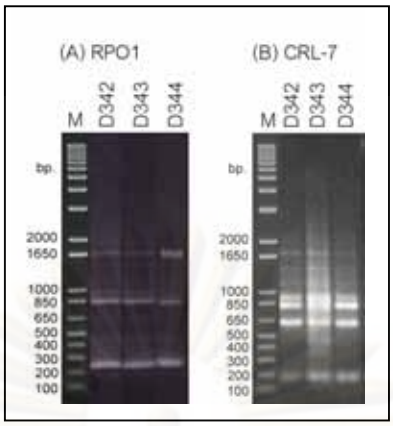









(A) RPO1
(B) CRL-7
M
D331
bp.
2000
1650
1000
850
650
500
400
300
200
100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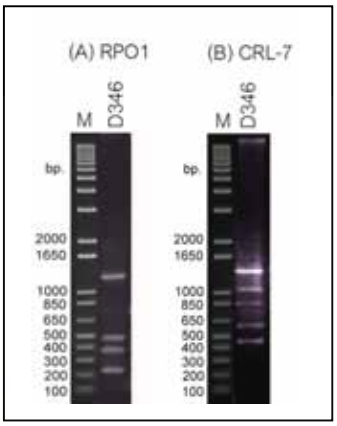
(B) CRL-7
M
D342
D343
D344
bp.
2000
1650
1000
850
650
500
400
300
200
100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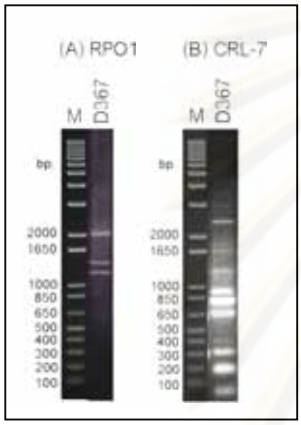
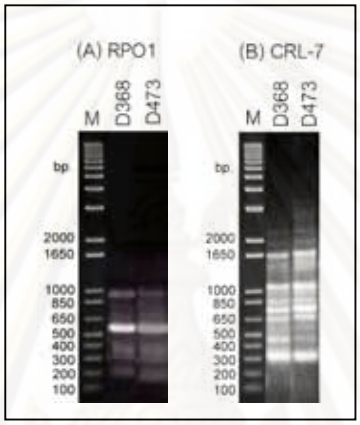
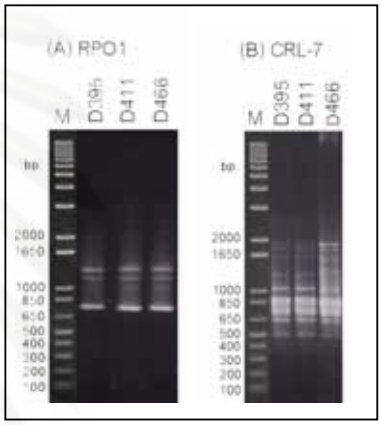
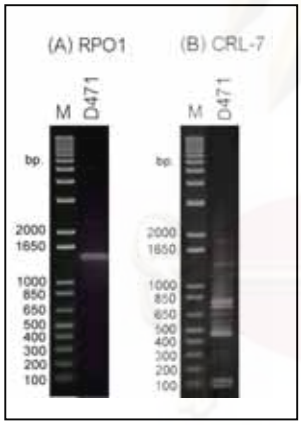
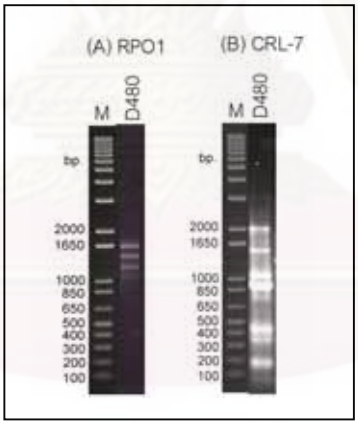
(B) CRL-7
M
D346
bp.
2000
1650
1000
850
650
500
400
300
200
100


