POLYGENETIC DIVERSITY OF RHIZOBIAL STRAINS ISOLATED FROM DIVERSED ECOSYSTEMS IN THAILAND

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ความหลากหลายทางพันธุกรรมของไรโซเบียมที่แยกได้จากดินในระบบนิเวศวิทยา
ที่แตกต่างกันของประเทศไทย

นางสาวดนยา มาศปถวี

Polygenetic Diversity of Rhizobial Strains Isolated from Diversed Ecosystems in Thailand

Suranaree University of Tcehnology Council has approved this submitted in partial fulfillment of the requirements for the Master's Degree

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ดนยา มาศปถวี : ความหลากหลายทางพันธุกรรมของไรโซเบียมที่แยกได้จากดินในระบบ
นิเวศวิทยาที่แตกต่างกันของประเทศไทย
(POLYGENETIC DIVERSITY OF RHIZOBIAL STRAINS ISOLATED FROM
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ความหลากหลายทางพันธุกรรมของไรโซเบียมที่แยกได้จากดินในระบบนิเวศวิทยาที่แตก
ต่างกันในประเทศไทย ทำโดยสุ่มตัวอย่างดินจาก 3 ภาคของประเทศไทย ได้แก่ ภาคกลาง ภาคเหนือ
และภาคตะวันออกเฉียงเหนือ ในแต่ละภาคจะเก็บตัวอย่างดินจาก 11 พื้นที่ที่มีระบบนิเวศต่าง ๆ กัน
ไป โดยนำมาศึกษาประชากรประจำถิ่นของไรโซเบียม โดยใช้วิธี MPN plant infection technique
กับพืชดัชนี (ถั่วเซราโต) โดยจะคัดเลือกตัวอย่างเชื้อไรโซเบียมพื้นที่ละ 4 สายพันธุ์ การทดลองที่
ศึกษา ได้แก่ 1) การนับจำนวนประชากรไรโซเบียม 2) ลักษณะทางสรีรวิทยาและกายภาพ 3) การ
สร้างปมและประสิทธิภาพการตรึงไนโตรเจนกับพืชดัชนี (ถั่วเซราโต) 4) การต้านทานต่อสาร
ปฏิชีวนะ 8 ชนิด 6) การเกิดปมกับพืชตระกูลถั่ว 3 ชนิด และ 6) การเพิ่มปริมาณ DNA โดยวิธี
PCR ชนิด RAPD จากผลการทดลองพบว่า ประชากรไรโซเบียมมีความหนาแน่นค่อนข้างสูงในฤดู
ฝน โดยเฉพาะในพื้นที่เชิงเขาและพื้นที่ไร่สลับข้าวของทุกภาค อย่างไรก็ตามพบว่าประชากรไรโซ-
เบียมส่วนใหญ่จะอยู่ในกลุ่ม *Bradyrhizobium* ซึ่งมีสายพันธุ์ไรโซเบียมที่สร้าง indole acetic acid
(IAA) ได้ประมาณ 37% ของไรโซเบียมทั้งหมด ไรโซเบียมที่ศึกษาสามารถสร้างปมกับพืชดัชนีได้
ทุกสายพันธุ์และมีความสามารถในการตรึงไนโตรเจนได้ในช่วงที่กว้าง รูปแบบการต้านทานสาร
ปฏิชีวนะพบว่า มีความหลากหลายค่อนข้างสูง โดยสามารถแบ่งตามรูปแบบการต้านทานได้ถึง 123
กลุ่ม การสร้างปมกับพืชตระกูลถั่ว 3 ชนิด พบว่าส่วนใหญ่สามารถสร้างปมได้กับพืชในกลุ่ม
Cowpea จากการศึกษาการเพิ่มปริมาณ DNA โดยวิธี PCR ชนิด RAPD พบว่ากลุ่มไรโซเบียมแสดง
ความสัมพันธ์ภายในกลุ่มค่อนข้างสูง จากผลการทดลองทั้งหมดสามารถสรุปได้ว่าประชากรไรโซ-
เบียมที่สุ่มได้จากดินในประเทศไทย ส่วนใหญ่จะอยู่ในสกุล *Bradyrhizobium* มากกว่าสกุลอื่น ๆ ใน
Family Rhizobiaceae

สาขาวิชา เทคโนโลยีชีวภาพ	ลายมือชื่อนักศึกษา _____
ปีการศึกษา 2542	ลายมือชื่ออาจารย์ที่ปรึกษา _____
	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____
	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

DONNAYA MATPATAWEE : POLYGENETIC DIVERSITY OF RHIZOBIAL

STRAINS ISOLATED FROM DIVERSED ECOSYSTEMS IN THAILAND

THESIS ADVISOR : PROFESSOR Dr. NANTAKORN BOONKERD, 113 PP.

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To investigate the genetic diversity of rhizobia isolated from various ecosystems in Thailand, soil samples were taken from Central, North and North Eastern part of Thailand. The soil samples in each part were collected from 11 different type of ecosystems to investigate the native population of rhizobia. Enumeration of rhizobial population was conducted by MPN plant infection method using host index plant (siratro). Only 4 isolates from each type of ecosystem were randomly selected for studying as follow, 1) population number of rhizobia 2) morphological and physiological aspects 3) nodule formation and N_2-fixing ability with host index plant (siratro) 4) antibiotic resistant profiles 5) host-dependent cross-inoculation group and 6) DNA amplification by using primer such as RAPD. The results obtained from this study indicated that a high level of rhizobial population was found in rainy season especially at the foot hill of mountain and rice in rotation with other crops areas. However, found that most of rhizobia in Thailand were belonged to slow-grower group and 37% of total isolates were able to produce indole acetic acid (IAA). Most of rhizobial isolates could nodulate plant index and performed broad range of N_2-fixing efficiency. The antibiotic resistant profies using 8 kinds of antibiotic could differentiate them into 123 groups. By using host dependent cross-inoculation group with 3 plants found that rhizobial isolates could nodulate with cowpea more than other plants. DNA characterization using PCR-RAPD products showed that there were closely related among rhizobial isolates. The results indicated that major rhizobial population in Thai soil was most likely belonged to genus *Bradyrhizobium* rather than other genera in Family Rhizobiaceae.

สาขาวิชา เทคโนโลยีชีวภาพ   ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2542   ลายมือชื่ออาจารย์ที่ปรึกษา _____

           ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

           ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

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Donnaya Matpatawee

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LIST OF ABBREVIATIONS

ADP	adenosine 5'- diphosphate
AMP	adenosine 5'- monophosphate
ARA	acetylene reduction activities
ATP	adenosine 5'- triphosphate
BNF	biological nitrogen fixation
bp	base pair
^{o}C	degree celcius
cm	centimeter
CV	coefficient of variation
dATP	deoxyadenosine 5' triphosphate
dCTP	deoxycytidine 5' triphosphate
dGTP	deoxyguanosine 5' triphosphate
DNA	deoxyribonucleic acid
DMRT	duncan's new multiple range test
dTTP	deoxythymine 5' triphosphate
EDTA	ethylene diamine teraacetic acid
e. g.	for example
et al.	et alia (and others)
g	gram
h	hour
kDa	kilodalton
l	litre
M	molar
μM	micromolar
μm	micrometer
μmol	micromole
μl	microlitre

LIST OF ABBREVIATIONS (CONTINUED)

μg	microgram
mg	milligram
min	minute
ml	millilitre
mM	millimolar
N	normal
NAD	nicotinamide adenine dinucleotide
NADH	reduced NAD
nm	nanometer
OD	optical density
pmol	picomole
RAPD	random amplification polymorphism DNA
RNA	ribonucleic acid
rDNA	ribosomal DNA
rRNA	ribosomal RNA
rpm	revolution per minute
UV	ultraviolet
vol/vol	volume per volume

1. INTRODUCTION

1.1 Biological Nitrogen Fixation (BNF)

The complex biochemical reactions whereby the inert atmospheric nitrogen is enzymatically reduced into a utilizable form for the plant by the nitrogenase enzyme complex of the bacteroids is called biological nitrogen fixation (BNF). BNF on the other hand, play an importance role by maintain the life because it involves the enzymatic reduction of N_2 to ammonia. The ammonia produced then can be incorporated by enzymatic means for the growth and maintenance of the cell. N_2 fixing is unique to bacteria; animals and plants that fix must do so in association with bacteria. An understanding of biological N_2 is essential to elucidate the dynamics of the global nitrogen cycle. With the exception of water; nitrogen is generally considered the most limiting nutrient for growth of plants in their natural environment; therefore, input of nitrogen via biological N_2 fixation often has profound ecological effects. Capability of fixing N_2 is recognized as a process of great agronomic importance, and a variety of leguminous plants and some nonleguminous plants can obtain their nitrogen from the air by symbiotic association with microorganisms. In addition to these rather practical applications of research on biological N_2 fixation, there has been a considerable development of new technology and information from research on N_2 fixation that is benefiting other areas of biological research. The main compartments were the atmosphere, the soil, the aquatic environment and the biomass, prokaryotes, plants and animals. The microorganisms that could fix nitrogen from atmosphere as summarized in Table 1. However, in this thesis a nitrogen fixing bacteria, rhizobia were selected for study their genetic diversity under different ecosystems.

The process of BNF by rhizobia consist of the infection, the nodule formation, the N_2 fixation (Nitrogenase gene and leghemoglobin), the N_2 assimilation and the translocation were explained in the next title.

Table 1. Genera of nitrogen-fixing bacteria (Spent, J.I. and Spent, P. 1990)

Family Genus Species	General comments
Acetobacteriaceae	
Acetobacter diazotrophicus	Isolated from sugar cane: grows at low pH and on high concentrations of sucrose: can form acetate from ethanol (Gillis *et al*, 1989)
Azotobacteraceae	Soil, water, leaf and root surfaces; all species fix N_2 aerobically, but generally more efficiently at low pO_2
Azotobacter beijerinckii, chirococcuin paspali, vinelandii *Azomonas insignis, macrocytogenes* *Azotococcus agilis*	Alkaline soils: generally produce some extracellular slime
Beijerinckia derxii, fluminensis, indica, mobilis *Derxia gummosa*	Acid soils: not temperate regions: produce abundant slime
Xanthobacter autotrophicus flavus	May fix N_2 autotrophically (H_2, CO_2) and heterotrophically (sucrose)
Bacillaceae *Bacillus macerans polymyxa clostridium ≥12 spp*	Widespread: aerobic or facultatively anaerobic Fixation less common Most strains fix N_2 anaerobically Soil, fresh and salt water, sediments, intestines, faeces; some strains fix N_2 anaerobically or microaerophilically; some reduce Fe
Desulfotomaculum orientis, ruminis	Intestines, rumens: strict anaerobes; convert SO_4^{2-} to S^-: some strains fix N_2
Beggiatoaceae	Marine and freshwater gliding, filamentous, sulphide oxidizing bacteria
Beggiatoa alba	Fixes micro-aerophilically (Polman and Larkim, 1988)
Thiothrix Vitreoscilla	Preliminary reports only
Chlorobiaceae *Chlorobium*	Green sulphur bacteria
thiosulfatophilium, limicola, phaeobacteroides *Pelodictyon luteulum*	Moist and muddy soils, salt and freshwater. Strict anaerobes: fixation of N_2 rare

Table 1. (continued)

Family
    Genus
        Species
	General comments

Chloroflexaceae
    Chloroflexus
        aurantlcus
	Filamentous green gliding bacteria
	Can fix N_2 anaerobically (Gallon and Chaplin, 1987)

Chromatiaceae
    Amoebobacter
        roseus
    Chromatium
        gracile, minus,
        minutissimum
        vinosum, violescens,
        warmingii, weissei
    Ectothiorhodospira
        shaposhnikovii
    Thiocapsa
        pfennigii, roseopercicina
    Thiocystis
        violacea
	Purple sulphur bacteria
	
	Habitats as for Chlorobiaceae. Strict anaerobes: fixation of N_2 rare

Corynebacteriaceae
    Arthrobacter
        fluorescens
	Soils, mud: several strains shown to fix N_2 autotrophically (H_2, CO_2), as well as heterotrophically (sucrose) in presence of O_2

Enterobacteriaceae
    Citrobacter
        freundii
    Enterobacter
        aerogenes, agglomerans,
        cloacae
	All originally isolated from intestinal flora, now reported from various habitats including leaf, nodule and bark surfaces: only a few strains actively fix N_2. Nitrogenase synthesis and activity anaerobic or micro-aerophilic

Enterobacteriaceae (cont.)
    Escherichia
        intermedia
    Klebsiella
        aerogenes, pneumoniae
    Erwinia
        herbicola
	A soil or plant pathogenic organism

Methanomonadaceae
    Methylobacter
    Methylococcus
    Methylocystis
    Methylomonas
    Methylosinus
        trichosporum
	Soil, water: utilize methane: growth and N_2 fixation aerobic

Table1. (continued)

Family
   Genus
      Species
	General comments

Pseudomonadaceae
   Pseudomonas
      diazotrophicus,
      pseudoflava,
      saccharophila, stutzeri
	See Jenni et al. (1989)

Rhizobiaceae
   Azorhizobium
   Bradyrhizobium
   Rhizobium
   Mesorhizobium
   Sinorhizobium	

Rhodospirillaceae
   Rhodomicrobium
      vannielii
   Rhodopseudomonas
      acidophila, capsulata,
      gelatinosa, globiformis,
      palustris, sphaeroides,
      viridis
   Rhodospirillum
      fulvum, molischianum,
      photometricum, rubrum,
      tenue
	Purple non-sulphur bacteria
	
	Predominantly aquatic, facultative anaerobes: generally micro-aerophilic when grown on combined nitrogen. Require light if grown anaerobically: under these conditions some strains fix N_2

Spirillaceae
   Aquaspirillum
      fasciculus, perigrenum
   Azospirillum
      amazonica, brasilense,
      halopraeferans, lipoferum
   Herbaspirillum
   Campylobacter
	Obligate aerobe associated with roots of grasses etc., where it may fix N_2 micro-aerophilically
	
	Some strains may fix N_2 in association with roots

Streptomycetaceae *Frankia*	

Thiobacteriaceae
 	
   Thiobacillus
      ferro-oxidans
	Acid waters with high iron content. Chemolithotrophic, Oxidizing Fe^{2+} and S compounds: growth aerobic and N_2 fixation micro-aerophilic

Vibrionaceae
   Vibrio
      diazotrophicus,
      natriegens, sp
	Marine, probably halophytic; may use agar as sole C source (Shieh, Simidu and Maruyama, 1987, 1988)

Table 1. (continued)

Family
  Genus
    Species
	General comments

Uncertain family	
Alcaligenes	Soil, hydrogen-oxidizing
latus	
Desulfovibrio	Wet soils, fresh water and salt water with high organic
desulfuricans, gigas,	content. Not all strains actively fix N_2
vulgaris	

1.2 Infection and Nodule Formation

Rhizobia are soil bacteria belonging to the alpha subdivision of the Proteobacteria. The reason for the great interest in this group of bacteria is their capability to form nitrogen-fixing symbiosis with leguminous plants. Fixation of atmospheric dinitrogen into ammonia takes place in specific organs, root nodules, or in special cases, between the symbiotic partners (Fisher and long 1992; Spaink 1995; van Rhijn and Vanderleyden 1995; Denarie et al. 1996). The free-living rhizobia in the soil could infect into roots by 3 ways, hairs, wounds or cracks and between cells of intact epidermises.

1.21 Hair infection

This occurs in the most widely studied crop plants and has frequently been reviewed (e.g. Bauer, 1981). Methods are now being developed to study root hairs separately from their parent roots. These should allow rapid progress to be made in defining those features of legume hairs which are necessary for infection. It has been shown by Mort and Grover (1987) that walls of legume hairs may have a higher pectin content than those of other plants studied. In order to penetrate these walls there is good circumstantial evidence that localized digestion must take place (Callham and Torrey, 1981). However, in spite of much effort, there is little evidence to suggest that the cellulases and pectinases familiar in plant pathogenic bacteria have a major role to play. Indeed, these enzymes applied artificially may destroy specificity (Al-Mallah, Davey and Cocking, 1987). A much more subtle process is indicated, spatially confined within the curl of a

root hair and closely associated with hair metabolism as suggested by an enlarged nucleus which moves near to the point of rhizobial entry and rapid protoplasmic streaming.

Once the hair wall is breached a tubular structure, the infection thread, is formed. This consists of newly synthesized host cell wall material surrounding the rhizobia which are themselves embedded in a matrix which may by partly of bacterial origin but which has been shown by Bradley et al. (1998) also to contain host glycoproteins. The whole structure is surrounded by a host membrane continuous with the root hair plasma-lemma. In root hairs where infections have been observed, micrographs indicate that only primary wall layers are present. The thread wall has often been thought to be of primary structure, but evidence from use of wall degrading enzymes is accumulating to show this may not be the case (Higashi, Kushiyama and Abe, 1987). Indeed, if rhizobia produce enzymes which degrade wall components as part of the entry procedure (Callaham and Torrey, 1981) it may necessary for infection threads to contain nonprimary elements if their integrity is to be retained (Sprent and Faria, 1988).

1.2.2 Wound (crack) infection

Detailed studies have been made for *Arachis* and *Stylosanthes* (Chandler, 1978; Chandler, Date and Roughley, 1982). In both plants, rhizobia gain entry at the point where lateral roots emerge. Nodules appear macroscopically to be in the axils of lateral roots, a location common in most general of the legume tribe Dalbergieae and all those examined from the Aeschynomeneae (Sprent, Sutherland and Faria, 1989). Since all these nodules share a similar internal arrangement of cells, notably the absence of uninfected (interstitial) cells in the infected region, it is reasonable to inter they share a common mode of entry. Collapse of cell walls, as seen in *Stylosanthes*, may represent a hypersensitive reaction on the part of the plant, more like parasitism than symbiosis in the mutualistic sense.

Wound entry may be common in nature especially in some legume tribes. This alone suggests it is a property principally of the host, a point of view supported by the observation that the same rhizobial strain nodulates *Vigna* species via hairs and *Arachis* via wounds (e.g. Sen and Weaver, 1984). However, genetically engineered agrobacteria and rhizobia may obtain entry by wounds and induce fix⁻ (non-nitrogen-fixing) nodules, usually lacking intracellular rhizobia, in plants which normally have a root hair infection. These observations underline similarities between agrobacteria and rhizobia – the former always enter via wounds. However, the tumours

resulting from these engineered changes do not differentiate into functional nodules, indicating that some other plant and/or rhizobial limitation has been invoked. Whereas agrobacteria can gain entry via artificially induced wounds, this has not so far been shown for rhizobia. It is possible that the natural changes which take place when a lateral root emerges will involve modifications to cell walls which predispose them to rhizobial attack.

A slightly different type of wound infection is found in the non-legume *Parasponia*. Here infection also occurs through a ruptured epidermis, the rupture in this case results from division of root cortical cells (Bender et al. 1987).