(A) RPO1
(B) CRL-7
M
D367
bp.
2000
1650
1000
850
650
500
400
300
200
100


(A) RPO1
(B) CRL-7
M
D368
D473
bp.
2000
1650
1000
850
650
500
400
300
200
100


(A) RPO1
(B) CRL-7
M
D395
D411
D466
bp.
2000
1650
1000
850
650
500
400
300
200
100


(A) RPO1
(B) CRL-7
M
D471
bp.
2000
1650
1000
850
650
500
400
300
200
100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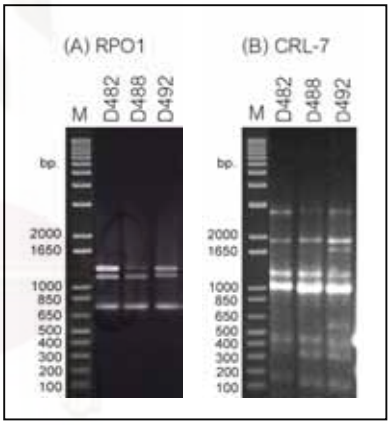
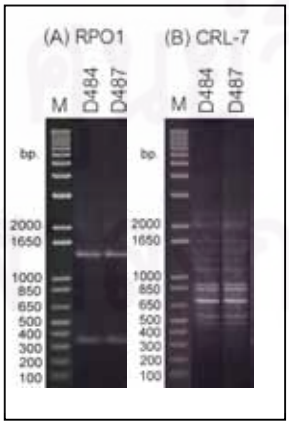
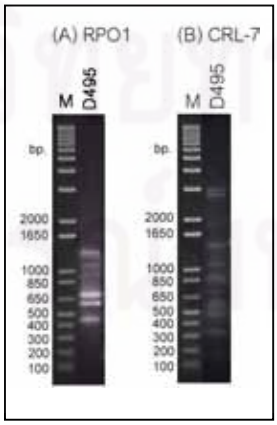
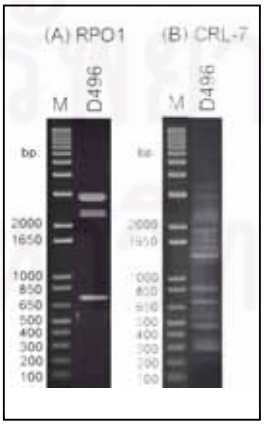
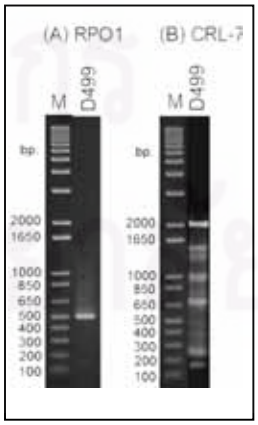
(B) CRL-7
M
D480
bp.
2000
1650
1000
850
650
500
400
300
200
100


(A) RPO1
(B) CRL-7
M
D482
D488
D492
bp.
2000
1650
1000
850
650
500
400
300
200
100

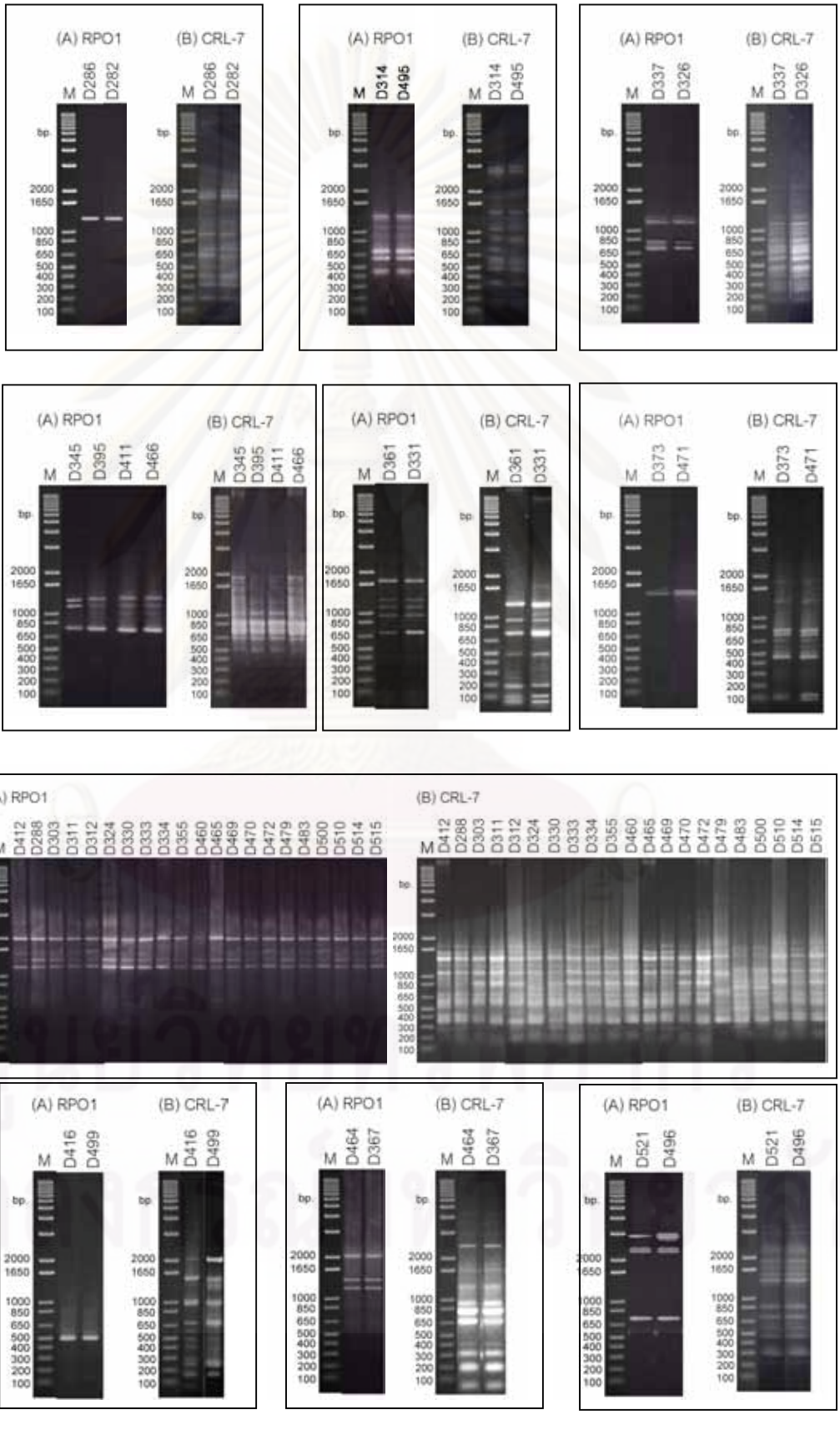

(A) RPO1
(B) CRL-7
M
D484
D487
bp.
2000
1650
1000
850
650
500
400
300
200
100


(A) RPO1
(B) CRL-7
M
D495
bp.
2000
1650
1000
850
650
500
400
300
200
100


(A) RPO1
(B) CRL-7
M
D496
bp.
2000
1650
1000
850
650
500
400
300
200
100


(A) RPO1
(B) CRL-7
M
D499
bp.
2000
1650
1000
850
650
500
400
300
200
100

There are 10 strains has the same fingerprints as 23 slow-growing soybean rhizobium strains isolated by Emampaiwong (2006) from root nodules of soybeans grown in soils from Bang Rakam district, Phitsanulok province.