1.2.3 Epidermal infection

There have been sporadic reports of direct infection through undamaged epidermises of legumes normally infected via hairs (Dart, 1977). Many nodulating legumes, especially woody species, do not regularly produce hairs, nor do they have nodules associated with lateral roots (Sprent, Sutherland and Faria,1987; 1989). Since nodules develop on young and apparently undamaged roots, epidermal infection seem likely and this has been confirmed in *Mimosa scabrella* by Faria, Hay and Sprent (1988). Here rhizobia penetrate between epidermal cells in a manner similar of the non-legume *Elaeagnus*. The mode of infection is host controlled. Direct epidermal infection is probably common among legumes which have neither 'typical' root hairs nor wound infections.

After infection process, the nodule formation was occurred. There are at least three ways in which cells may become infected.

1. Each individual cell in a young nodule becomes infected by a branch of the infection thread. Some cells (called interstitial cells) remain uninfected. This pattern is seem in nodules of indeterminate growth such as those of pea (Newcomb, 1981; Fig. 1). The infection process continues throughout the life of the nodule, in cells newly formed by the nodule meristem.
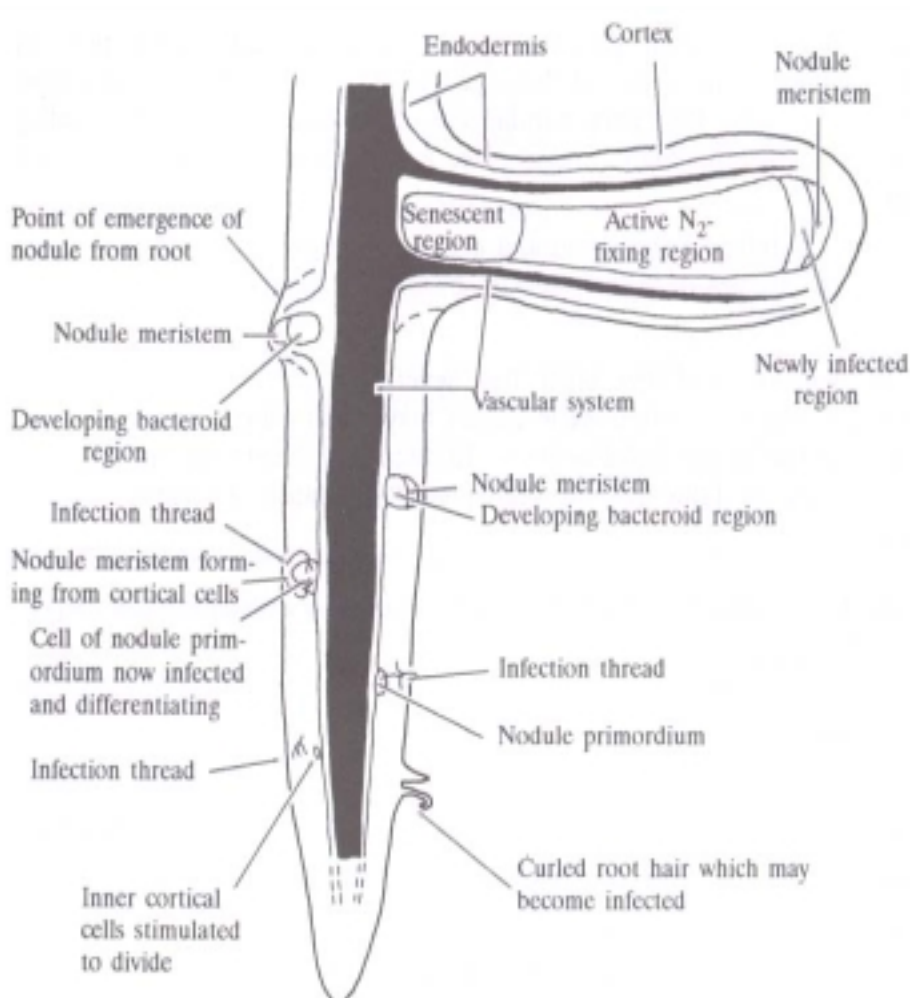
Figure 1. A typical sequence of events leading to the formation of an indeterminate nodule as found in legumes such as peas and clovers. Not drawn to an exact scale. At the time of emergence nodules vary (with species) from 1-3 mm in diameter (Sprent, J.I and Sprent, P. 1990).

2. A limited number of cells may become infected by branches of the infection thread. These cells and the bacteria contained within them divide and then enlarge, forming a nodule of limited growth of the soybean/*Vigna* type (Newcomb, 1981; Sprent and Thomas, 1984). Infected cells tend to occur in groups interspersed with interstitial cells (Fig. 2).
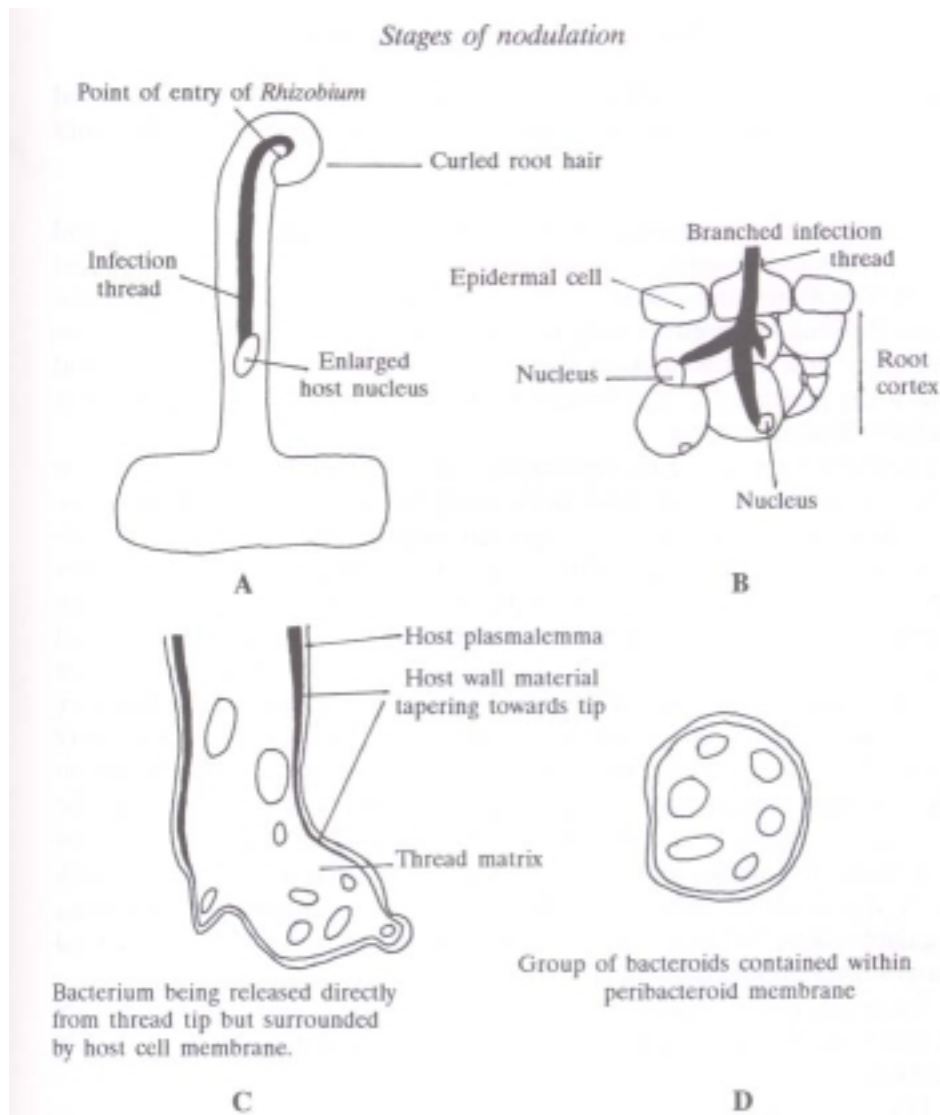
Figure 2 Stages in infection and formation of nitrogen fixing cells in a 'typical' legume' A. Curled root hair with infection thread. B. Transverse section of the cortex region of a root to show branching of infection thread and its association with cell nuclei. C. and D. show release of bacteria from infection thread. The thread matrix may contain host-generated glycoprotein (Sprent, J.I and Sprent, P. 1990).

3. One or a few cells may be infected by an undefined 'zoogloea' (not an organized infection thread). A number of infection attempts may abort (the host cell die) before infection is achieved. Infected cells divide repeatedly but neither infection threads nor interstitial cell occur.

The consequent uniformity of infection central tissue is seen typically in nodules of the aeschynomenoid type described by Sprent and Faria (1998) and found in such genera as *Arachis, Stylosanthes, Aeschynomene* and *Dalbergia*. This implies that infection threads are not an essential stage in nodule formation.

1.3 Nitrogen Fixation

The components of N_2-fixation process were nitrogenase enzyme and leghemoglobin, in nodules of soybean and pea, nitrogenase synthesis normally follows very shortly after bacteria are released from infection threads. The enzyme nitrogenase is a complex of two enzymes, an Fe-containing protein and an Fe-Mo protein (Gallon and Chaplin 1987). It is responsible for the conversion (reduction) of atmospheric N into NH^+_4 and is synthesized in the cytosol of the bacteroids. The legume utilizes NH_4^+ to convert certain precursor metabolites (e.g., α-ketoglutarate, phosphoenpyruvate) into amino acid, which, in turn, are synthesized into proteins (Haaker and Klugkist 1987). At approximately the same time leghaemoglobin is produced in host cells giving active nodules a distinct pink colour. Leghaemoglobin acts to maintain a high flux of O_2 at a low concentration (Appleby, 1984). The process of nitrogen fixation as below.

$$3H_2 + N_2 + 8H^+ + 8e + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi$$

1.4 Nitrogen Assimilation

The assimilation of N_2 fixed in the bacteriods is exported through the inner and outer membrane of the microsymbiont and through the peribacteroid membrane into the host plant cytosol. The pathways of ammonium assimilation was occured in the host plant cytoplasm. The major enzymes involved catalyze the following reactions:

NH3 Assimilation

Glutamine synthetase
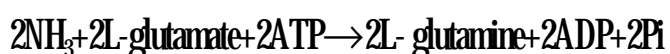
$$2NH_3 + 2L\text{-glutamate} + 2ATP \rightarrow 2L\text{-glutamine} + 2ADP + 2Pi$$

Glutamate synthase

$$2\text{-oxaloglutarate} + L\text{-glutamine} + NADH + H^+ \rightarrow 2L\text{-glutamate} + NAD^+$$

Asparate aminotransferase

$$L\text{-glutamate} + \text{oxaloacetate} \rightarrow L\text{-asparate} + 2\text{-oxoglutarate}$$

Asparagine synthetase

L-aspatate+L-glutamate-ATP$\rightarrow$L-asparagine+L-glutamate+AMP-PPi

Overall reaction

$2NH_3$+3ATP+oxaloacetate+NADH+$H^+$$\rightarrow$L-asparagine+AMP+2ADP+$2P_1$+$NAD^+$+PPi
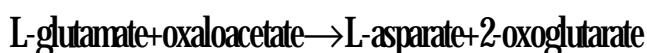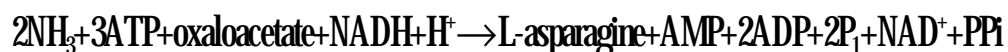
The overall costs for the fixation and assimilation of 1 mol N_2 is thereby 19 ATP. Compared to the 3 mol ATP per mol CO_2 fixed in C3 plant photosynthesis or 5 ATP in C4 plant photosynthesis, and to assimilation of other inorganic anions, N_2 fixation is by far the most costly primary fixation process in terms of energy.

Glutamate synthetase (GS) is an octameric enzyme with a molecular weight of 860,000. Four different subunits (alpha, beta, gramma, and delta) have been identified in nodules of *Phaseolus vulgaris* (Bennett et al 1988). The subunit delta is a plastidic form, the three others are cytosolic subunits. From these subunits two different nodule GS forms are synthesized, whereas roots contain only a single GS. Leaves also contain two isoenzymes, the cytosolic GS 1 and the chloroplast-associated form GS2. Reguratory compounds for GS (e.g., GTP and AMP) affect both enzymes in the same way, whereas glucosamine-6-phosphate only inhibits GS2 (Hirel et al. 1980 and Lara et al. 1984). Compared to roots, GS activity in nodules is about 15 times greater and represents more than 2% of the soluble nodule protein (Mcdermott et al. 1976). GS1 was expressed normally in nodules formed by *Bradyrhizobium* strains defective in nitrogenase activity, indicating that GS1 induction is not dependent on nitrogenase. The data on subunits of GS in soybean nodules are less clear than those for *Phaseolus* beans.

The next enzyme in the assimilation pathway is glutamine-2-oxoglutarate-amino-transferase (GOGAT); two NADH-dependent forms have been found in nodules of *Phaseolus vulgaris*, whereas in alfalfa nodules only one isozyme was detected (Anderson, M. P., et al. 1965). In contrast to GS, GOGAT seem to be a monomer with a high molecular mass of about 230 kDa. The K_m values for glutamine, 2-oxogultarate and NADH are 446 μM, 33 μM, and 42 μM, respectively (Anderson et al. 1989). Expression of the nodule NADH – GOGAT in other plant organs is very low. Antibodies against the ferredoxin-dependent GOGAT do not cross-react with the NADH-dependent GOGAT, indicating that these enzymes are distinctly different. Besides the NADH-dependent GOGAT there is also a soybean nodule ferredoxin-dependent GOGAT that is readily recognized by antibodies against rice leaf ferredoxin-GOGAT.

The enzyme for formation of aspartate from glutamate is the aspartate aminotransferase (AAT). Two immunologically different forms have been detected in nodules of alfalfa (AAT-1 and AAT-2) with molecular masses of 42 and 40 kDa (Farnham et al. 1990). AAT form 2 is a dominant form in nodules. In roots about 60% of activity is present in AAT-1. Two forms of AAT have also been found in soybeans (Ryan et al. 1972) and in peas (Appels et al. 1989). AAT-2 is not a nodulin, but a nodule enhanced protein with about a six-fold increase from root to nodules on a fresh weight basis (Vance et al. 1990). The nodule-enhanced form of AAT (synonymous with glutamate oxaloacetate transaminase/GOT) has much higher K_m values for glutamate (27 mM), oxaloacetate (0.14 mM), and aspartate (4.9 mM) as compared with the root form with 7.9 mM, 0.019 mM for the three substrates (Appels et al. 1989).

The central role of asparagine synthetase in amide-exporting nodules can be easily demonstrated by determination of amino acid concentrations in the cytosol (Ta et al. 1986). The concentration of asparagine in the host plant cytosol was 44 mM. Ammonia concentration was 4.3 mM, glutamate concentration, 0.2 mM; and alanine concentration, 2.2 mM. The concentration of all amino acid was below 1 mM. Asparagine synthetase activity increased 170-fold in nodules of soybeans compared to cortex tissue (Huber et al. 1984). The apparent K_m values for the substrate aspartate Mg-ATP, and glutamine are 0.24 mM, 0.076 mM, and 0.16 mM, respectively. The K_m for ammopnium ions as a N donor is 40 times larger than that for glutamine.

In ureide-exporting legumes (e.g., soybeans and *Phaseolus* beans) glutamine, glycine, and aspartate are used for purine synthesis in plastids of the infected cells. Purine nucleotide is transformed to xanthine and uric acid, which is transformed by peroxisomes in neighboring uninfected cells to allantoin. This is further transformed in the smooth ER of uninfected cells to allantoic acid (Mellor et al. 1990). The nodulin uricase II, located in the peroxisomes of the uninfected cells, is involved in this metabolism (Nguyen et al. 1985). There is a strong correlation between N_2-fixation rates and the total N and ureides in soybeans. It is interesting that allantoin amino hydrolase responds to inhibition of N_2-fixation and urate transport by NH_4NO_3 (Blevins et al. 1988).

1.5 Rhizobial Characteristics

Rhizobia or root nodule bacteria are medium-sized, rod shaped cells, gram-negative, 0.5-0.9 μm in width and 1.2-3.0 μm in length. They do not from endospore and mobile by single polar flagellum or two to six peritrichous flagella. Uneven gram is frequently encountered with rhizobia, depending on the age of the culture. Cells from a young culture and nodule bacteroids usually show even gram staining while older and longer cells give a banded appearance with unstained areas. These unstained areas have been identified to be large granules of polymeric beta-hydroxybutyric acid (PHBA). The PHBA is refractile under phase-contrast microscopy. Rhizobia are predominantly aerobic chemoorganotrophs and are relatively easy to culture (Allen, 1980). They grow well in the presence of O_2 and utilize relatively simple carbohydrates and amino acid compounds. With the exception of a few strains, they have not been found to fix atmospheric nitrogen in the free-living form except under special conditions. Optimal growth condition are of most strains at a temperature range of 25^0-30^0C and pH of 6.0-7.0. Despite their usual aerobic metabolism, many strains are able to grow well under microaerophilic conditions at O_2 tensions of less than 0.01 atm (Graham and Parker 1964). Generally, most rhizobia produce white colonies, but those that nodulate *Lotononis bainesii* produce a characteristic red nonheme carotenoid pigment when cultured in yeast-mannitol (YM) medium. Most rhizobia only absorb congored (diphenyldiazo-bis-α-nephthylaminesulfonate) dye, which is included in culture media for isolating rhizobia. However, if the culture medium is not buffered, acid-producing rhizobia cause the dye to turn purple. Other interesting and useful characteristics of rhizobia are other growth reactions in the standard YM medium containing bromthymol blue (BTB) as the pH indicator. Fast growing rhizobia produce an acid reaction in the YM medium containing bromthymol blue (pH 6.8) while slow growers produce an alkaline reaction (Date, 1979).

The basic knowledge on the rhizobial identification by different standards such as 1) some morphological and physiological aspects 2) serological marker 3) antibiotic resistant profile 4) nitrogen fixation potential 5) host-dependent cross-inoculation group 6) molecular biology techniques, etc. For instant of some morphological and physiological aspects as growth rate that *Rhizobium* was fast-grower and *Bradyrhizobium* was slow-grower, colony type, acid-base reaction on YM medium containing bromthymol blue. This technique could segregate only 2 groups were fast-grower that could produce acid while slow-grower that could produce base, and IAA

production which could use for separate only soybean nodulation rhizobia (Somasegaran et al. 1965 and Norris, 1964). The serology, indirect procedures for identifying rhizobia by antigen-antibody reaction are highly reacts only with the antigen that elicited its formation. Bacterial strains contain naturally occurring mutants which are resistant to certain antibiotics. This resistant may be used for the recognition of rhizobial strains. The cross inoculation group system of classifying rhizobia has been subjected to much criticism, because it is not a taxonomic one, it is the best practical system currently available (Burton, 1965). The molecular biology techniques, the new technique for study genotypic and phylogeny of rhizobia also has been developing from time to time.