APPENDIX E

Determination with the Biolog test kit of the ability or inability to use 95 carbon and nitrogen sources by 5 soybean rhizobium strains (D361, D373, D388, D416, and D467).

DWD < A_1 + 25%A_1 = - (not utilize that source)

A_1 + 25%A_1 < DWD < 0.25 = + (utilize that source)

DWD > 0.25 < 0.50 = ++ (utilize that source)

DWD > 0.50 = +++ (utilize that source)

A_1 = Dual Wavelength Data (DWD) of control well

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	D361			Consensus results from 3 determinations
				1st	2nd	3rd	
α-Cyclodextrin	-	-	-	-	+	+	+
Dextrin	+	+	+	+	+	-	+
Glycogen	-	-	+	-	+	+	+
Tween 40	+	+	+	+	+	+	+
Tween 80	+	+	+	+	+	+	+
N-Acetyl-D-Galactosamine	-	-	-	-	-	-	-
N-Acetyl-D-Glucosamine	-	-	-	-	-	-	-
Adonitol	-	-	-	+	+	+	+
L-Arabinose	+	+	+	+	+	+	+
D-Arabitol	+	-	-	+	+	+	+
D-Cellobiose	-	-	-	+	-	-	-
i-Erythritol	-	-	-	-	-	+	-
D-Fructose	-	+	-	+	+	+	+
L-Fucose	-	-	-	+	+	+	+
D-Galactose	-	+	+	+	+	+	+
Gentiobiose	-	-	-	-	-	-	-
α-D-Glucose	-	-	+	+	+	+	+
m-Inositol	-	-	-	-	-	-	-
α-D-Lactose	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	D361			Consensus results from 3 determinations
				1st	2nd	3rd	
Lactulose	-	-	-	-	-	-	-
Maltose	-	-	+	-	-	-	-
D-Mannitol	+	+	+	-	+	+	+
D-Mannose	+	+	+	+	-	+	+
D-Melibiose	-	-	-	-	-	+	-
β-Methyl-D-Glucoside	+	-	-	-	-	-	-
D-Psicose	-	-	-	-	-	-	-
D-Raffinose	+	-	-	-	-	-	-
L-Rhamnose	+	-	+	-	+	-	-
D-Sorbitol	-	-	-	+	+	+	+
Sucrose	+	-	-	-	-	-	-
D-Trehalose	-	-	-	-	-	-	-
Turanose	+	-	-	-	-	-	-
Xylitol	+	-	-	-	-	-	-
Pyruvic Acid Methyl Ester	+	+	+	++	++	+	+
Succinic Acid Mono-Methyl-Ester	+	+	+	+	+	+	+
Acetic Acid	+	-	+	++	+	+	+
Cis-Aconitic Acid	-	-	-	+	+	+	+
Citric Acid	+	-	-	+	+	+	+
Formic Acid	-	-	-	-	-	-	-
D-Galactonic Acid Lactone	+	-	-	-	-	-	-
D-Galacturonic Acid	+	-	-	-	-	-	-
D-Gluconic Acid	+	+	+	+	+	+	+
D-Glucosaminic Acid	-	-	-	-	-	-	-
D-Glucuronic Acid	+	-	-	-	-	-	-
α-Hydroxybutyric Acid	+	+	+	-	+	+	+
β-Hydroxybutyric Acid	+	+	+	++	++	++	+