Eventually, with the development of molecular sequencing technique especially of the 16S rRNA gene, the situation has changed. The most prominent person in initiating this revolution study of microbial phylogeny and evolution was Carl Woese who from the sixties on has relentlessly worked towards defining the natural order of microbial life (Woese 1987; Woese 1994; Morell 1997).

The current status of rhizobial taxonomy as shown in Table 2, a few years after the work of Young et al. (1996), the first phylogenetic trees based on close to full-length 16S sequences were published for rhizobia and related organisms (Willems and Collins, 1993; Yanagi and Yamasato 1993). These trees showed that rhizobia can be devided into three clusters: 1) the fast- or moderate-growing rhizobia in the genera *Rhizobium, Sinorhizobium, Mesorhizobium* and *R. galegae,* which is located in the branch containing serveral *Agrobacterium* species, 2) the slow-growing *Bradyrhizobium* and 3) the stem nodulating *Azorhizobium* Sequencing of the full length of the 16S rRNA gene is now required for the description of new species. The correlation between full-length sequences and various shorter fragments has in general proved to be good and very useful when used for initial strain recognition and screening of large numbers of isolates (Oyaizu et al. 1993; Laguerre et al, 1993; So et al. 1994; van Rossum et al. 1995; Hernandez-Lucas et al. 1995; Urtz and Elkan 1996).

Table 2. Currently described species of rhizobia (Young and Haukka, 1996)

Species	Representative strains
Rhizobium	
Rhizobium leguminosarum	ATCC 10004
bv. viciae, trifolii and phaseoli	
Rhizobium tropici type B	CIAT 899
type A	CFN 299
Rhizobium etli	CFN 42, CE-3
Rhizobium hainanense	CCBAU 57015
Sinorhizobium	
Sinorhizobium meliloti	ATCC 9930
Sinorhizobium fredii	USDA 205
Sinorhizobium teranga	ORS 1009
Sinorhizobium saheli	*ORS 609*
Sinorhizobium medicae	A 321, CC169
Mesorhizobium	
Mesorhizobium loti	NZP 2213
Mesorhizobium huakuii	CCBAU 2609
Mesorhizobium ciceri	UPM-Ca7
Mesorhizobium mediterraneum	UPM-Ca36
Mesorhizobium tianshanense	CCBAU 3306
[Rhizobium]	
[Rhizobium] galegae	HAMBI 540
Bradyrhizobium	
Bradyrhizobium japonicum	ATCC 10324
Bradyrhizobium elkanii	USDA 76
Bradyrhizobium liaoningense	2281
Azorhizobium	
Azorhizobium caulinodans	ORS 571

Before the development of molecular methods, bacteria used to be classified according to their phenotypic features. Nowadays, however, the taxonomy and phylogeny of bacteria is based increasingly on genotypic characteristics, that is on the genetic constitution of the organism. The bacterial genome can be divided into two parts: chromosomal DNA and plasmid DNA. It has become apparent that the phylogenies of these two components do not necessarily agree. This is especially interesting, because in fast-growing rhizobia the symbiotic genes commonly reside in plasmids, which can transfer between strains. Thus instead of being a permanent association, the chromosomal background-plasmid combinations can vary. However, also symbiotic genes located in the chromosome have been shown to be transferable. There are many techniques that have been uesed for studying the chromosomal diversity of rhizobia, for example multilocus enzyme electrophoresis (MLEE), whole-cell protein analysis, pulsed-field gel electrophoresis(PFGE), DNA-DNA hybridization, DNA restriction fragment length polymorphism (RFLP), 16S rRNA gene sequencing restriction fragment length polymorphism of PCR-amplified DNA fragments (PCR-RFLP), PCR with random or arbitrary primers (RAPD, AP-PCR) etc. In this thesis, The PCR-RAPD was selected to study phylogenetic tree-biodiversity for distinguish rhizobial isolates from soil samples.

1.6 Genes Involving in Nodulation and Nitrogen Fixation

1.6.1 Nodulation genes

Nodulation genes *nod* (also *nol* and *noe*) are essential for the first steps of infection and nodule formation in a *rhizobium*-legume symbiosis. Plant secreted flavonoid compounds first activate reguratory *nodD*, which in turn controls the transcription of the other rhizobial *nod* genes. The *nod* genes products are involved in the synthesis of lipo-chitooligosaccharide (LCO) signal, or Nod factors (Truchet et al.1991; Demarie et at. 1996; Debelle et al. 1997). Nod factors, in turn, induce various root responses including root hair deformation and cortical cell division. The structural *nod* genes responsible for production of Nod factors can be classified into two groups, the common and host specific genes. The common *nodABC* have been found in all rhizobia studied and they are structurally and functionally conserved. In most species the *nodABC* genes are part of a single operon (van Rhijn and Vanderleyden 1996). However, in *R. etli, nodA* is separated by 20 Kb from the *nodBC* genes

(Vazquez et al. 1991) and in *Mesorhizobium loti nod B* is found on an operon separate from *nod ACIJ* (Scott et al. 1996). Studies on *nodABC* genes have led to the following model of how Nod factors are synthesised (Ritsema et al. 1996). Nod*C* is involved in the biosynthesis of a chitin oligosaccharide backbone from UDP-N-acetylglucosamine precursors. Subsequently, the non-reducing terminal saccharide of the chitin oligosaccharide is de-N-acetylated by Nod*B*, resulting in a free amine. The role of Nod*A* in the transfer of fatty acids resulting in a core LOC. The host specificity of Nod*A* in the Nod factor synthesis was recently reported by several groups (Debelle et al. 1996; Ritsema et al. 1996; Roche et al. 1996). Their studies showed that the common nod genes can not be freely exchanged between species without affecting the host-range, which is contrary to what was previously believed. Thus allelic variation in *nodA*, as well as in *nodB* and *nodC*, contribute the control of host range via variation in the Nod factor structure (Relic et al. 1994).

In addition to the common *nod* genes, the host-specific nod genes play an important role in Nod factor synthesis. These genes, which include *nodEF, nodH, nodQ, nodSU* etc., add particular 'decoration' to the LCO backbone. The substitutions can take place both at the nonreducing and reducing end and they include, for example, methylation, fatty acid substitutions, acetylation, carbamylation (Denarie et al. 1996). Mutation and complementation experiment have been used to study the role of particular host-specific *nod* genes in nodulation of certain legumes. For example, several studies have been published where the host–range of a *rhizobial* strains has been extended to a tropical tree species *Leucaena leucocephala*. In nodulation of this tree species *nodS*, coding for a methyltransferase, seems to be an essential gene (Lewin et al. 1990; Krishnan et al. 1992; waelkens et al.1995). However, van Rhijn et al. (1996) found that in *Rhizobium* sp. BR816 (Brazilian isolate from *L. leucocephala*) *nodO* might have the same function as *nodS* in *Sinorhizobium* sp. NGR234 and in *R. tropici*, and when *nodO* was transferred to *R.etli* CE-3 this gained the ability to modulate *L. teucocephala*.

Thus, the host-range of any rhizobial species seem to be, at least partly, determined by the range of produced Nod factors it produces. The board host range of *Sinorhizobium* sp. strain NGR234 has been found to be mediated by the synthesis of a whole family of LCO signals, with differing 'decoration' of the backbone (Price et al., 1992). However, all strains produce a mixture of Nod factors, and whether this diversity is important to broadening the host range or to

diversification of signalling to the same host is unknown. Reciprocally, the ability of plants to be nodulated by a narrow or broad spectrum of rhizobia can be a consequence of the plant's capability to recognize different types of bacterial signals. For example, efficient infection of alfalfa requires rhizobia that produce Nod factors with three precise substitutions, whereas, in contrast, *Phaseolus* can be nodulated by various *Rhizobium* species that produce quite different Nod factors (Demarie et al. 1996).

The regulatory NodD protein plays an important roles in activation of other *nod* genes. In the presence of plant flavonoid signals, The NodD binds to conserved DNA sequences, called *nod* boxes, located upstream of the inducible *nod* operon and acts as transcriptional activator of these genes (Rostas et al. 1986; van Rhijn and Vanderleyden 1995). The domain topology of NodD suggests that the N-terminus of the protein (corresponds to the 5' end in the DNA sequence) bind to the *nod* box, whereas the C-terminus interacts with the flavonoid inducer (Gyorgypal et al. 1991a). Consequently, the 5' end of NodD is more highly conserved than the 3' end.

1.6.2 Nitrogen fixation genes

The genes coding for and controlling the nitrogen-fixing apparatus in rhizobia are designated as *nif* and *fix*. The *nif* genes are structurally homologous to the *Klebsiella pneumoniae* genes, which were the first characterised nitrogen fixation genes. The *fix* genes are essential for nitrogen fixation in rhizobia, but do not have homologous counterparts in *K. pneumoniae* (Fischer 1994). In rhizobia, the *nif* and *fix* genes are organized in distinct clusters whose structure and genomic location are species specific. The recently published complete nucleotide sequence of the *Sinorhizobium* sp. NGR234 symbiotic plasmid pNGR234a reveals a single large cluster, which contains all 43 *nif* and *fix* genes, but no *nod* genes (Freiberg et al. 1997). In *S. meliloti* the nitrogen fixing genes are divided into two clusters I and II, which are both located on the same megaplasmid. Cluster I is interrupted by a cluster of nod genes, including *nodABC* (Fisher 1994). *B. japonicum*, on the other hand, has no plasmids and thus the symbiotic genes are located on the chromosome. Its *nif* and *fix* genes are divided into four clusters, which can contain also *nod* genes and are dispersed along about 1,000 Kb of the chromosome (Fisher 1994). The study by Barbour et al. (1985) on *S. fredii* suggested that the species might have both plasmid- and chromosome-borne multiple *nif* genes.

Phylogentic studies have been conducted on different nitrogen-fixation genes e.g. *nifD* (Ueda et al.1995b), but *nifH* has been the one most commonly used. *nifH* is often part of a *nifHDK* operon, where *nifD* and *nifK* code for the α and β subunits of component I in nitrogenase, respectively, and *nifH* codes for two identical subunits of component II, nitrogenase reductase. The nitrogenase enzyme complex, comprising the components I and II is responsible for the actaul dinitrogen fixation. *nifHDK* genes are highly conserved, *nifH* might be even the most conserved translated gene in bacteria (Ruvkun and Ausubel 1980; Norel and Elmerich 1987). In rhizobia *nifH* can exist in multiple copies: *R. etli* has three (Quinto et al. 1985), *A. caulinodans* two (Norel and Elmerich 1978) and *Sinorhizobium sp.* NGR234 two copies (Badenoch-Jones et al. 1989). However, sequencing the multiple copies has shown that they were or closely related. Several studies conducted on *R. etli* strain CFN42 have indicated that recombination between *nif* gene reiterations can lead to high-frequency amplification and deletion events taking place in the pSym plasmid (Quinto et al. 1982; Martinez et al 1985; Quinto et al. 1985; Brom et al. 1991; Romero et al. 1991; Flores et al. 1993; Romero et al. 1995). Possibly this ability to rearrange can facilitate rapid and reversible adaptation to environmental factors that require expression of the specific genes, like *nif*, carried in tandem amplifications. Although reiteration of the Rhizobiaceae genome reiterated sequences have been found to enhance the frequency of rearrangements in the *rhizobium* genome and thus produce variation between rhizobial line ages descending from a common ancestor (Flores et al. 1987, 1988; Martinez et al. 1990).

1.7 Impact of Rhizobial Biodiversity

Rhizobium is one of nitrogen-fixing microorganism that was found in the most of area. It showed the crucial role in BNF which very important in agricultural field especially the food legume. Study of range of nodules out with the normal group of economically important crop and forage legumes has not only given new insights into primitive features of nodule structure, but parallel studies on their rhizobia are beginning to give clues as to how rhizobia may have evolved their endosymbiotic life-style. Currently, the basic knowledge on the diversity, taxonomy and phylogeny of the rhizobia has been very scared, in the other hand, a lot of research tried to study biodiversity in term of phylogeny that can be defined simply as an evolutionary history of organism or gene.

1.7.1 Identification rhizobia in term of genotypic diversity and phylogeny

The research by Young and Haukka (1996), who study diversity and phylogeny of rhizobia that used the sequence of gene for the 16S or small subunit of ribosomal RNA (SSU rRNA) to classified rhizobia. The result from this study support the well-established subdivision of rhizobia into three genera: *Rhizobium, Bradyrhizobium and Azorhizobium* and these all lie within the alpha subdivision of the *Proteobacteria*. The backbone of phylogenetic picture that just represented is the comparison of SSU rRNA sequence. This approach has revolutionized our understanding of bacteria evolution and classification over the last few years. However, any method has limitations, and in the case of SSU sequences the limitations are particularly apparent at the find end of phylogenetic scale. The sequence are very useful for assigning species to genera and for defining the relationships among genera, but frequently do not have the power to sort out the relationships between close species. Some of the sources of this limitation are illustrated by the following cases.

It is usually assumed that ribosomal rRNA genes are not transferred between species, and that their evolution in different lineages is therefore indestructing valid phylogenies. However, Sneath (1993) presented a detailed analysis of a set of sequences that showed clear evidence for recombination between SSU genes in different lineages of the genus *Aeromonas*, and similar phenomena are apparent in the data for rhizobia. Either there has been substantial parallel evolution in different lineages or, more likely, recombination has generated 'hibrid' sequences. In either case, the result is anomalously short branch lengths on the tree and reduced confidence in the phylogeny.

There is a substantial number of differences between the SSU sequences of the type strains of *R. leguminosarum* and *R. etli*. Eardly et al. (1995) used restriction enzymes to distinguish these two types in a large sample of bean rhizobia that were also characterized for other chromosomal genes by multilocus enzyme electrophoresis. They found that some strains that had an enzyme type that clustered with *R. etli* nevertheless had the SSU pattern typical of *R. leguminosarum* and vice versa. Thus the differences in SSU sequence do not necessarily indicate a correspondingly large divergence of the genome as a whole. Either the two SSU types have both been inherited by both species from their common ancestor, of there is still some gene exchange within this group.

The rhizobia have for many years been placed, with the agrobacteria and phyllobacteria, in their own family, the Rhizobiaceae. However, it is now clear that this family, defined almost entirely by the interactions of these bacteria with plants, is not natural. DNA sequence comparisons indicate that each of recognized rhizobial genera has close relatives that are placed in quite different 'families'.

Several authors have constructed phylogenies base on *nod* gene sequences and have concluded that they are not congruent with those based on SSU rRNA (Gyorgypal, Kiss and Kondorosi, 1991; Dobert, Breil and Triplett, 1994; Lindstrom et al., 1995a). For example, the *nodABC* and D genes of *S. fredii* and *S. meliloti* are not closely related; they are closer to those of *B. japonicum* on the one hand and to *R. leguminosarum* biovars *trifolii* and *viciae* on the other. This makes sense in terms of their respective host plant ranges, and is more likely to reflect lateral transfer than convergent evolution since this genes the so-called ' common *nod* genes' are not major determinants of host range. Analysis of *nifH* sequences indicated that they, too, do not necessarily reflect the phylogeny of the rhizobia that carry them (Eardly, Yound and Selander, 1992), Strengthening genes have move between bacterial lineages. If they often move together, this would explain why *nif* genes and *nod* genes are often tightly linked, even though there no functional necessary for this. The history of these genes will have to be worked out separately from that of the bacteria that now carry them.

1.8 Aims of the Study

The objectives of this thesis were to find the answer to the solutions were **the suitable of environment and ecosystem for rhizobia to maintain sustainably, the effect on the population of rhizobia after that environment and ecosystem were changed and polygenetic diversity and dominant native strains of rhizobia under diversed ecosystems.**

The answers receiving from this study would be able to bring about the management for proper utilization of nitrogen fixing microorganisms in increasing soil fertility in sustainable agriculture and forestry.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Rhizobial isolates

Rhizobial isolates were obtained from soil samples which were collected from 11 areas in the Central, the North and the North Eastern part of Thailand. Only 4 isolates from each area were randomly selected for further study.

2.1.2 Culture media

Composition per litre of each medium was as following;

A. Yeast manitol medium(YM)

The medium containing :	Na_2HPO_4	0.5 g
	: D-manitol	10.0 g
	: Yeast extract	0.4 g
	: NaCl	1.0 g
	: $MgSO_4.7H_2O$	0.2 g
	pH 6.8	

B. Tryptone yeast extract medium(TY)

The medium containing :	Tryptone	5.0 g
	: Yeast extract	3.0 g
	: $CaCl_2.2H_2O$	0.1 g
	: $MgSO_4.7H_2O$	0.2 g
	pH 6.8	

C. HM medium

The medium containing :	Na_2 Glutamate	1.0 g
	: Na_2HPO_4	0.125 g
	: $NaSO_4$	0.25 g
	: NH_4Cl	0.32 g
	: $MgSO_4.7H_2O$	1.8 g
	: $FeCl_3$	0.004 g

	: $CaCl_2.2H_2O$	0.013 g
	: HEPES	1.3 g
	: MES	1.1 g
	: Yeast extract	1.0 g
	:L- arabinose	1.0 g
	pH 6.8	

D. N-free medium

The medium containing :	KH_2PO_4	0.1 g
	:$MgSO_4.7H_2O$	0.12 g
	:KH_2PO_4	0.1 g
	:$Na_2HPO_4.2H_2O$	0.15 g
	:Ferric citrate	0.005 g
	:Mn, Cu, Zn, B, Mo	traces
	(From stock containing : 0.5% Bo, 0.05% Mn, 0.005% Zn, 0.005% Mo and 0.002% Cu)	
	pH 6.5	

E. Tris-TMRT medium

The medium containing :	D-manitol	10.0 g
	: Yeast extract	0.2 g
	: $CaCl_2 .2H_2O$	0.2 g
	: $MgSO_4.7H_2O$	0.25 g
	: Tris base	1.21 g
	: L-tryptophan	0.061 g
	pH 6.8	

21.3 Chemical

All chemicals used were laboratory grade, or otherwise specified.

A. For morphological and physiological aspect

- Bromthymol Blue (BTB) : Stock solution 0.5 g/100 ml ethanol

Add 5 ml stock/ liter YMA

Final concentration of BTB ;

25 ppm.

- CongoRed (CR) : Stock solution 0.25 g/100 ml distilled water

Add 10 ml stock/ liter YMA

Final concentration of CR ;

25 ppm.

- 0.01 M $FeCl_3$ in 35% $HClO_4$

B. For acetylene reduction assay (ARA measurement)

- Calcium carbide

- gases : Compresser air   From Thai Industrial Gas Limited.

Hydrogen   From Thai Industrial Gas Limited.

Ethylene standard   From Thai Industrial Gas Limited.

Nitrogen   From Thai Industrial Gas Limited.

Calcium carbile

- Column :   Porapak N

C. For antibiotic resistant profiles

- YMA containing congored

- Antibiotics (as summarized in Table 3)

Table 3 The 8 kinds of antibiotic and suspension used in this study

Antibiotic	Concentration in medium (μg/ml)	Suspended in
Carbenicillin (Car)	500 μg/ml	Distilled water
Chloramphenicol (Chl)	500 μg/ml	Distilled water
Erythromycin (Ery)	250 μg/ml	50% ethanol
Kanamycin (Kan)	100 μg/ml	Distilled water
Nalidixic (Nal)	50 μg/ml	0.35N NaOH
Tetracycline (Tet)	100 μg/ml	50% ethanol
Trimetroprim (Tmp)	50 μg/ml	70% ethanol
Streptomycin (Str)	100 μg/ml	Distilled water

D. For PCR-RAPD amplification

- 20% NaClO

- 1 M NaCl

- Reagent for PCR reaction :10X buffer production from Promega

                                    :dNTP mixture production from Promega

                                    :MgCl$_2$ production from Promega

                                    :*Taq* polymerase production from Promega

                                    :primer production from Bioservice unit

- Reagent for gel running gel of PCR product

                                    :TBE buffer

                                    :Ethidium bromide for gel staining

                                    :Agarose gel

Table 4 Preparing of TBE buffer

Buffer	Concentration	Amount of chemicals for prepared TBE 1 L
Tris-borate, pH 8.3	89 mM Tris OH	10X: 108 g Tris base
(TBE)	89 mM boric acid	55 g boric acid
	2.5 mM EDTA	3 g Na$_2$ EDTA

E. For the host-dependent cross-inoculant group

- HM medium

- H$_2$O$_2$ 20%

- Alcohol 70%

2.1.4 Other materials

- Growth pouch

- Growth rack

- Siratro seed

- Soybean seed

- Cowpea seed

- Sesbania seed

- Petridish and petridish box

- Test tube

- Plastic bag

- Ice box

- Standard lab

2.1.5 Other equipments

- Gas chromatography
- UV transilluminator
- Hot air oven
- Incubator
- Autoclave
- Electrophoresis set
- Gel documentation
- Refrigerator $4^{\circ}C$
- Freezer $-20^{\circ}C$
- Deep freezer $-70^{\circ}C$
- Laminar flow
- Shaking and shaking water bath
- Water bath
- Microwave oven
- Centrifuge
- Light shelf

2.2 Methods

2.2.1 Soil samples collection

The soil samples were collected from three regions, the North, the North Eastern and the Central part of Thailand. In each region, soil samples were collected from highest elevation as on the top of the mountain, in the middle and at the foot hill of mountain. Soil samples were also collected from the flat area of agricultural practice as field crop cultivation, rice cultivation, rice in rotation with other crops, uncultivated area, undisturb forest, forest clearance for crop cultivation for 1-2 years, 3 years and in the areas where intensive agricultural production using high rate of pesticides and fertilizers. Soil samples were collected from rainy season and dry season for further analysis. The information of temperature in air and soil, the character of the environment and the type of plant around site selection were recorded.

2.2.2 Enumeration of rhizobia

The most-probable-number (MPN) count by using index plant as siratro was used to determine the number of viable and infection rhizobia from soil sample in four fold dilution (Somasegaran and Hoben, 1994). The amount of soil sample, 250 g was weighted and separated into 2.0 litre flask containing steriled water 750 ml and shake vigorously for 5 min after close flask with a steriled rubber stopper. This give 4^1 dilution of rhizobial inoculant in steriled water. Continue the serial dilutions using 1.0 ml aliquots of inoculant suspension and 3.0 ml sterile water in tubes to obtain dilutions 4^2 to 4^9. The inoculant was inoculated on seeding of siratro on growth pouches 1 ml of each dilution (from 4^1 to 4^9) to each one of four replicates in each set. Nodulation may be evident after 2 weeks. Make the final observation after 3 weeks and record presence(+) or absence (-) of nodules. The four fold dilutions was using for estimation of the number of rhizobia by log number of cell at 1-5% level by DMRT.

2.2.3 Rhizobial isolation

The nodule from enumeration rhizobia process was selected for isolated rhizobial strains by surface sterilized and crushed the nodule. Bacteroid from nodule was streaked on YMA+congored medium and purified before further study.

2.2.4 Characterization of rhizobia

A. Some morphological and physiological aspects

- Colony and acid-base production characteristics

Each rhizobial isolates was streaked on YMA containing bromthymol blue and incubated at $30^\circ C$. The colony type was observed after single colony occurring such as large colony (diameter of colony more than 2 mm), medium colony (diameter of colony between 1- 2 mm) and small colony (diameter of colony less than 1 mm), including the other characters as dry colony (gum and surface) and wet colony (slimey)(Mpepereki et al.1996). The fast-grower rhizobia can produced acid (change the color of agar from green to yellow color) and colony forming within 2-4 days, while the slow-grower can produced alkaline (change the color of agar from green to blue color) and formed colony within 4-8 days (Somasegaran et al.1982).

- Indole Acetic Acid (IAA) production assay

Strains were grown at $28^\circ C$ in the dark cultured for 10 days on Tris-

TMRT broth. IAA accumulated in the culture was determined by adding 2 ml of 0.01 M $FeCl_3$ in 35% $HClO_4$ to 1 ml of supernatant of the culture. The color reaction was detected after 30 min incubation in the dark at 30° C, and compared with positive control (pink to orange) and negative control (yellow).

B. Nodulation and N_2 fixing ability with index plant (siratro)

Siratro seeds were surface-sterilized by hot water at 85-90° C and waiting for the swelling of seed treatment. Seeds were placed on wet sterilized cotton plate and kept in the dark for 1-2 days. Seedlings were grown in sterilized growth pouches supplemented with N-free medium. The rhizobial inoculants were inoculated to seedlings at the amount of 1 ml (10^6-10^7 cells)/seed and moved to the light-shelf that set up 12 h in light and 12 h in the dark. After 45 days the root nodules were detected and nitrogen fixing activity by using acetylene reduction assay. The value of plant dry weight, nodules weight and amount of nodules were also collected.

C. Antibiotic resistant profiles

Each rhizobial strain was grown in YMB until the mid of log phase (OD_{600nm}=0.4-0.6). The strains were tested in YMA-congored medium containing 8 kinds of antibiotic (Table 2). The 10 μl of cultures were dropped on every antibiotic medium and observed after 4-7 days of incubation at 30° C, after that compared with the control condition (without additional of antibiotics). The results were reported in the form of resistant (r) if colony was usually grown, susceptible if colony could not be grown (s) and susceptible with mutation (s_m) if colony was unusually grown.

D. The host-dependent cross-inoculation group

The rhizobial isolates were cultured in HM medium about 5 days, while 3 seedplants as soybean, cowpea and sesbania seed were surface-sterilized by 20% H_2O_2, 70% alcohol and rinsed with sterilized DW. The seed were cultured on petridish with wet cotton in the dark about 2 nights. Seedlings were grown in sterilized growth pouches that filled with N-free medium. The rhizobial inoculants were inoculated to seedings at amount of 1 ml (10^6-10^7 cells/seed) and moved to the light-shelf that set up 12 h in light and 12 h in the dark in each day. The data were recorded after the plants were nodulated by rhizobial inoculants.

E. PCR-RAPD amplification and phylogenetic tree

DNA template was extracted from 1.5 ml of HM broth rhizobial culture In microcentrifuge tube 1.5 ml, centrifuged at 12,000 rpm 5 min and removed the supernatant. The pellet was cleaned by suspended in 300 μl of sterile distilled water, centrifuged at 12,000 rpm 5 min and removed supernatant once more. The pellet was resuspended in 100 μl of sterile distilled water, boiling for 5 min for and put in ice immediately for 3 min. The last step was centrifuged at 12,000 rpm for 5 min once again and the supernatant was used for DNA template 1 –2 μl followed by the final volume of PCR reaction (about 50 ng). The PCR component and condition were shown in Table 5

Table 5 Primer sequence and condition of the PCR-RAPD reaction

Component	Condition
10X buffer (1X)	1) 94°C 1 min, 2) 36°C 1 min,
dNTP mixture (0.25 mM each)	3) 72°C 2 min, and 4) recycle step
MgCl$_2$ (1.5 mM)	1-3 44 cycles
Primer RAPD (50 pmol) ($^{5'}$GTT TCG CTC C $^{3'}$)	
Taq-polymerase (1 unit)	

The PCR products were separated by gel electrophoresis, using 1.2% agarose gel at 80-100 volt.

Phylogenetic tree was constructed from PCR-RAPD band of rhizobial isolates by using Neighbor-joining and UPGMA methods by Nei and Saitou 1987.

3 RESULTS

3.1 Determination Rhizobial Population Number

To investigate the kind of environment suitable for rhizobial establishment, population dynamics of rhizobia in undisturbed and agricultural practice conditions. Site selection was conducted in three regions, the Central, the North and the North Eastern. In each region, soil samples were collected from highest elevation as on the top of mountain, in the middle and at the foot hill of mountain. The another group of collected soil sample were from flat area of agricultural practice as field crop cultivation, rice cultivation, rice in rotation with other crops and uncultivated area. Soil samples were collected every two months until completion for 3 years. On the other hand, to elucidate the occurrence of rhizobial population upon changing the environment and cropping system, soil samples were collected from undisturb forest, forest clearance for crop cultivation for 1-2 and 3 years and in the areas where intensive agricultural production using high rate of pesticides and fertilizer. Rhizobial number from all collected soil was enumerated from number of nodule forming in siratro on the basis of MPN-plant infection approach.

3.1.1 The number of rhizobia in the Central region

The result obtained from this region found that population number of rhizobial isolates in rainy season higher than in dry season. In highest elevation areas showed high amount of rhizobial number at foot hill of mountain in both of seasons. Because this area also found legume plant as leucaena, the normal plant in area. However, low rhizobial population at highest mountain and middle mountain was observed in particular dry season. These results could be concluded that the suitable environment in highest elevation area was the foot hill of the mountain. In flat area of agricultural practice, high number of rhizobia at rice in rotation with other crops in both of seasons were found. The other areas where shown low number of rhizobial isolates as rice cultivation from both of seasons and uncultivated in dry season were indicated the unsuitable environment in these areas. For comparison among field crop cultivation, rice cultivation, rice in rotation with other crops and uncultivated areas found that in rice in rotation with other crops showed high rhizobial population even in dry season. This area always cultivated

legume plant as mung bean indicated that the influence by legume plant could maintain rhizobia in ecosystem. In Table 7, to study the effect on the population of rhizobia after environment and ecosystem changed, the majority of rhizobial population in both of cropping system and undisturb forest were not different. The rhizobial population in rainy season was higher than dry season, except in forest clearance for crop cultivation for 1-2 years because this area was cultured mung bean only in dry season. The intensive agricultural production using high rate of pesticides and fertilizers area was not be found rhizobial population. This area where the leguminous plant had never been cultivated and always used pesticides and fertilizers suggested that the environment changed by using high rate of pesticides and fertilizers was high effect to rhizobial population if did not cultivate legume plant to rotated with normal plants. The data in Table 6 and 7 could confirm that the suitable season for rhizobial population in natural was rainy season and using leguminous plant in agricultural system could maintain rhizobial isolates in ecosystems.

Table 6 Rhizobial population in soil from the Central region (log number of cell/g soil)

Areas	Rainy season (September/1997)	Dry season (January/1998)
1. Highest mountain	1.32 cd[1/]	0.40 b
2. Middle mountain	2.05 bc	0.98 b
3. Foot hill of mountain	3.45 a	2.45 a
4. Field crop cultivation	1.80 bcd	1.01 b
5. Rice cultivation	0.73 d	0.45 b
6. Rice in rotation with other crops	2.68 ab	2.37 a
7. Uncultivated	2.56 ab	0.57 b
CV(%)	37.4	46.7

1/ Values followed by the same letter are not significantly different at 1% and 5% level
    by DMRT.

Table 7 Rhizobial population from cropping system and undisturb forest areas
in the Central region (log number of cell/g soil)

Areas	Rainy season (September/1997)	Dry season (January/1998)
1. Undisturb forest	1.56b[1]	0.75a
2. Forest clearance for crop cultivation for 1-2 years	2.08a	2.46a
3. Forest clearance for crop cultivation for 3 years	2.53a	2.00a
4. Intensive agricultural production using high rate of pesticides and fertilizers	0	0
CV(%)	11.7	25.4

1/ Values followed by the same letter are not significantly different at 1% and 5% level
by DMRT.

3.1.2 The number of rhizobia in the North region

The rhizobial population in soil from the North region was summarized in Table 8. The highest elevation area was displayed highest population at the foot hill of mountain in rainy season and in this season was mostly higher population than in dry season. This data showed the suitable environment in rainy season and indicated the critical condition, the highest mountain was draught. The flat area of agricultural practice was shown highest population in rice in rotation with other crops which increased even the dry season. This area showed high effect by crop rotation with legume plant as soybean could maintain rhizobial population. While the other areas found that in rainy season promoted higher population than in dry season. Among the agricultural practice area, rice cultivation system seemed to be the most inadequate ecosystem since less rhizobial population number was established. From Table 9 which displayed rhizobial population from cropping system and undisturb areas in the North region, the high population was found in dry season especially in undisturb forest. The data from this area not clear. Without any rhizobial population in these areas was found in forest clearance for crop cultivation for 1-2 years. This data showed that the effect from cropping system for 1-2 years in dry season to rhizobial distribution was found. Rhizobial populations in forest clearance or crop cultivation for 3 years and intensive agricultural production using high rate of pesticides and fertilizers were maintained in these areas.

Table 8 Rhizobial population in soil from the North region (log number of cell/g soil)

Areas	Rainy season (September/1997)	Dry season (January/1998)
1. Highest mountain	1.42 cd[1]/	0
2. Middle mountain	1.82 bcd	2.01 b
3. Foot hill of mountain	3.02 a	1.56 bc
4. Field crop cultivation	2.19 abc	1.81 b
5. Rice cultivation	1.09 d	0.62 c
6. Rice in rotation with other crops	2.90 a	3.13 a
7. Uncultivated	2.38 ab	2.07 b
CV(%)	28.4	35.7

1/ Values followed by the same letter are not significantly different at 1% and 5% level by DMRT.

Table 9 Rhizobial population from cropping system and undisturb forest areas in the North region (log number of cell/g soil)

Areas	Rainy season (September/1997)	Dry season (January/1998)
1. Undisturb forest	1.023 a[1]/	3.80 a
2. Forest clearance for crop cultivation for 1-2 years	1.480 a	0
3. Forest clearance for crop cultivation for 3 years	0.930 a	0.98 b
4. Intensive agricultural production using high rate of pesticides and fertilizers	1.107 a	1.50 b
CV(%)	52.3	24.8

1/ Values followed by the same letter are not significantly different at 1% and 5% level by DMRT.

3.1.3 The number of rhizobia in the North Eastern region

The results of rhizobial population in the North Eastern region were displayed in Table 10. In highest elevation area showed higher population in rainy season than dry season. Generally, in rainy season the population number of rhizobia in each area was not significantly different. However, less population found from rice in rotation with other crops and uncultivated areas in dry season was not significantly reduced when compared with in rainy season. This data could explain the effect by leguminous plant as mung bean that rotated with rice in rice in rotation with other crops area could maintain rhizobial population but the data of uncultivated area not clear. In Table 11, displayed the effect by cropping system and undisturb forest, the results suggested that in rainy season higher population than dry season was found. The forest clearance for crop cultivation for 1-2 years in dry season not found rhizobial population because the legume was cultivated only in rainy in this area. On the other hand, the forest clearance for crop cultivation for 3 years showed closely amount of rhizobial since this area cultivated mung bean in both of seasons.

Table 10 Rhizobial population in soil from the North Eastern region (log number of cell/g soil)

Areas	Rainy season (September/1997)	Dry season (January/1998)
1. Highest mountain	1.70 a[1]	0.97 a
2. Middle mountain	1.56 a	0.61 a
3. Foot hill of mountain	1.96 a	0.71 a
4. Field crop cultivation	1.74 a	1.06 a
5. Rice cultivation	1.11 a	0.57 a
6. Rice in rotation with other crops	1.82 a	1.28 a
7. Uncultivated	1.67 a	1.41 a
CV(%)	34.6	58.8

[1] Values followed by the same letter are not significantly different at 1% and 5% level by DMRT.

Table 11 Rhizobial population from cropping system and undisturb forest areas
in the North Eastern region (log number of cell/g soil)

Areas	Rainy season (September/1997)	Dry season (January/1998)
1. Undisturb forest	2.45ab[1]	0.41b
2. Forest clearance for crop cultivation for 1-2 years	2.53a	0
3. Forest clearance for crop cultivation for 3 years	1.62ab	1.11a
4. Intensive agricultural production using high rate of pesticides and fertilizers	1.42b	0.15b
CV(%)	27.2	58.9

1/ Values followed by the same letter are not significantly different at 1% and 5% level
by DMRT.

The comparision among 3 regions found that the suitable area in highest elevation of rhizobial distribution was the foot hill of mountain in rainy season and high average number was found in rainy season more than dry season, except some areas as middle mountain in North Eastern region. To compare about 1.5-2.5 Km from highest to foot hill of mountain in every region found that the majority of rhizobial population varied by level of the height. Rhizobial population from foot hill of mountain was found highest and reduced in middle and highest mountain, respectively. These results indicated the influence by moisture in highest elevation areas. While the flat area of agricultural practice also found different patterns of rhizobial population as highest population area particularly in Central and North. The total amount of rhizobial population in every region displayed that the North Eastern region was lowest than other regions. In the rice in rotation with other crops area was less effect by environment than other areas in every regions because this area cultivated leguminous plant in rotation crop system. Rhizobial population from cropping system and undisturb forest areas were shown the high average in rainy season than dry season and the undisturb area not along with season in every region. Focusing into clearance for crop cultivation for 1-2 years in dry season found that these areas very high effect to population especially in the North and the North Eastern region. While in clearance for crop cultivation for 3 years was not effect. Rhizobial population in intensive

agricultural production using high rate of pesticides and fertilizers showed low average population in every region especially in the central not found rhizobia in both of seasons. This result indicated that using high rate of pesticides and fertilizers without rotation with legume plant were high effect to rhizobial population.

3.2 Isolation and Characterization of Rhizobia

The 200 rhizobial isolates were obtained from various soil samples in different seasons in Thailand. In this experiment, rhizobia were roughly characterized in term of morphological and physiological aspects. Colony characteristic, acid-base production and Indole Acetic Acid (IAA) was conducted to preliminary determine diversity of rhizobia group from soil in Thailand as shown in Table 12.