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	D361			Consensus results from 3 determinations
				1st	2nd	3rd	
γ-Hydroxybutyric Acid	+	+	+	++	++	+	+
p-Hydroxy Phenylacetic Acid	+	-	-	++	-	+	+
Itaconic acid	-	-	+	-	-	-	-
α-Keto Butyric Acid	+	-	+	-	-	-	-
α-Keto Glutaric Acid	+	-	+	+	+	+	+
α-Keto Valeric Acid	+	+	+	-	+	+	+
D,L-Lactic Acid	+	+	+	+	++	+	+
Malonic Acid	+	-	+	+	+	+	+
Propionic Acid	+	-	+	-	-	-	-
Quinic Acid	-	+	-	+	+	+	+
D-Saccharic Acid	+	+	+	-	-	+	-
Sebacic Acid	+	-	+	-	-	-	-
Succinic Acid	+	+	+	+	+	+	+
Bromosuccinic Acid	+	+	+	+	+	+	+
Succinamic Acid	+	+	+	++	++	+	+
Glucuronamide	-	-	-	-	+	+	+
L-Alaninamide	+	+	-	-	-	-	-
D-Alanine	+	+	-	+	+	+	+
L-Alanine	+	+	+	-	+	-	-
L-Alanyl-glycine	-	-	-	-	-	-	-
L-Asparagine	+	-	-	-	-	-	-
L-Aspartic Acid	-	+	+	+	+	+	+
L-Glutamic Acid	-	-	-	-	-	-	-
Glycyl-L-Aspartic Acid	-	-	-	-	-	-	-
Glycyl-L-Glutamic Acid	-	-	+	+	-	+	+
L-Histidine	-	-	-	-	-	-	-
Hydroxy-L-Proline	-	-	-	-	-	-	-
L-Leucine	+	-	+	-	+	+	+
L-Ornithine	-	-	+	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	D361			Consensus results from 3 determinations
				1st	2nd	3rd	
L-Phenylalanine	+	-	+	+	-	+	+
L-Proline	+	-	-	-	-	+	-
L-Pyroglutamic Acid	+	-	+	+	+	+	+
D-Serine	-	+	-	-	-	-	-
L-Serine	+	-	-	-	-	-	-
L-Threonine	-	-	-	-	-	-	-
D,L-Carnitine	-	-	-	-	-	-	-
γ-Amino Butyric Acid	-	-	-	+	-	-	-
Urocanic Acid	-	-	-	+	-	+	+
Inosine	-	-	-	-	-	-	-
Uridine	-	-	-	-	-	-	-
Thymidine	-	-	-	-	-	-	-
Phenyethyl-amine	-	-	-	-	-	-	-
Putrescine	-	-	-	-	-	-	-
2-Aminoethanol	-	-	-	-	-	-	-
2,3-Butanediol	-	-	-	-	-	-	-
Glycerol	+	+	+	+	+	+	+
D,L-α-Glycerol Phosphate	+	-	-	-	-	-	-
α-D-Glucose-1-Phosphate	-	-	-	-	-	-	-
D-Glucose-6-Phosphate	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	D373			Consensus results from 3 determinations
				1st	2nd	3rd	
α-Cyclodextrin	-	-	-	-	-	+	-
Dextrin	+	+	+	+	+	+	+
Glycogen	-	-	+	+	-	+	+
Tween 40	+	+	+	+	-	+	+
Tween 80	+	+	+	+	-	+	+
N-Acetyl-D-Galactosamine	-	-	-	-	-	-	-
N-Acetyl-D-Glucosamine	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-
L-Arabinose	+	+	+	+	-	+	+
D-Arabitol	+	-	-	+	-	+	+
D-Cellobiose	-	-	-	-	-	-	-
i-Erythritol	-	-	-	-	-	+	-
D-Fructose	-	+	-	+	-	+	+
L-Fucose	-	-	-	-	-	-	-
D-Galactose	-	+	+	+	-	-	-
Gentiobiose	-	-	-	-	-	-	-
α-D-Glucose	-	-	+	+	-	-	-
m-Inositol	-	-	-	-	-	-	-
α-D-Lactose	-	-	-	-	-	-	-
Lactulose	-	-	-	-	-	-	-
Maltose	-	-	+	-	-	-	-
D-Mannitol	+	+	+	+	-	+	+
D-Mannose	+	+	+	+	-	+	+
D-Melibiose	-	-	-	-	-	-	-
β-Methyl-D-Glucoside	+	-	-	-	-	-	-
D-Psicose	-	-	-	-	-	-	-
D-Raffinose	+	-	-	-	-	-	-
L-Rhamnose	+	-	+	-	-	-	-
D-Sorbitol	-	-	-	+	-	-	-
Sucrose	+	-	-	-	-	-	-
D-Trehalose	-	-	-	-	-	-	-
Turanose	+	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	D373			Consensus results from 3 determinations
				1st	2nd	3rd	
Xylitol	+	-	-	-	-	-	-
Pyruvic Acid Methyl Ester	+	+	+	++	+	+	+
Succinic Acid Mono-Methyl-Ester	+	+	+	+	-	-	-
Acetic Acid	+	-	+	+	-	+	+
Cis-Aconitic Acid	-	-	-	-	-	-	-
Citric Acid	+	-	-	+	-	+	+
Formic Acid	-	-	-	-	-	-	-
D-Galactonic Acid Lactone	+	-	-	-	-	-	-
D-Galacturonic Acid	+	-	-	-	-	-	-
D-Gluconic Acid	+	+	+	+	-	-	-
D-Glucosaminic Acid	-	-	-	+	-	-	-
D-Glucuronic Acid	+	-	-	-	-	-	-
α-Hydroxybutyric Acid	+	+	+	-	-	-	-
β-Hydroxybutyric Acid	+	+	+	++	+	+	+
γ-Hydroxybutyric Acid	+	+	+	+	+	+	+
p-Hydroxy Phenylacetic Acid	+	-	-	-	-	-	-
Itaconic acid	-	-	+	-	-	-	-
α-Keto Butyric Acid	+	-	+	-	-	-	-
α-Keto Glutaric Acid	+	-	+	+	+	+	+
α-Keto Valeric Acid	+	+	+	+	-	-	-
D,L-Lactic Acid	+	+	+	+	+	+	+
Malonic Acid	+	-	+	+	-	+	+
Propionic Acid	+	-	+	-	-	-	-
Quinic Acid	-	+	-	+	-	-	-
D-Saccharic Acid	+	+	+	+	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	D373			Consensus results from 3 determinations
				1st	2nd	3rd	
Sebacic Acid	+	-	+	-	-	-	-
Succinic Acid	+	+	+	+	-	+	+
Bromosuccinic Acid	+	+	+	+	-	+	+
Succinamic Acid	+	+	+	+	+	+	+
Glucuronamide	-	-	-	-	-	-	-
L-Alaninamide	+	+	-	-	-	+	-
D-Alanine	+	+	-	+	-	-	-
L-Alanine	+	+	+	-	-	-	-
L-Alanyl-glycine	-	-	-	-	-	-	-
L-Asparagine	+	-	-	-	-	-	-
L-Aspartic Acid	-	+	+	+	-	-	-
L-Glutamic Acid	-	-	-	-	-	-	-
Glycyl-L-Aspartic Acid	-	-	-	-	-	-	-
Glycyl-L-Glutamic Acid	-	-	+	-	-	-	-
L-Histidine	-	-	-	-	-	-	-
Hydroxy-L-Proline	-	-	-	-	-	-	-
L-Leucine	+	-	+	+	-	-	-
L-Ornithine	-	-	+	-	-	-	-
L-Phenylalanine	+	-	+	+	-	+	+
L-Proline	+	-	-	+	-	+	+
L-Pyroglutamic Acid	+	-	+	+	-	-	-
D-Serine	-	+	-	-	-	-	-
L-Serine	+	-	-	-	-	-	-
L-Threonine	-	-	-	-	-	-	-
D,L-Carnitine	-	-	-	-	-	-	-
γ-Amino Butyric Acid	-	-	-	-	-	-	-
Urocanic Acid	-	-	-	-	-	-	-
Inosine	-	-	-	-	-	-	-
Uridine	-	-	-	-	-	-	-
Thymidine	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	D373			Consensus results from 3 determinations
				1st	2nd	3rd	
Phenyethyl-amine	-	-	-	-	-	-	-
Putrescine	-	-	-	-	-	-	-
2-Aminoethanol	-	-	-	-	-	-	-
2,3-Butanediol	-	-	-	-	-	-	-
Glycerol	+	+	+	+	+	+	+
D,L-α-Glycerol Phosphate	+	-	-	+	-	-	-
α-D-Glucose-1-Phosphate	-	-	-	-	-	-	-
D-Glucose-6-Phosphate	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	D388			Consensus results from 3 determinations
				1st	2nd	3rd	
α-Cyclodextrin	-	-	-	-	+	-	-
Dextrin	+	+	+	+	+	+	+
Glycogen	-	-	+	-	-	-	-
Tween 40	+	+	+	+	+	+	+
Tween 80	+	+	+	+	+	+	+
N-Acetyl-D-Galactosamine	-	-	-	-	-	-	-
N-Acetyl-D-Glucosamine	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-
L-Arabinose	+	+	+	+	+	+	+
D-Arabitol	+	-	-	+	+	+	+
D-Cellobiose	-	-	-	-	-	-	-
i-Erythritol	-	-	-	-	-	-	-
D-Fructose	-	+	-	+	+	+	+
L-Fucose	-	-	-	-	-	-	-
D-Galactose	-	+	+	+	+	+	+
Gentiobiose	-	-	-	-	-	-	-
α-D-Glucose	-	-	+	+	+	+	+
m-Inositol	-	-	-	-	-	-	-
α-D-Lactose	-	-	-	-	-	-	-
Lactulose	-	-	-	-	-	-	-
Maltose	-	-	+	-	-	-	-
D-Mannitol	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+
D-Melibiose	-	-	-	-	-	-	-
β-Methyl-D-Glucoside	+	-	-	-	-	-	-
D-Psicose	-	-	-	-	-	-	-
D-Raffinose	+	-	-	-	-	-	-
L-Rhamnose	+	-	+	-	-	-	-
D-Sorbitol	-	-	-	-	-	-	-
Sucrose	+	-	-	-	-	-	-
D-Trehalose	-	-	-	-	-	-	-
Turanose	+	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	D388			Consensus results from 3 determinations
				1st	2nd	3rd	
Xylitol	+	-	-	-	-	-	-
Pyruvic Acid Methyl Ester	+	+	+	++	++	++	+
Succinic Acid Mono-Methyl-Ester	+	+	+	++	+	+	+
Acetic Acid	+	-	+	++	+	+	+
Cis-Aconitic Acid	-	-	-	-	-	-	-
Citric Acid	+	-	-	-	-	-	-
Formic Acid	-	-	-	+	-	+	+
D-Galactonic Acid Lactone	+	-	-	+	+	+	+
D-Galacturonic Acid	+	-	-	+	-	-	-
D-Gluconic Acid	+	+	+	+	+	+	+
D-Glucosaminic Acid	-	-	-	-	-	-	-
D-Glucuronic Acid	+	-	-	-	-	-	-
α-Hydroxybutyric Acid	+	+	+	+	+	+	+
β-Hydroxybutyric Acid	+	+	+	++	++	++	+
γ-Hydroxybutyric Acid	+	+	+	++	++	+	+
p-Hydroxy Phenylacetic Acid	+	-	-	+	-	+	+
Itaconic acid	-	-	+	+	+	+	+
α-Keto Butyric Acid	+	-	+	+	+	+	+
α-Keto Glutaric Acid	+	-	+	+	+	+	+
α-Keto Valeric Acid	+	+	+	-	+	+	+
D,L-Lactic Acid	+	+	+	++	+	++	+
Malonic Acid	+	-	+	-	-	-	-
Propionic Acid	+	-	+	+	+	+	+
Quinic Acid	-	+	-	-	-	-	-
D-Saccharic Acid	+	+	+	++	++	+	+