3.2.1 Colony and acid-base production characteristics

The colony forming characteristic such as medium wet, small dry, medium dry and small wet (Mpepereki et al. 1997) were used to distinguish among strains. Most of the strains able to form colony within 3-8 days, but mainly found in 5th day of cultivation. The results of acid-base reaction indicated that 99% of rhizobia in Thailand were classified into slow-grower group (*Bradyrhizobium*) (Fig. 3) and the color reaction of fast-slow grower on medium as shown in Fig. 4 (slow-grower) and Fig. 5 (fast-grower). Along with this criteria, all rhizobia could be grouped into 2 genera, *Rhizobium* and *Bradyrhizobium*

Table 12. The percentage of some mophological and physiological aspects of rhizobia in rainy season and dry season

Areas	Type of colony		Fast/Slow grower		IAA population	
1. Highest mountain						
- in the Central	Sd	58%	Slow grower	100%	+	72%
	Sw	14%				
	Md	14%				
	Mw	14%				
- in the North	Sd	100%	Slow grower	100%	+	100%
- in the North Eastern	Sd	63%	Slow grower	100%	+	37%
	Md	37%				
2. Middle mountain						
- in the Central	Sd	64%	Slow grower	88%	+	25%
	Sw	12%	Fast grower	12%		
	Mw	12%				
	Lw	12%				
- in the North	Mw	50%	Slow grower	100%	+	38%
	Md	25%				
	Sd	25%				
- in the North Eastern	Sd	58%	Slow grower	100%	+	58%
	Md	29%				
	Sw	13%				
3. Foot hill of mountain						
- in the Central	Mw	75%	Slow grower	100%	+	13%
	Md	15%				
- in the North	Mw	50%	Slow grower	100%	+	38%
	Md	38%				
	Sw	12%				
- in the North Eastern	Sd	50%	Slow grower	100%	+	25%
	Mw	62%				
	Md	12%				
	Sw	12%				
4. Field crop cultivation						
- in the Central	Mw	38%	Slow grower	100%	-	100%
	Md	25%				
	Sd	25%				
	Sw	1%				
- in the North	Md	76%	Slow grower	100%	+	50%
	Mw	12%				
	Sd	12%				

Table 12. (continued)

Areas	Type of colony		Fast/Slow grower	IAA population
- in the North Eastern	Mw	76%	Slow grower 100%	+ 13%
	Md	12%		
	Sd	12%		
5. Rice cultivation				
- in the Central	Sw	43%	Slow grower 100%	+ 43%
	Mw	27%		
	Md	15%		
	Sd	15%		
- in the North	Mw	72%	Slow grower 100%	+ 15%
	Sw	14%		
	Sd	14%		
- in the North Eastern	Mw	86%	Slow grower 100%	+ 29%
	Sw	14%		
6. Rice in rotation with other crops				
- in the Central	Sd	38%	Slow grower 100%	+ 50%
	Mw	38%		
	Md	24%		
- in the North	Sd	50%	Slow grower 100%	+ 63%
	Md	25%		
	Mw	25%		
- in the North Eastern	Md	50%	Slow grower 100%	+ 25%
	Mw	25%		
	Sw	25%		
7. Uncultivated				
- in the Central	Sd	43%	Slow grower 100%	+ 25%
	Md	43%		
	Mw	14%		
- in the North	Md	50%	Slow grower 100%	- 100%
	Sd	25%		
	Mw	25%		
- in the North Eastern	Mw	50%	Slow grower 100%	+ 38%
	Md	25%		
	Sw	25%		
8. Undistrub forest				
- in the Central	Md	25%	Slow grower 100%	+ 25%
	Mw	25%		
	Sd	25%		
	Sw	25%		

Table 12. (continued)

Areas	Type of colony		Fast/Slow grower	IAA population
- in the North	Md	50%	Slow grower 100%	+ 25%
	Mw	25%		
	Sd	25%		
- in the North Eastern	Md	67%	Slow grower 100%	+ 33%
	Sd	33%		
9. Forest clearance for crop cultivation for 1-2 years				
- in the Central	Mw	100%	Slow grower 100%	+ 25%
- in the North	Md	50%	Slow grower 100%	+ 100%
	Sd	50%		
- in the North Eastern	Md	100%	Slow grower 100%	+ 50%
10. Forest clearance for crop cultivation for 3 years				
- in the Central	Md	50%	Slow grower 100%	+ 75%
	Mw	25%		
	Sd	25%		
- in the North	Mw	50%	Slow grower 100%	+ 50%
	Md	25%		
	Sd	25%		
- in the North Eastern	Mw	75%	Slow grower 100%	+ 25%
	Md	25%		
11. Intensive agricultural production using high rate of pesticides and fertilizers				
- in the North	Md	33%	Slow grower 100%	+ 50%
	Sw	33%		
	Mw	17%		
	Sw	17%		
- in the North Eastern	Md	60%	Slow grower 100%	+ 20%
	Mw	20%		
	Sd	20%		

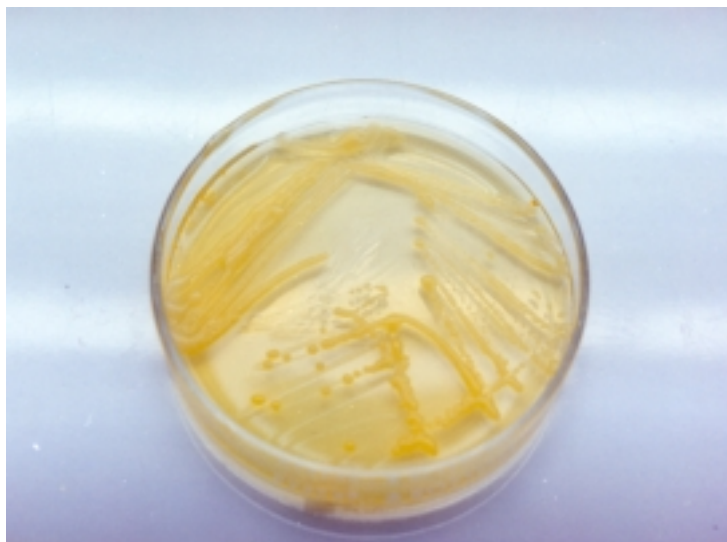Sd = Small dry   Sw = Small wet   Mw = Medium wet   Md = Medium dry
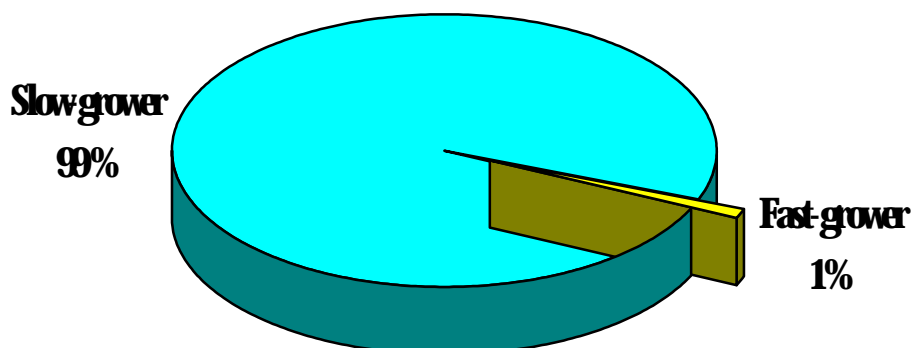
Figure 3 The percentage of fast and slow-grower rhizobial population isolated from soil in Thailand.
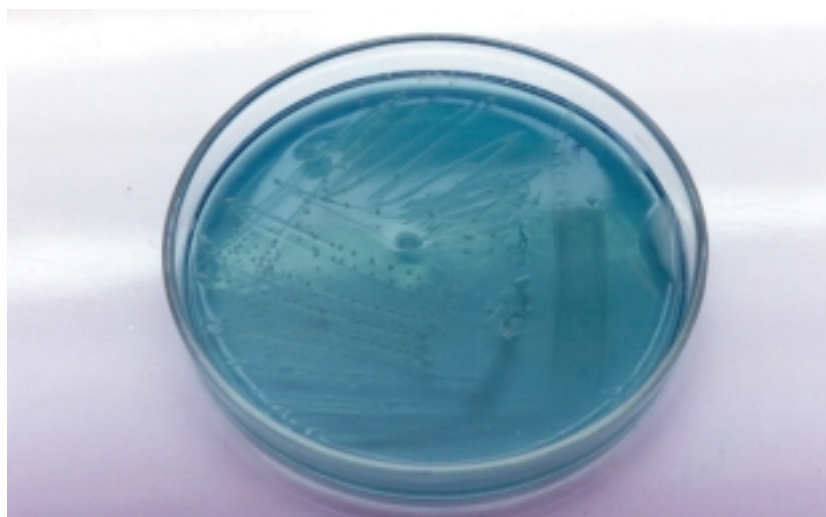
Figure 4 The alkaline reaction by slow-grower rhizobia on YMA + BTB medium.

Figure 5. The acid reaction by fast-grower rhizobia on YMA + BTB medium.

3.2.2 Indole Acetic Acid (IAA) production assay

The 37% out of total isolates of rhizobia were able to produce IAA and number of slow grower isolates could produce IAA rather than fast-grower and found IAA from this group only 1% while found rhizobia unable to poduced IAA 63% (Fig. 6). However, IAA production was used for separated soybean nodulation isolates as *Bradyrhizobium elkanii* from *B. japonicum* due to *B. elkanii* could be produced higher IAA as shown in Fig. 7. This information also used for identify soybean nodulation rhizobial isolates in the next experiment.

Rhizobia unable to produced IAA 63%

IAA from fast-grower 1%

IAA from slow-grower 36%
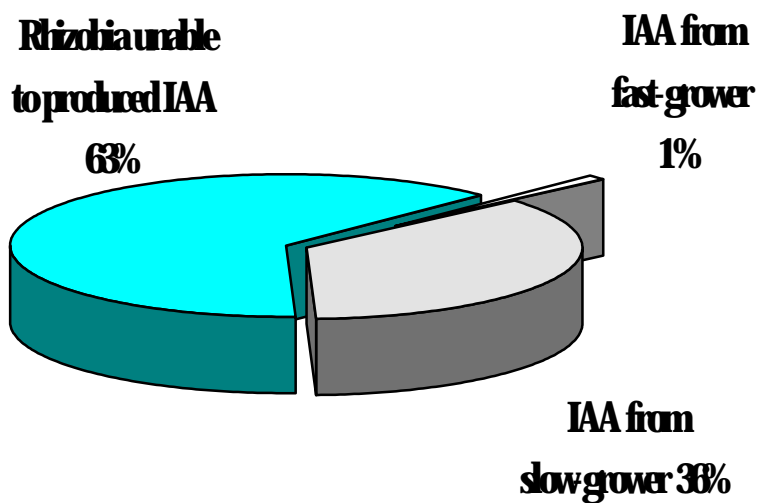
Figure 6 The percentage of IAA production from fast-grower and slow-grower rhizobial strains.

1   2   3   4

Figure 7. IAA production from rhizobial strains.
1. control (without rhizobia)
2. IAA production +++
3. IAA production ++
4. IAA production +

### 3.3 Nodulation and N$_2$-Fixing Ability with Index Plant (Siratro)

In this experiment, every rhizobial strains could nodulate and showed N$_2$-fixing ability with the plant index (Fig. 8, 9). The acetylene reduction assay (ARA) was used to investigate N$_2$-fixing efficiency and wide range in N$_2$-fixing efficiency (0.01-258.68 μmol C$_2$H$_4$/g nod/h) was detected. The maximum average of ARA which showed in this data found only 2 isolates ($\geq$ 100 μmol C$_2$H$_4$/g nod/h). While the medium average (10-99 μmol C$_2$H$_4$/g nod/h) found 31 rhizobial strains. Almost rhizobia about 179 rhizobial strains could detected ARA in minimum range ($\leq$ 9 μmol C$_2$H$_4$/g nod/h). Main population of rhizobia could reduce C$_2$H$_2$ in range between 1 to 20 μmol C$_2$H$_4$/g nod/h. The results obtained from this study was used for confirming the activity of rhizobia that isolated from soil samples and provision the data of N$_2$-fixing ability in further experiment. The range of ARA was summarized in Figure 10.

Figure 8 Rhizobial inoculation with plant index (siratro) growing in growth pouches.

A   B

Figure 9 A. control (without rhizobia).
B. plant index with rhizobial inoculant.
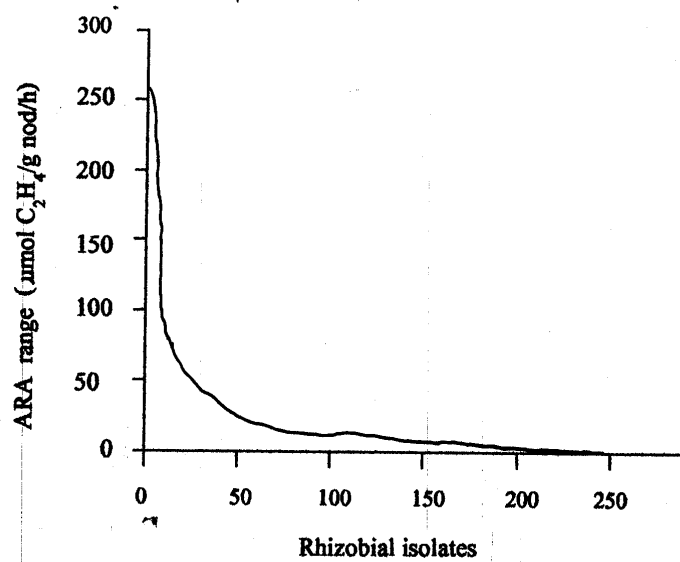
Figure 10 The range of ARA from rhizobial isolates.

3.4 Antibiotic Resistant Profiles

The intrinsic antibiotic resistant profiles of rhizobial isolates against 8 kinds of antibiotic exhibited highly diversification and almost of them could be differentiated into 123 groups. The result obtain from this study showed high resistant in trimetroprim, high susceptible in kanamycin and high susceptible with mutated colony in chloramphenicol. If classify between season, they could differentiate into 65 patterns in rainy season and 58 patterns in the dry season as shown in appendix A in Table 5A, 6A. In rainy season, found highly resistant in trimetroprim 73%, highly susceptible in erythromycin 33%, and highly susceptible with mutated colony in chloramphenicol 87% while in dry season, found highly resistant in trimetroprim 69%, highly susceptible in kanamycin 40%, and highly susceptible with mutated colony in chloramphenicol 72%.
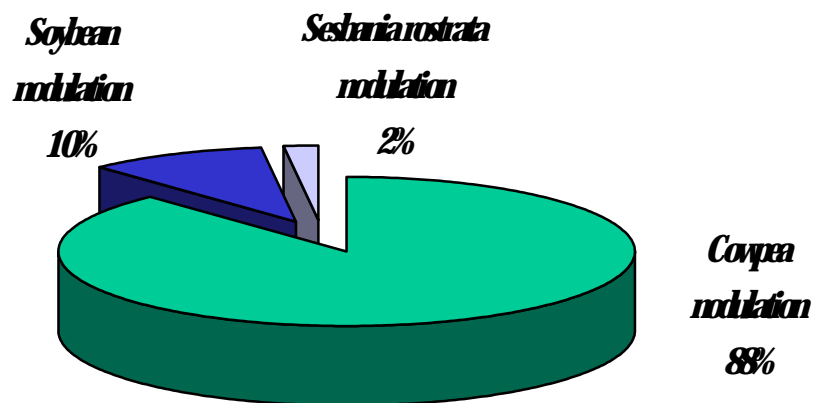
3.5 The Host-Dependent Cross-Inoculation Group

The host-dependent cross-inoculation group by rhizobial isolates from dry season and rainy season with 3 plants were displayed in Table 7A, 8A (appendix A). From 197 isolates found that 175 isolates could nodulate with cowpea, 23 isolates could nodulate with soybean and *Sesbania rostrata*. These results indicated that almost rhizobial isolates were belong to *Bradyrhizobium* sp. Since their nodulation in particular plant as cowpea (Fig. 11). From this result, soybean nodulated rhizobia might be able to separate into 2 groups by IAA production. The soybean nodulated rhizobia could produce higher IAA were *B. elkanii* while the common soybean nodulated rhizobia were *B. japonicum* (Fahrmam, 1993). This study might be able to identify rhizobia into 3 groups : 1) *Bradyrhizobium* sp. 88% 2) *B. japonicum* 8% and 3) *B. elkanii* 4% (Fig. 12).
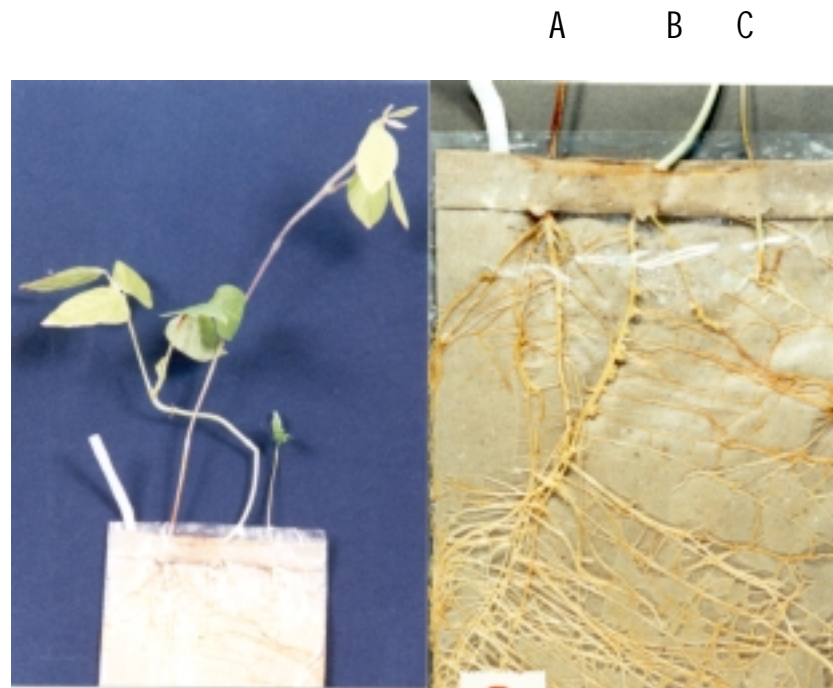
Figure 11. The host-dependent cross-inoculation group by rhizobial isolates.
   A. Soybean
     B. Cowpea (nodulation)
     C. *Sesbania rostrata*

Figure 12. The percentage of host-dependent cross-inoculation group.

3.6 PCR-RAPD Amplification and Phylogenetic Tree

To analyze the rhizobial diversification among group, the DNA fingerprint approach was applied in this study on the basis of DNA amplification with random primer. Prior to application PCR-RAPD to rhizobial isolates, the PCR-RAPD products from various reference strains *R. leguminosarum*, *R. tropici*, *B. japonicum* and *Azorhizobium* sp. were compared with an isolate from this study strain IICM$_3$-1 as shown in Fig. 13.
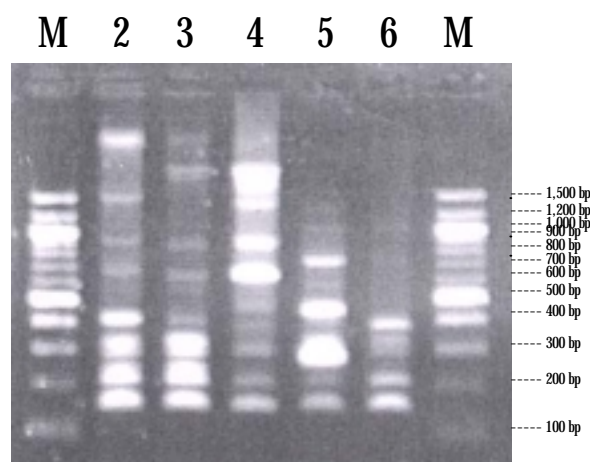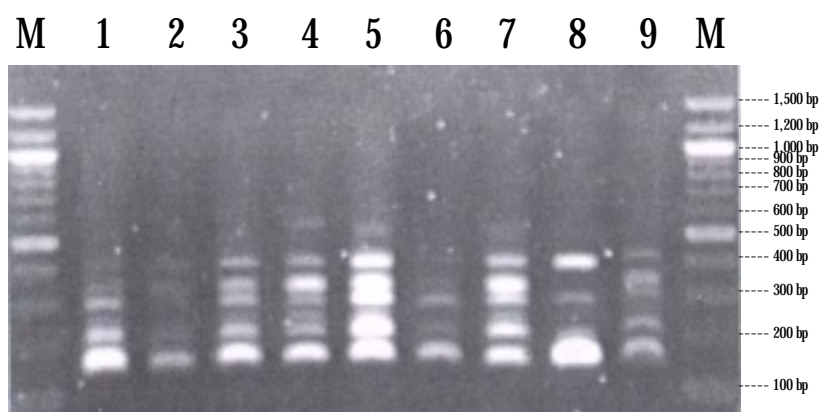

Figure 13 PCR-RAPD pattern of rhizobial reference strains and some isolates from Thai soil. M 100 bp Ladder marker, lane 2; *Rhizobium leguminosarum*, lane 3; *R. tropici*, Lane 4; *Bradyrhizobium japonicum*, lane 5; *Azorhizobium* sp. and lane 6; IICM$_3$-1.

The results of PCR-RAPD patterns of rhizobia from 11 areas were compared between rainy season and dry season as following discribed.

3.6.1 PCR-RAPD of rhizobia from the highest mountain area

The PCR products were mainly found in size of 150-390 bp. For comparison of PCR-RAPD patterns from rhizobial strains established in 2 seasons among 3 regions, the Central, the North and the North Eastern found that there were 9 different types from 9 randomly selected strains in the rainy season (Fig. 14) and found 8 different types from 9 strains in the dry season as shown in Fig 15. The similar strains was found 1 strain as lane 6 in rainy season with 1 strain as lane 9 in dry season and found the similar strains in dry season as lanes 6 and 7. The phylogenetic tree of rhizobia from rainy season and dry season in Central region also displayed in Fig. 16, that could explain almost of rhizobial strains were closely related and strain IIICM$_1$-2 was

different from the group. However, rhizobia in this area were separated from *B. japonicum* In Figure 17, phylogentic tree of rhizobia in the North showed most distinct among strains while in Fig. 18, phylogenetic tree in the North Eastern, found the similar strain in dry season as IIINEM$_1$-1 and IIINEM$_1$-3 and found strain INEM$_1$-1 in rainy season was similar to strain IIINEM$_1$-4 from dry season. This results could illustrate that some rhizobial strain such as INEM$_1$-1 could be the dominant native strain which able to persisted across the season.

Figure 14 PCR-RAPD patterns of rhizobial isolates from highest mountain area in rainy season. M, 100 bp Ladder marker; Lane 1, ICM$_1$-1; Lane 2, ICM$_1$-4; Lane 3, INM$_1$-1; Lane INM$_1$-2; Lane 5, INM$_1$-4; Lane 5, INEM$_1$-1, Lane 7, INEM$_1$-2; Lane 8, INEM$_1$-3 and Lane 9, INEM$_1$-4.
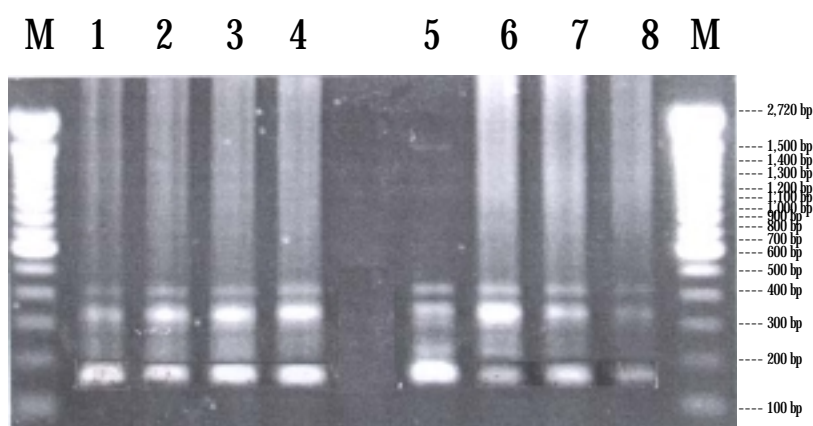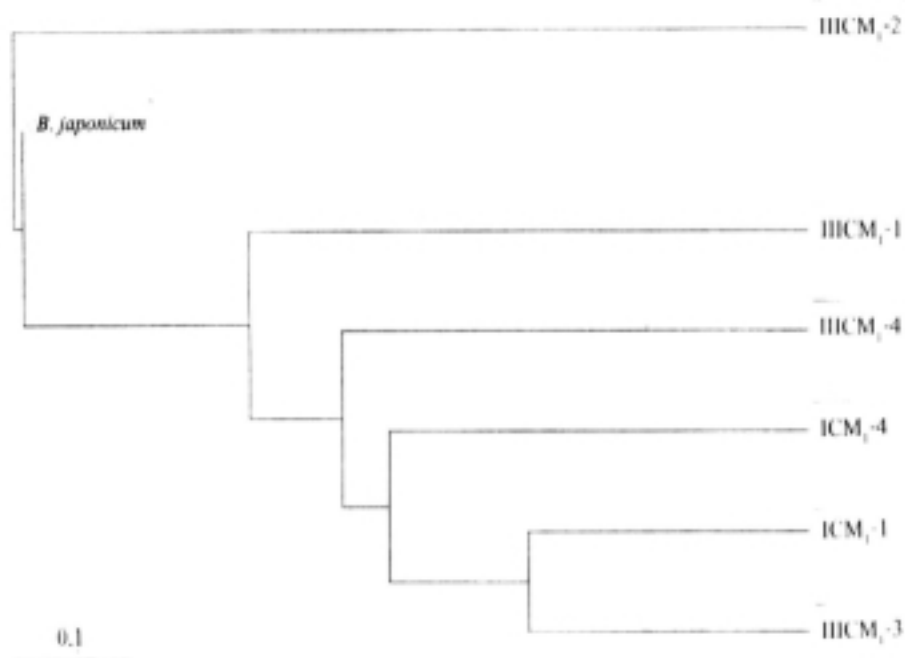

Figure 15 PCR-RAPD patterns of rhizobial isolates from highest mountain area in dry season M, 100 bp Ladder marker; Lane 1, IIICM$_1$-1; Lane 2, IIICM$_1$-2; Lane 3, IIICM$_1$-3; Lane 4, IIICM$_1$-4; Lane 5, IIINEM$_1$-1; Lane 6, IIINEM$_1$-2; Lane 7, IIINEM$_1$-3 and Lane 8, IIINEM$_1$-4.

Figure16 Phylogenetic tree of rhizobial isolates compared between the rainy season and dry season in the Central region, highest mountain.
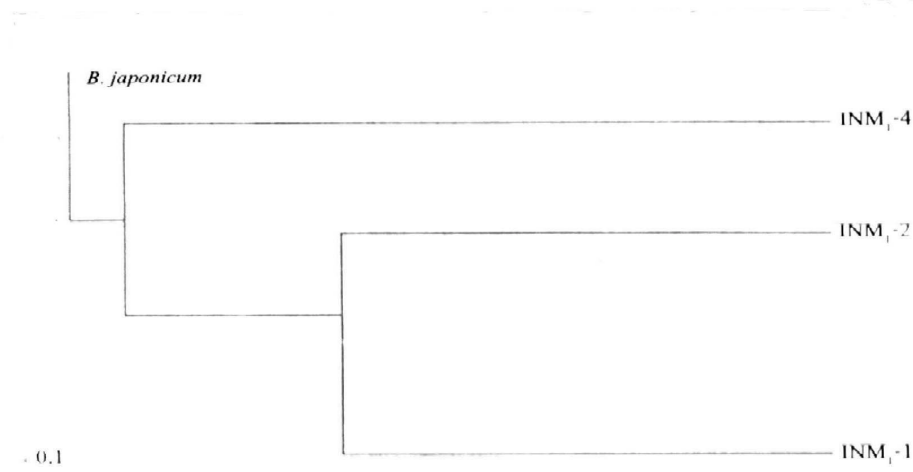
Figure17. Phylogenetic tree of rhizobial isolates in the rainy season in the North region, highest mountain.

Figure 18 Phylogenetic tree of rhizobial isolates compared between the rainy season and dry season in the North Eastern region, highest mountain.

3.6.2 PCR-RAPD of rhizobia from the middle mountain area

The PCR-RAPD pattern at middle mountain from 3 regions were compared between 2 seasons as shown in Fig. 19 and 20. Many strains of rhizobia were closely related especially in Fig. 20 found highly similar among strains. The phylogenetic tree of rhizobial isolates between rainy and dry season in the Central (Fig. 21) indicated that only 1 strain was different from group such as strains ICM_2-3, and 1 strain (ICM_2-4) was closer to *B. japonicum* than other strains. In dry season strains such as $IIICM_2$-1, $IIICM_2$-3, $IIICM_2$-4 which were closly related supposed to be the dominant strain in this period of time. Figure 22, phylogenetic tree of rhizobial isolates between rainy and dry season in the North was found that strain INM_2-3 was different from the group and almost rhizobia in this group were closely related, and different from *B. japonicum*. The similar strains were found in both of seasons as INM_2-1, INM_2-2 and INM_2-4 from rainy season and strains $IIINM_2$-2 and $IIINM_2$-4 from dry season. This result could be described that rhizobia in rainy season and dry season were closely related in each group. Figure 23, phylogenetic tree of rhizobial isolates between rainy and dry season in the North Eastern found nearly strain with *B. japonicum* as $INEM_2$-2. The similar strains were found in dry season

as strains IIINEM$_2$-2 and IIINEM$_2$-4 and found different strain from group as INEM$_2$-1. The isolates from dry season were closely related among group more than isolates in rainy season.
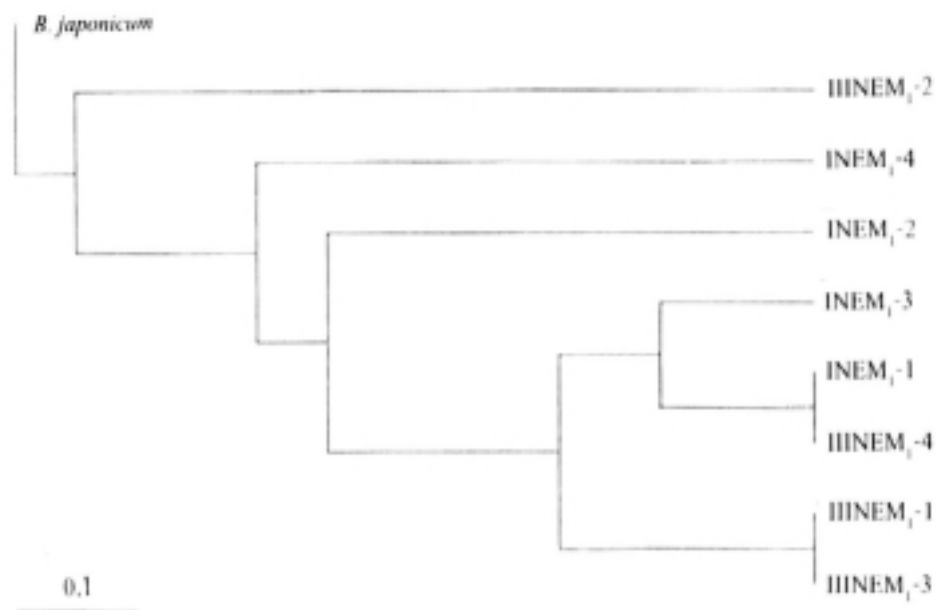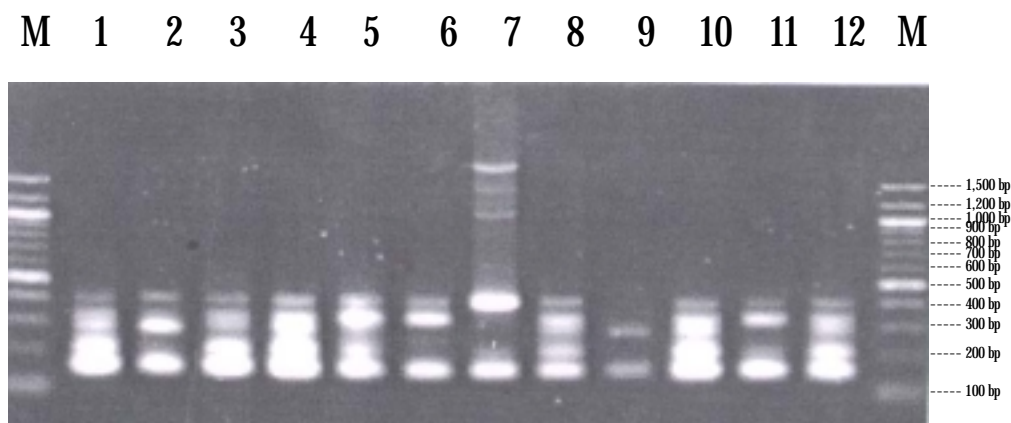

Figure 19   PCR-RAPD patterns of rhizobial isolates from middle mountain area in rainy season
M, 100 bp Ladder marker; Lane 1, ICM$_2$-1; Lane 2, ICM$_2$-2; Lane 3, ICM$_2$-3; Lane 4, ICM$_2$-4; Lane 5, INM$_2$-1; Lane 6, INM$_2$-2; Lane 7, INM$_2$-3; Lane 8, INM$_2$-4; Lane 9, INEM$_2$-1; Lane 10, INEM$_2$-2; Lane 11, INEM$_2$-3 and Lane 12, INEM$_2$-4.

Figure 20   PCR-RAPD patterns of rhizobial isolates from middle mountain area in dry season
M, 100 bp Ladder marker; Lane 1, IIICM$_2$-1; Lane 2, IIICM$_2$-2; Lane 3, IIICM$_2$-3; Lane 4, IIICM$_2$-4; Lane 5, IIINM$_2$-1; Lane 6, IIINM$_2$-2; Lane 7, IIINM$_2$-3; Lane 8, IIINM$_2$-4; Lane 9, IIINEM$_2$-2; Lane 10, IIINEM$_2$-3 and Lane 11, IIINEM$_2$-4.
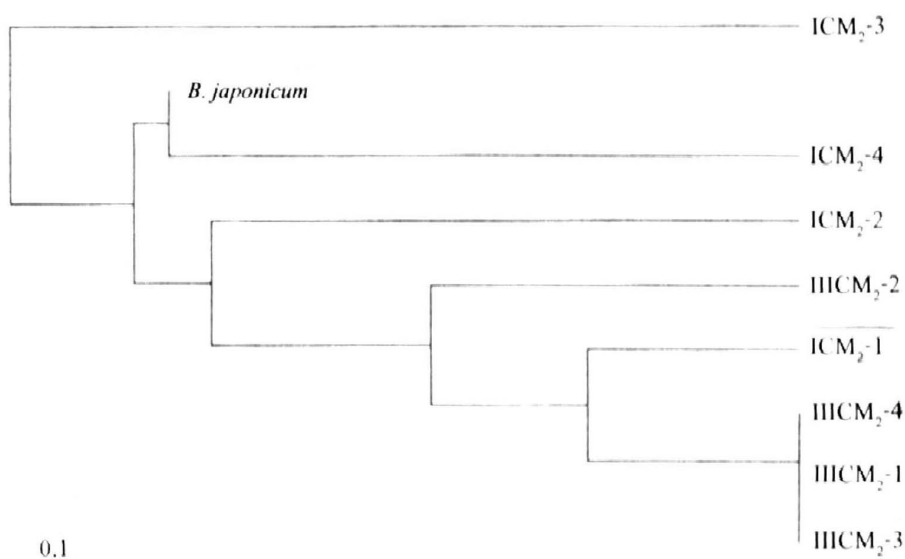
Figure 21. Phylogenetic tree of rhizobial isolates compared between rainy season and dry season in the Central region, middle mountain area.
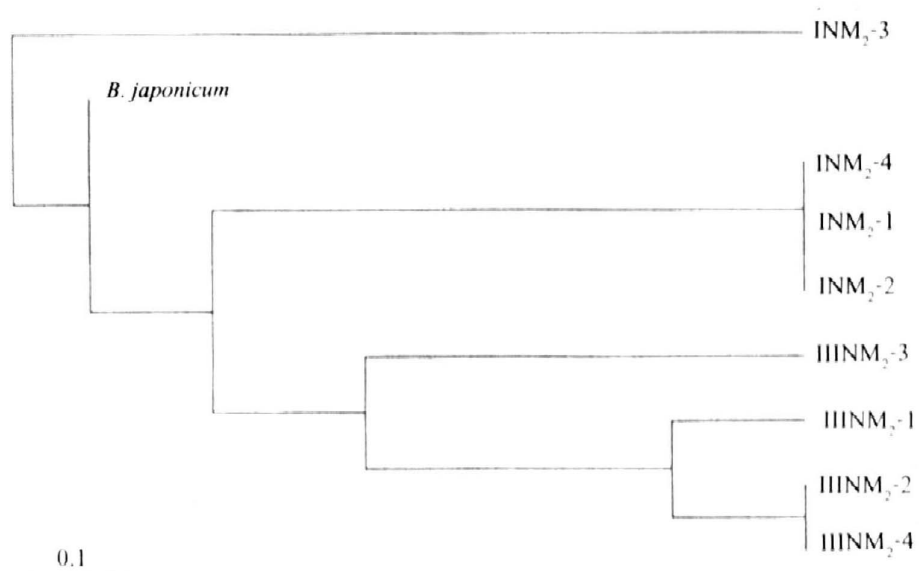
Figure 22. Phylogenetic tree of rhizobial isolates compared between rainy season and dry season in the North region, middle mountain area.