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	D388			Consensus results from 3 determinations
				1st	2nd	3rd	
Sebacic Acid	+	-	+	-	-	-	-
Succinic Acid	+	+	+	++	++	+	+
Bromosuccinic Acid	+	+	+	++	+	+	+
Succinamic Acid	+	+	+	++	++	++	+
Glucuronamide	-	-	-	-	-	-	-
L-Alaninamide	+	+	-	+	-	+	+
D-Alanine	+	+	-	-	-	-	-
L-Alanine	+	+	+	-	-	-	-
L-Alanyl-glycine	-	-	-	+	+	+	+
L-Asparagine	+	-	-	-	-	-	-
L-Aspartic Acid	-	+	+	+	-	-	-
L-Glutamic Acid	-	-	-	-	+	+	+
Glycyl-L-Aspartic Acid	-	-	-	-	-	-	-
Glycyl-L-Glutamic Acid	-	-	+	-	-	-	-
L-Histidine	-	-	-	-	-	-	-
Hydroxy-L-Proline	-	-	-	-	-	-	-
L-Leucine	+	-	+	+	+	+	+
L-Ornithine	-	-	+	-	-	-	-
L-Phenylalanine	+	-	+	+	+	+	+
L-Proline	+	-	-	+	+	+	+
L-Pyroglutamic Acid	+	-	+	+	+	+	+
D-Serine	-	+	-	-	-	-	-
L-Serine	+	-	-	-	-	-	-
L-Threonine	-	-	-	+	+	+	+
D,L-Carnitine	-	-	-	-	-	-	-
γ-Amino Butyric Acid	-	-	-	+	+	+	+
Urocanic Acid	-	-	-	+	+	+	+
Inosine	-	-	-	-	-	-	-
Uridine	-	-	-	-	-	-	-
Thymidine	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	D388			Consensus results from 3 determinations
				1st	2nd	3rd	
Phenyethyl-amine	-	-	-	-	-	-	-
Putrescine	-	-	-	-	-	-	-
2-Aminoethanol	-	-	-	-	-	-	-
2,3-Butanediol	-	-	-	-	-	-	-
Glycerol	+	+	+	+	+	+	+
D,L-α-Glycerol Phosphate	+	-	-	-	-	-	-
α-D-Glucose-1-Phosphate	-	-	-	-	-	-	-
D-Glucose-6-Phosphate	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	D416			Consensus results from 3 determinations
				1st	2nd	3rd	
α-Cyclodextrin	-	-	-	-	+	-	-
Dextrin	+	+	+	-	+	+	+
Glycogen	-	-	+	-	-	-	-
Tween 40	+	+	+	-	++	++	+
Tween 80	+	+	+	++	++	++	+
N-Acetyl-D-Galactosamine	-	-	-	-	-	-	-
N-Acetyl-D-Glucosamine	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-
L-Arabinose	+	+	+	++	++	++	+
D-Arabitol	+	-	-	-	+	+	+
D-Cellobiose	-	-	-	-	-	-	-
i-Erythritol	-	-	-	-	-	-	-
D-Fructose	-	+	-	-	+	+	+
L-Fucose	-	-	-	-	+	+	+
D-Galactose	-	+	+	-	+	+	+
Gentiobiose	-	-	-	-	-	-	-
α-D-Glucose	-	-	+	-	+	+	+
m-Inositol	-	-	-	-	-	-	-
α-D-Lactose	-	-	-	-	-	-	-
Lactulose	-	-	-	-	-	-	-
Maltose	-	-	+	-	-	-	-
D-Mannitol	+	+	+	-	+	+	+
D-Mannose	+	+	+	-	+	+	+
D-Melibiose	-	-	-	+++	-	-	-
β-Methyl-D-Glucoside	+	-	-	-	-	-	-
D-Psicose	-	-	-	-	-	-	-
D-Raffinose	+	-	-	-	-	-	-
L-Rhamnose	+	-	+	-	+	+	+
D-Sorbitol	-	-	-	-	-	-	-
Sucrose	+	-	-	-	-	-	-
D-Trehalose	-	-	-	-	-	-	-
Turanose	+	-	-	-	+	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	D416			Consensus results from 3 determinations
				1st	2nd	3rd	
Xylitol	+	-	-	-	-	-	-
Pyruvic Acid Methyl Ester	+	+	+	++	++	++	+
Succinic Acid Mono-Methyl-Ester	+	+	+	++	++	++	+
Acetic Acid	+	-	+	+++	++	+	+
Cis-Aconitic Acid	-	-	-	-	-	-	-
Citric Acid	+	-	-	-	-	-	-
Formic Acid	-	-	-	++	++	++	+
D-Galactonic Acid Lactone	+	-	-	-	+	+	+
D-Galacturonic Acid	+	-	-	-	+	+	+
D-Gluconic Acid	+	+	+	-	+	+	+
D-Glucosaminic Acid	-	-	-	-	-	-	-
D-Glucuronic Acid	+	-	-	-	-	-	-
α-Hydroxybutyric Acid	+	+	+	-	+	+	+
β-Hydroxybutyric Acid	+	+	+	+++	+++	+++	+
γ-Hydroxybutyric Acid	+	+	+	++	++	++	+
p-Hydroxy Phenylacetic Acid	+	-	-	-	+	-	-
Itaconic acid	-	-	+	-	+	+	+
α-Keto Butyric Acid	+	-	+	-	+	+	+
α-Keto Glutaric Acid	+	-	+	-	+	-	-
α-Keto Valeric Acid	+	+	+	-	+	+	+
D,L-Lactic Acid	+	+	+	++	++	++	+
Malonic Acid	+	-	+	-	-	-	-
Propionic Acid	+	-	+	-	+	++	+
Quinic Acid	-	+	-	++	++	++	+
D-Saccharic Acid	+	+	+	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	D416			Consensus results from 3 determinations
				1st	2nd	3rd	
Sebacic Acid	+	-	+	-	+	+	+
Succinic Acid	+	+	+	++	++	++	+
Bromosuccinic Acid	+	+	+	++	++	++	+
Succinamic Acid	+	+	+	+++	++	+++	+
Glucuronamide	-	-	-	-	+	-	-
L-Alaninamide	+	+	-	-	-	-	-
D-Alanine	+	+	-	-	+	-	-
L-Alanine	+	+	+	-	+	-	-
L-Alanyl-glycine	-	-	-	-	-	-	-
L-Asparagine	+	-	-	-	-	-	-
L-Aspartic Acid	-	+	+	-	-	-	-
L-Glutamic Acid	-	-	-	-	+	+	+
Glycyl-L-Aspartic Acid	-	-	-	-	+	-	-
Glycyl-L-Glutamic Acid	-	-	+	-	+	-	-
L-Histidine	-	-	-	-	-	-	-
Hydroxy-L-Proline	-	-	-	-	-	-	-
L-Leucine	+	-	+	-	+	+	+
L-Ornithine	-	-	+	-	-	-	-
L-Phenylalanine	+	-	+	-	+	++	+
L-Proline	+	-	-	-	+	+	+
L-Pyroglutamic Acid	+	-	+	++	+	++	+
D-Serine	-	+	-	-	-	-	-
L-Serine	+	-	-	-	-	-	-
L-Threonine	-	-	-	-	-	+	-
D,L-Carnitine	-	-	-	-	-	-	-
γ-Amino Butyric Acid	-	-	-	-	+	-	-
Urocanic Acid	-	-	-	-	-	-	-
Inosine	-	-	-	-	-	-	-
Uridine	-	-	-	-	-	-	-
Thymidine	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	D416			Consensus results from 3 determinations
				1st	2nd	3rd	
Phenyethyl-amine	-	-	-	-	-	-	-
Putrescine	-	-	-	-	-	-	-
2-Aminoethanol	-	-	-	-	-	-	-
2,3-Butanediol	-	-	-	-	-	-	-
Glycerol	+	+	+	-	+	++	+
D,L-α-Glycerol Phosphate	+	-	-	-	-	-	-
α-D-Glucose-1-Phosphate	-	-	-	-	-	-	-
D-Glucose-6-Phosphate	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	D467			Consensus results from 3 determinations
				1st	2nd	3rd	
α-Cyclodextrin	-	-	-	-	-	-	-
Dextrin	+	+	+	+	+	+	+
Glycogen	-	-	+	-	-	+	-
Tween 40	+	+	+	+	++	+	+
Tween 80	+	+	+	+	++	+	+
N-Acetyl-D-Galactosamine	-	-	-	-	-	-	-
N-Acetyl-D-Glucosamine	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-
L-Arabinose	+	+	+	++	++	+	+
D-Arabitol	+	-	-	+	+	+	+
D-Cellobiose	-	-	-	-	-	-	-
i-Erythritol	-	-	-	-	-	-	-
D-Fructose	-	+	-	+	+	+	+
L-Fucose	-	-	-	+	+	+	+
D-Galactose	-	+	+	+	+	+	+
Gentiobiose	-	-	-	-	-	-	-
α-D-Glucose	-	-	+	+	+	+	+
m-Inositol	-	-	-	-	-	-	-
α-D-Lactose	-	-	-	-	-	-	-
Lactulose	-	-	-	-	-	-	-
Maltose	-	-	+	-	-	-	-
D-Mannitol	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+
D-Melibiose	-	-	-	-	-	-	-
β-Methyl-D-Glucoside	+	-	-	-	-	-	-
D-Psicose	-	-	-	-	-	-	-
D-Raffinose	+	-	-	-	-	-	-
L-Rhamnose	+	-	+	+	+	+	+
D-Sorbitol	-	-	-	-	-	-	-
Sucrose	+	-	-	-	-	-	-
D-Trehalose	-	-	-	-	-	-	-
Turanose	+	-	-	+	+	+	+