$INEM_2$-1
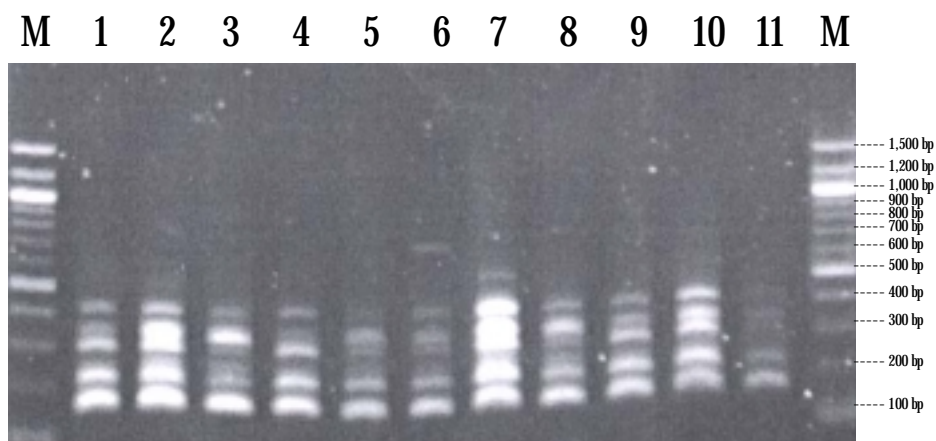
$INEM_2$-3

B. japonicum

$INEM_2$-2

$IIINEM_2$-3

$INEM_2$-4

$IIINEM_2$-2

$IIINEM_2$-4

0.1

Figure 23 Phylogenetic tree of rhizobial isolates compared between rainy season and dry season in the North Eastern, middle mountain area.

3.6.3 PCR-RAPD of rhizobia from foot hill of mountain area

The PCR patterns of rhizobia isolated from foot hill of mountain in rainy season was found different strains among 3 regions, except lanes 6 and 8 were closely related (Fig. 24) while PCR-patterns in dry season also found similar strains as lanes 1 and 2 and lanes 9 and 12 (Fig. 25). For comparison between 2 seasons, similar strains were found such as lanes 7 and 10 in rainy season with lane 7 in dry season and lane 10 in rainy season with lane 10 in dry season. The phylogenetic tree of rhizobia in the Central (Fig. 26) showed the comparison between 2 seasons and found that almost rhizobia separated from *B. japonicum* and found only 1 isolate as ICM_3-1 was different from the group while another isolate was closely related. This dendrogram displayed closely related strains as $IIICM_3$-1 and $IIICM_3$-2 in dry season and found the similar strains from different seasons as strains ICM_3-4 and $IIICM_3$-3 that could explain the persistence over the season isolate. Figure 26, phylogenetic tree in the North indicated that the strain $IIINM_3$-4 was close *B. joponicum* and found some strains as INM_3-2 and INM_3-4 were similar to strain $IIINM_3$-3 and could illustrate its establisment over the season isolate in this area. Fig. 27, the phylogenetic tree of rhizobia in the North Eastern found that strain $INEM_3$-1 was near to *B. japonicum* while the other strains were closely related among group and found similar strains as $IINEM_3$-1 and $IIINEM_3$-4 in the dry season and strain $INEM_3$-2 was similar to strain $IIINEM_3$-2 that could explain the persist over the season isolate as well.
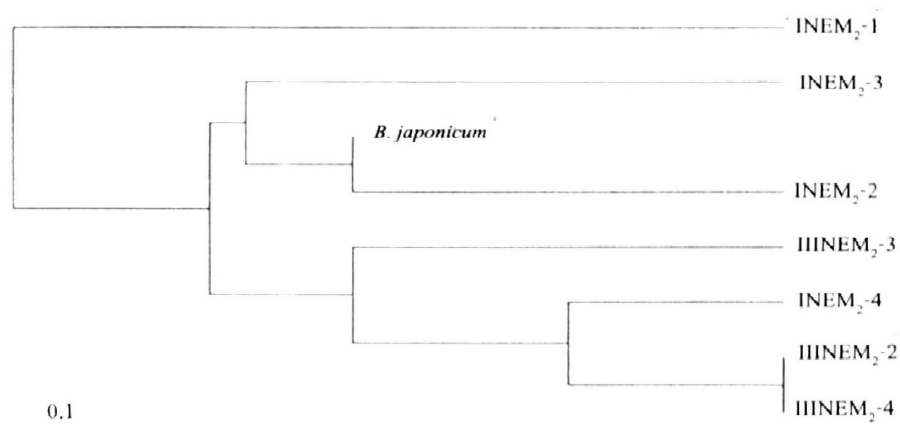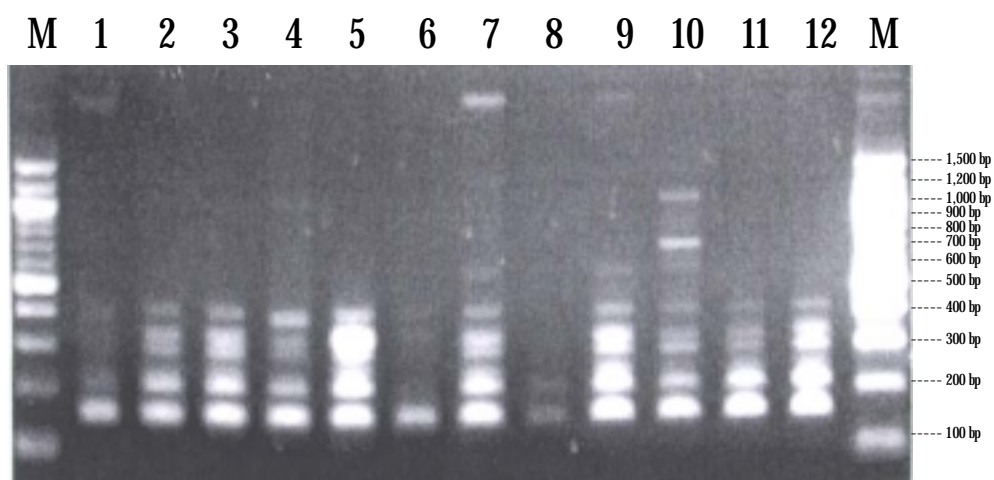
Figure 24 PCR-RAPD patterns of rhizobial isolates from foot hill of mountain area in rainy season

M, 100 bp Ladder marker; Lane 1, ICM₃-1; Lane 2, ICM₃-2; Lane 3, ICM₃-3; Lane 4, ICM₃-4; Lane 5, INM₃-1; Lane 6, INM₃-2; Lane 7, INM₃-3; Lane 8, INM₃-4; Lane 9, INEM₃-1; Lane 10, INEM₃-2; Lane 11, INEM₃-3 and Lane 12, INEM₃-4.
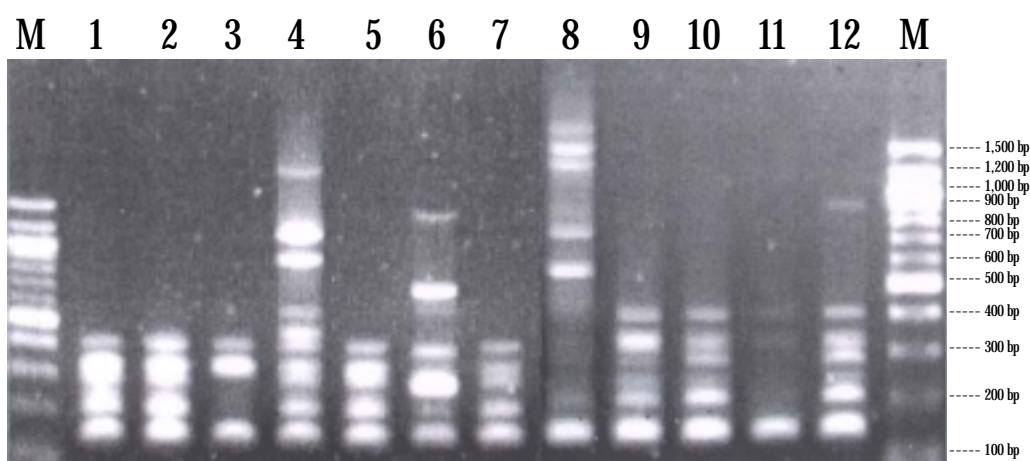
Figure 25 PCR-RAPD patterns of rhizobial isolates from foot hill of mountain area in dry season

M, 100 bp Ladder marker; Lane 1, IIICM₃-1; Lane 2, IIICM₃-2; Lane 3, IIICM₃-3; Lane 4, IIICM₃-4; Lane 5, IIINM₃-1; Lane 6, IIINM₃-2; Lane 7, IIINM₃-3; Lane 8, IIINM₃-4; Lane 9, IIINEM₃-2; Lane 10, IIINEM₃-3 and Lane 11, IIINEM₃-4.

Figure 26 Phylogenetic tree of rhizobial isolates compared between rainy season and dry season in the Central region, foot hill of mountain.
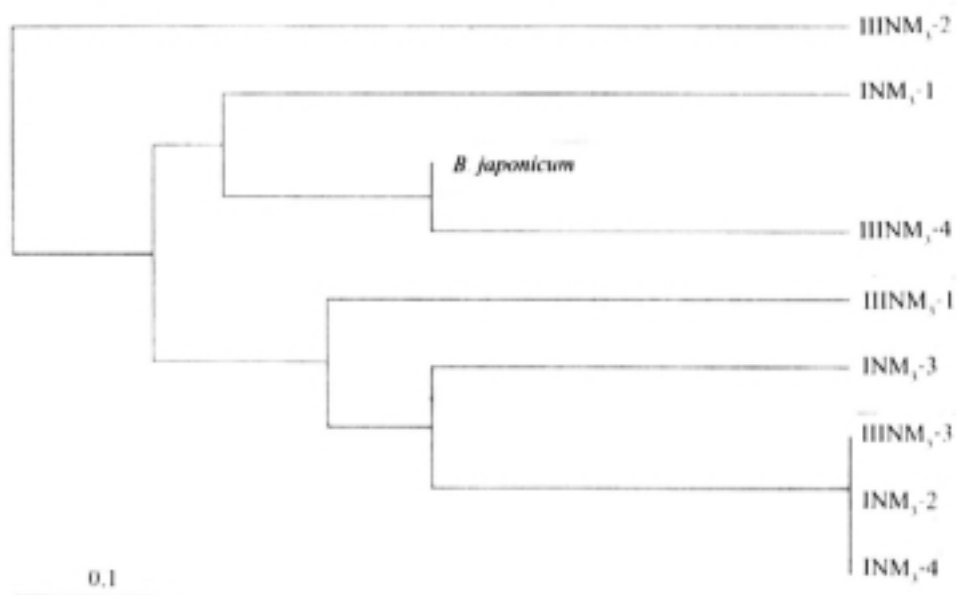

Figure 27. Phylogenetic tree of rhizobial isolates compared between rainy season and dry season in the North region, foot hill of mountai

Figure 28 Phylogenetic tree of rhizobial isolates compared between rainy season and dry season in the North Eastern region, foot hill of mountain.

3.6.4 PCR-RAPD of rhizobial from the field crop cultivation area

PCR patterns of rhizobia in field crop cultivation area in the riany season was found close to strains as in lanes 4 and 8 (Fig. 29) while in dry season found many similar strains in every regions as in lanes 1, 3 and 4 and lanes 5, 6 and 7 and lanes 10 and 11 (Fig. 30). The phylogenetic tree in the Central region showed closely related among group and found that many rhizobial strains was high similarity such as strains ICC4, IIICC1, IIICC3 and IIICC4 (Fig. 31). In Figure 32, phylogenetic tree in the North was found that rhizobial strains were closely related and found similar strains as IIINC1, IIINC2 and IIINC3. The last, Fig. 33, phylogenetic tree in the North Eastern found closely related rhizobia in dry season more than in rainy season and found similar strains as INEC2, IIINEC3 and IIINEC4. The phylogenetic tree in this area could be explained the less diversity of rhizobia group than in rainy season and found the dominat native strain as ICC4 and INEC2.
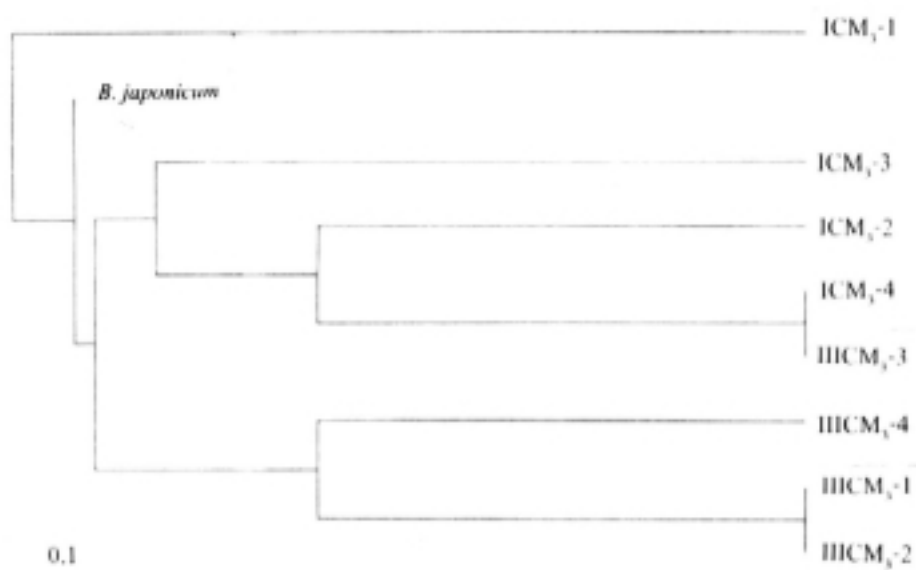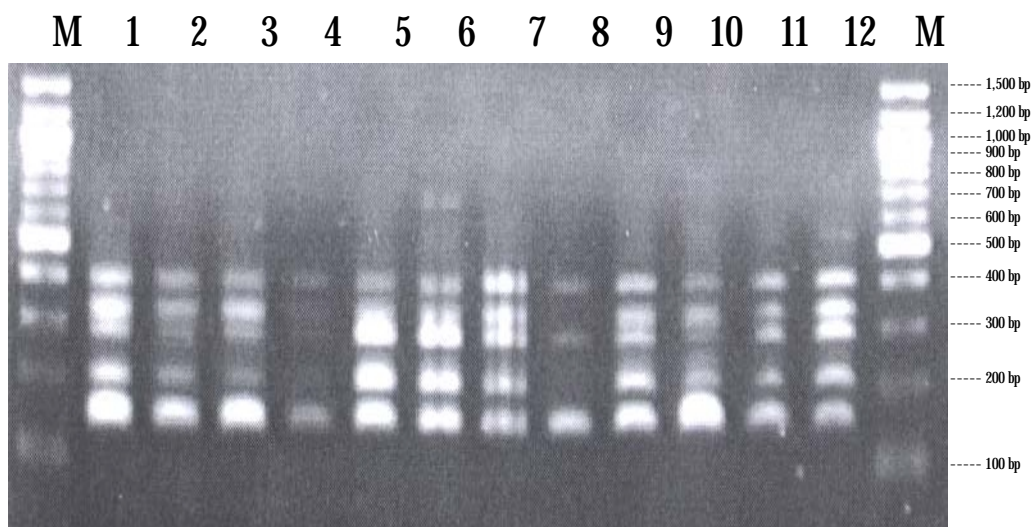
Figure 29 PCR-RAPD patterns of rhizobial isolates from field crop cultivation area in rainy season
M, 100 bp Ladder marker; Lane 1, ICC1; Lane 2, ICC2; Lane 3, ICC3; Lane 4, ICC4; Lane 5, INC1; Lane 6, INC2; Lane 7, INC3; Lane 8, INC4; Lane 9, INEC1; Lane 10, INEC2; Lane 11, INEC3 and Lane 12, INEC4.
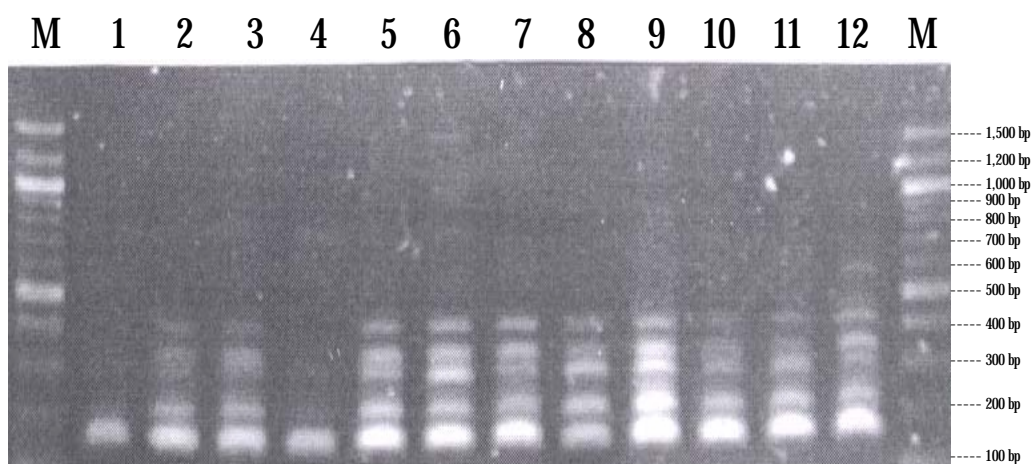