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	D467			Consensus results from 3 determinations
				1st	2nd	3rd	
Xylitol	+	-	-	-	-	-	-
Pyruvic Acid Methyl Ester	+	+	+	++	++	++	+
Succinic Acid Mono-Methyl-Ester	+	+	+	++	++	++	+
Acetic Acid	+	-	+	+	++	++	+
Cis-Aconitic Acid	-	-	-	-	-	-	-
Citric Acid	+	-	-	-	-	-	-
Formic Acid	-	-	-	-	+	+	+
D-Galactonic Acid Lactone	+	-	-	+	+	+	+
D-Galacturonic Acid	+	-	-	+	+	+	+
D-Gluconic Acid	+	+	+	+	+	+	+
D-Glucosaminic Acid	-	-	-	-	-	+	-
D-Glucuronic Acid	+	-	-	+	-	+	+
α-Hydroxybutyric Acid	+	+	+	-	+	+	+
β-Hydroxybutyric Acid	+	+	+	++	+	++	+
γ-Hydroxybutyric Acid	+	+	+	++	++	+	+
p-Hydroxy Phenylacetic Acid	+	-	-	-	+	+	+
Itaconic acid	-	-	+	+	+	+	+
α-Keto Butyric Acid	+	-	+	-	+	+	+
α-Keto Glutaric Acid	+	-	+	+	+	+	+
α-Keto Valeric Acid	+	+	+	-	+	+	+
D,L-Lactic Acid	+	+	+	+	++	++	+
Malonic Acid	+	-	+	-	-	-	-
Propionic Acid	+	-	+	+	+	+	+
Quinic Acid	-	+	-	++	++	++	+
D-Saccharic Acid	+	+	+	+	+	+	+

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	D467			Consensus results from 3 determinations
				1st	2nd	3rd	
Sebacic Acid	+	-	+	+	+	+	+
Succinic Acid	+	+	+	++	++	++	+
Bromosuccinic Acid	+	+	+	++	++	++	+
Succinamic Acid	+	+	+	++	++	++	+
Glucuronamide	-	-	-	+	+	+	+
L-Alaninamide	+	+	-	-	-	+	-
D-Alanine	+	+	-	+	+	+	+
L-Alanine	+	+	+	+	+	+	+
L-Alanyl-glycine	-	-	-	-	-	-	-
L-Asparagine	+	-	-	-	-	-	-
L-Aspartic Acid	-	+	+	+	+	+	+
L-Glutamic Acid	-	-	-	+	+	+	+
Glycyl-L-Aspartic Acid	-	-	-	-	+	+	+
Glycyl-L-Glutamic Acid	-	-	+	+	+	+	+
L-Histidine	-	-	-	-	-	-	-
Hydroxy-L-Proline	-	-	-	-	-	-	-
L-Leucine	+	-	+	+	+	+	+
L-Ornithine	-	-	+	-	-	-	-
L-Phenylalanine	+	-	+	+	+	+	+
L-Proline	+	-	-	+	+	+	+
L-Pyroglutamic Acid	+	-	+	+	++	+	+
D-Serine	-	+	-	-	-	-	-
L-Serine	+	-	-	-	-	+	-
L-Threonine	-	-	-	+	+	+	+
D,L-Carnitine	-	-	-	-	-	-	-
γ-Amino Butyric Acid	-	-	-	+	+	+	+
Urocanic Acid	-	-	-	-	-	-	-
Inosine	-	-	-	-	-	-	-
Uridine	-	-	-	-	-	-	-
Thymidine	-	-	-	-	-	-	-

Carbon/Nitrogen sources on Biolog GN2 MicroPlate	Consensus results from 7 determinations						
	B. elkanii NBRC 14791	*B. japonicum* NBRC 14783	*B. liaoningense* NBRC 100396	D467			Consensus results from 3 determinations
				1st	2nd	3rd	
Phenyethyl-amine	-	-	-	-	-	-	-
Putrescine	-	-	-	-	-	-	-
2-Aminoethanol	-	-	-	-	-	-	-
2,3-Butanediol	-	-	-	-	-	-	-
Glycerol	+	+	+	+	++	++	+
D,L-α-Glycerol Phosphate	+	-	-	-	-	+	-
α-D-Glucose-1-Phosphate	-	-	-	-	-	-	-
D-Glucose-6-Phosphate	-	-	-	-	-	-	-

BIOGRAPHY

Miss Nichanun Karbkesorn was born on November 18, 1984. She obtained a Bachelor of Science Degree in Biology from Srinakarinwirot University, Bangkok, Thailand, in 2006.

Publication

1) **ณิชานันท์ กาบเกษร** และ กาญจนา ชาญสง่าเวช. การใช้มัลติเพล็กซ์พีซีอาร์ทำนายการเข้าสร้างปมของไรโซเบียมถั่วเหลือง. หนังสือรวมบทความที่เสนอในการประชุมวิชาการเกษตรแห่งชาติ ครั้งที่ 5 " พลังงานทดแทนและความมั่นคงทางด้านอาหารเพื่อมนุษยชาติ ". 2-4 กรกฎาคม 2552. ณ โรงแรมอุบลอินเตอร์เนชั่นแนล. หน้า 451-458.

Presentation at Scientific Conference

1) Kanjana Chansa-ngavej, Ly Kim Pheng, Weerasak Chongfuengprinya, Thanpapha Chanthapetch, **Nichanun Karbkesorn**, and Darunee Pongpattarrine. 2008. Use of RAPD-PCR fingerprints to detect competitive ability of soybean rhizobial biofertilizer NA7 to nodulate soybean cv. Chiangmai 60. Abstract Book. The Science Forum 2008. March 13-14, 2008. Chulalongkorn University. p. 30.
2) **Karbkesorn, N.** and Chansa-ngavej, K. 2008. Feasibility study on the use of multiplex PCR to predict nodulation efficiency of soybean rhizobia isolated from Bang Rakam district, Phitsanulok province, Thailand. Abstract Book. The 13th Biological Science Graduate Congress, December 15-17, 2008. Singapore, p. 26.
3) **Karbkesorn, N.** and Chansa-ngavej, K. 2009. Optimization of mutiplex PCR conditions for the detection of fast- and slow-growing soybean rhizobia. Abstract Book. The 14th Biological Science Graduate Congress, December 10-12, 2009. Bangkok, Thailand, p.135.
4) **Karbkesorn, N.** and Chansa-ngavej, K. 2010. Multiplex PCR conditions for the prediction of nodulation property of slow-growing soybean rhizobia. Abstract Book. The Science Forum 2010. March 11-12, 2010. Chulalongkorn University. p. 78.