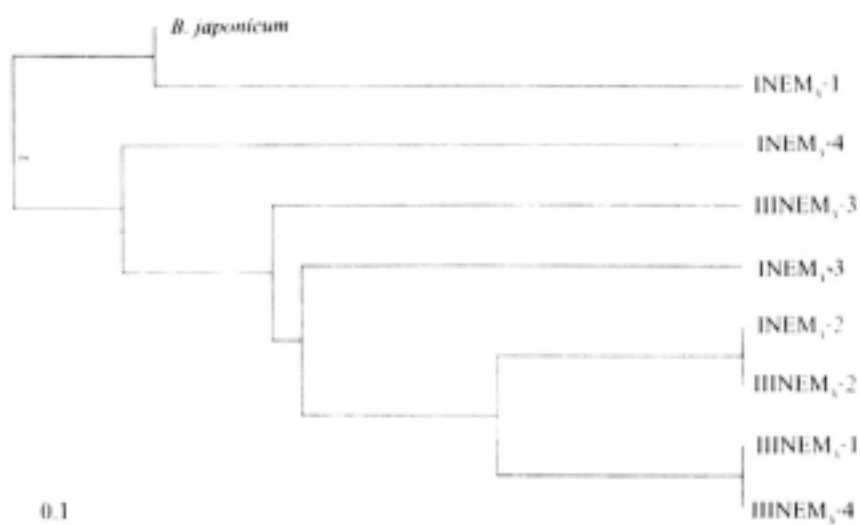**Figure 30** PCR-RAPD patterns of rhizobial isolates from field crop cultivation area in dry season
M, 100 bp Ladder marker; Lane 1, IIICC1; Lane 2, IIICC2; Lane 3, IIICC3; Lane 4, IIICC4; Lane 5, IIINC1; Lane 6, IIINC2; Lane 7, IIINC3; Lane 8, IIINC4; Lane 9, IIINEC1; Lane 10, IIINEC2; Lane 11, IIINEC3 and Lane 12, IIINEC4.
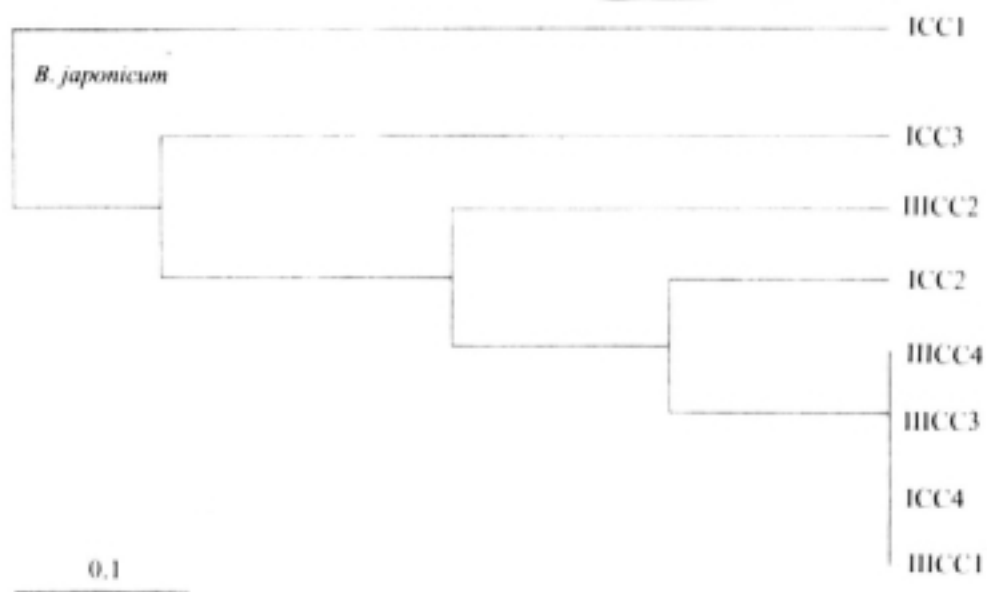
Figure 31. Phylogenetic Tree of rhizobial isolates compared between rainy season and dry season in the Central regoin, field crop cultivation area.
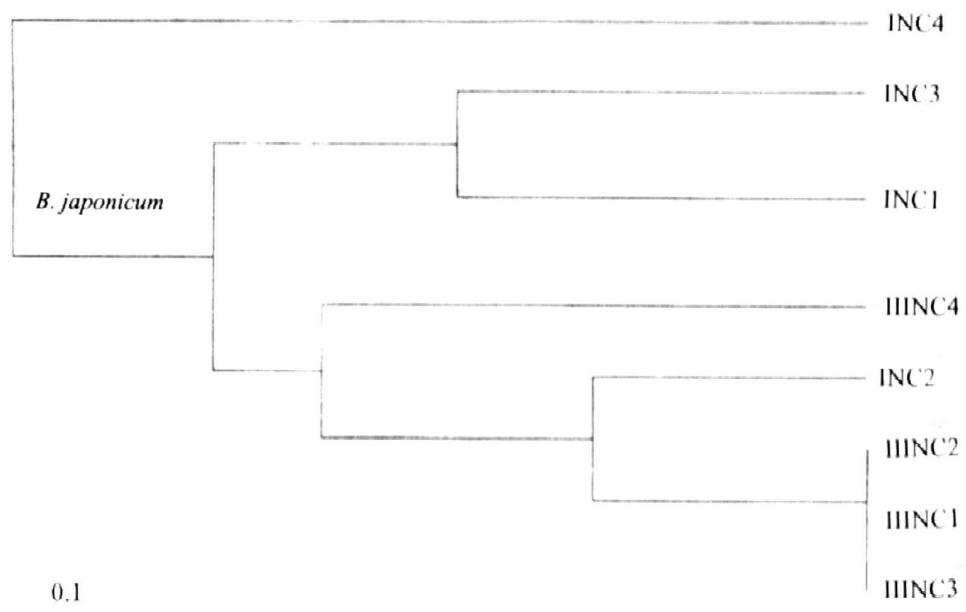
Figure 32. Phylogenetic Tree of rhizobial isolates compared between rainy season and dry season in the North region, field crop cultivation area.

Figure 33 Phylogenetic Tree of rhizobial isolates compared between rainy season and dry season in the North Eastern region, field crop cultivation area.

3.6.5 PCR-RAPD of rhizobia from rice cultivation area

PCR patterns of rhizobial isolates from rice cultivation area in the rainy season were compared between 3 regions and found that there were closely related strains as in lanes 6 and 7 (Fig. 34) while in the dry season found the similar strains as in lanes 9 and 10 (Fig. 35). The phylogenetic tree in the Central region showed the similar strains as ICR2, IIICR3 and IIICR4 that indicated the persistence of strains over season in this region and another strain was separated from group as strain IIICR2 (Fig. 36). The phylogenetic tree in the North region indicated the similar strains as INR2 and IIINR2 that displayed the persistence of strains over the season in this area (Fig. 36) and the last phylogenetic tree from the North Eastern found that some strains was somewhat nearly related to *B. japonicum* and this area was shown the closely related strains in dry season as IIINER1 and IIINER4 as well as strains in rainy season as INER3 and INER4 (Fig. 38).
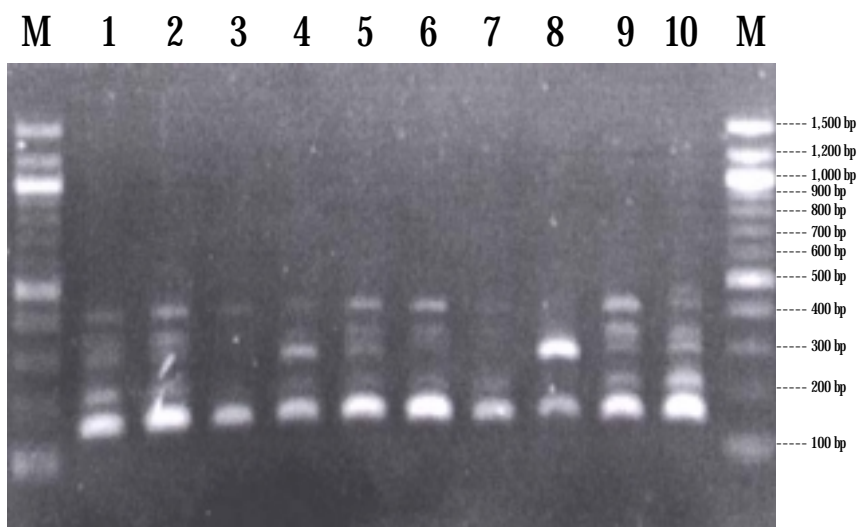
Figure 34 PCR-RAPA patterns of rhizobial isolates from rice cultivation area in rainy season
M, 100 bp Ladder marker; Lane 1, ICR1; Lane 2, ICR2; Lane 3, ICR4; Lane 4, INR1; Lane 5, INR2; Lane 6, INR4; Lane 7, INER1; Lane 8, INER2; Lane 9, INER3 and Lane 10, INER4.
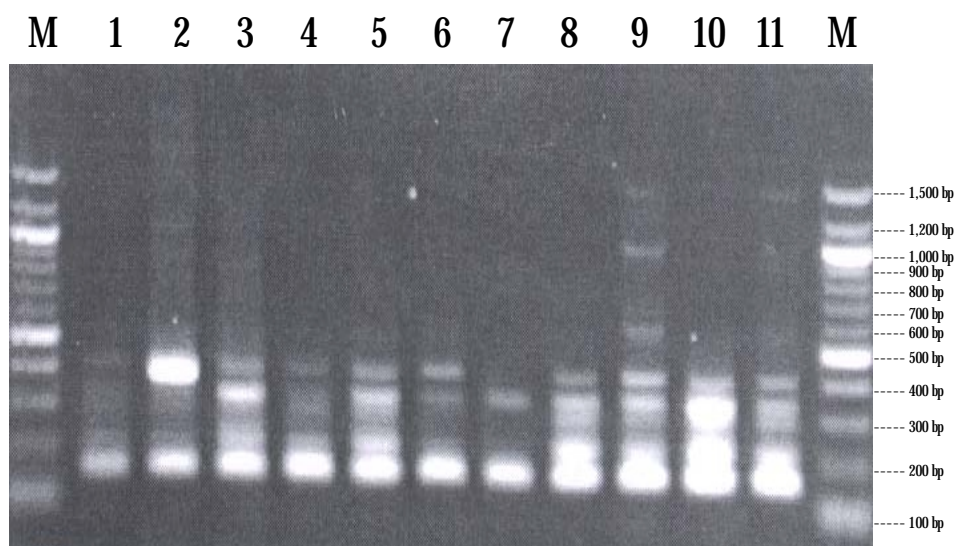
Figure 35 PCR-RAPA patterns of rhizobial isolates from rice cultivation area in dry season
M, 100 bp Ladder marker; Lane 1, IIICR1; Lane 2, IIICR2; Lane 3, IIICR3; Lane 4, IIICR4; Lane 5, IIINR1; Lane 6, IIINR2; Lane 7, IIINR3; Lane 8, IIINR4; Lane 9, IIINER1; Lane 10, IIINER3 and Lane 11, IIINER4.
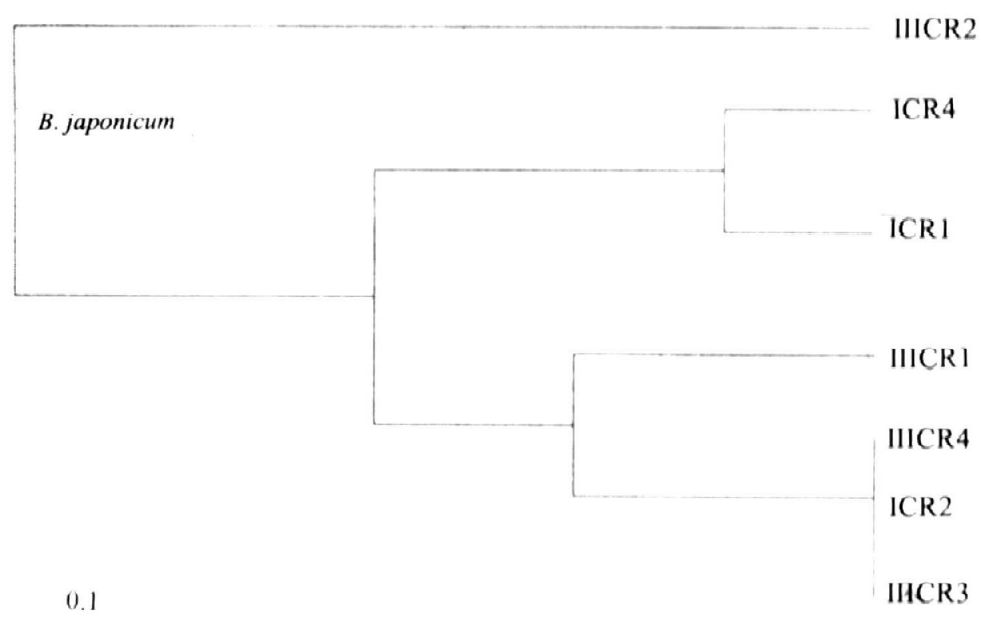
Figure 36 Phylogenetic tree of rhizobial isolates compared between rainy season and winter season in the Central, rice cultivation area.
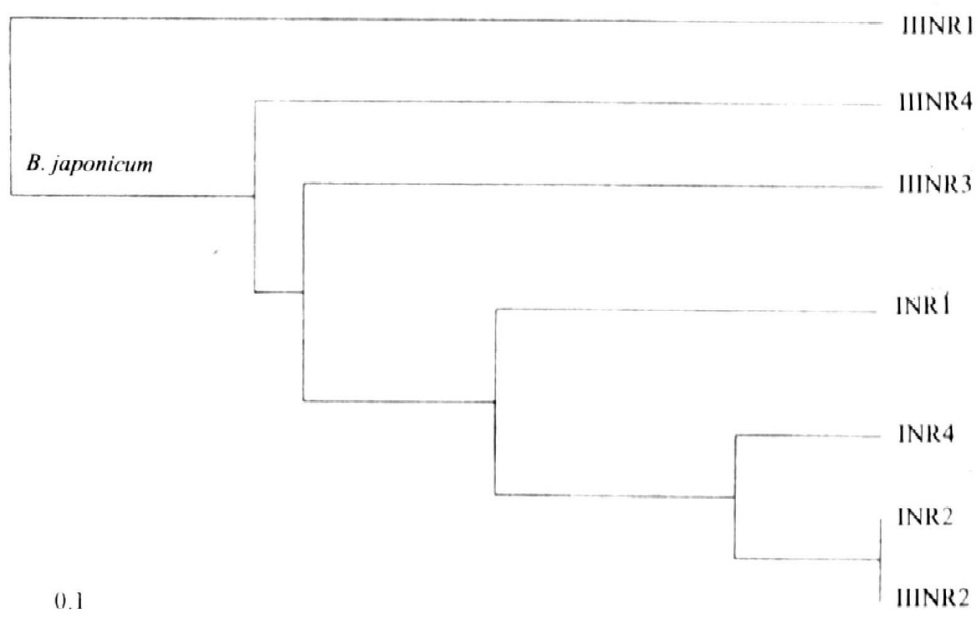
Figure 37. Phylogenetic tree of rhizobial isolates compared between rainy season and dry season in the North, rice cultivation area.
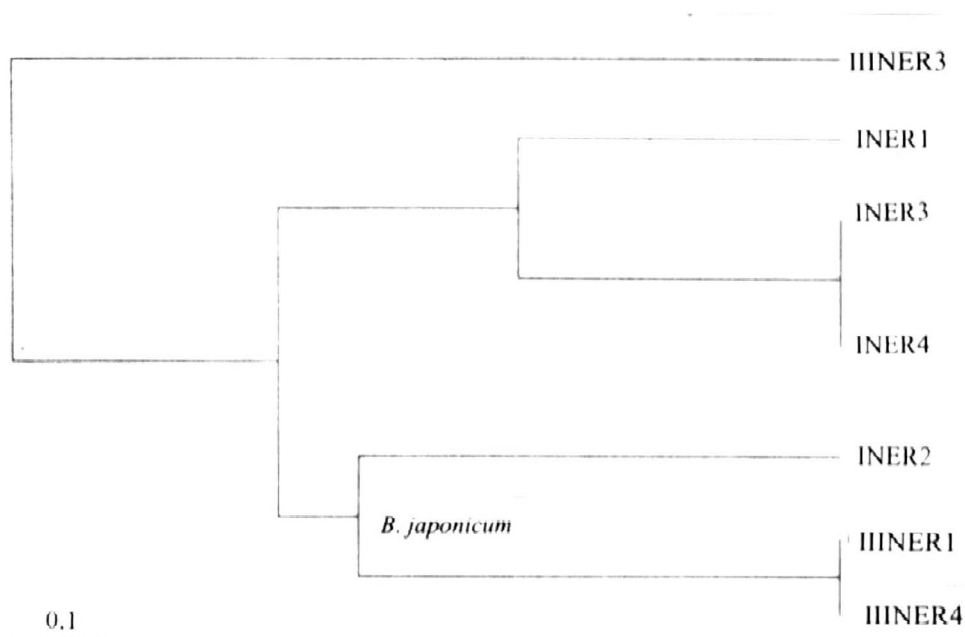
Figure 38 Phylogenetic tree of rhizobial isolates compared between rainy season and dry season in the North Eastern region, rice cultivation area.

3.6.6 PCR-RAPD of rhizobia from rice in rotation with other crops area

PCR patterns from this area was shown closely related strains in the rainy season as in lanes 9 and 10 (Fig. 39) while in the dry season found closely related strains as lanes 2 and 4, lanes 5, 7 and 8 (Fig. 40). The phylogenetic tree of rhizobia in the Central region found that the similar strain as ICCR4 from the rainy season that could persist over the new season and was similar to strains IIICCR2 and IIICCR4 in the dry season (Fig. 41) while the phylogenetic tree in the North was found the similar strains as INC2, IIINCR1, IIINCE3 and IIINCR4. This result showed the closely related strain in dry season more than rainy season and could explain the dominant native strain as INC2 that was high consistency in dry season (Fig. 42). The last phylogenetic tree in the North Eastern found the dominant native strains that could persist over the season as strain INECR4 and found the similar strains as INECR1 and INECR2 in rainy season (Fig. 43).
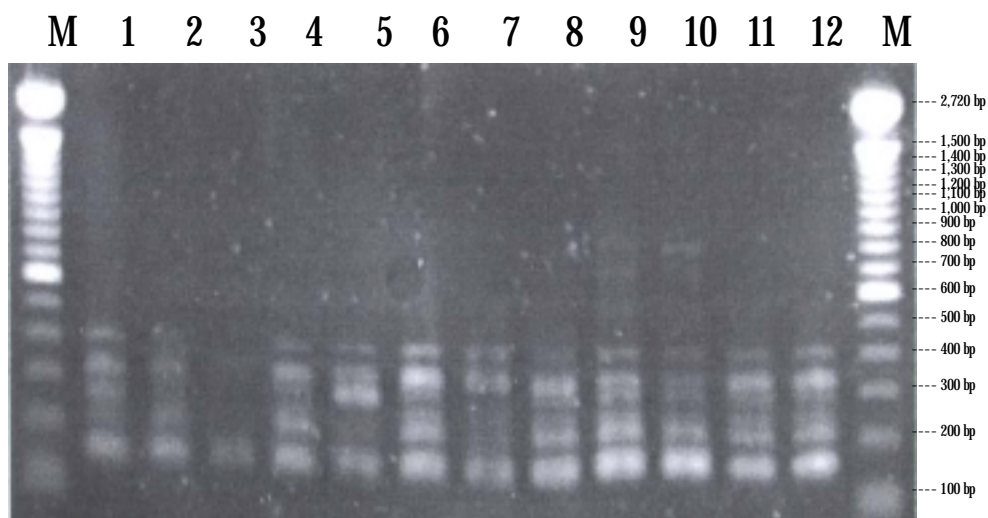
Figure 39 PCR-RAPD patterns of rhizobial isolates from rice in rotation with other crops in rainy season
M, 100 bp Ladder marker; Lane 1, ICCR1; Lane 2, ICCR2; Lane 3, ICCR3; Lane 4, ICCR4; Lane 5, INCR1; Lane 6, INCR2; Lane 7, INCR3; Lane 8, INCR4; Lane 9, INECR1; Lane 10, INECR2; Lane 11, INECR3 and Lane 12, INECR4.
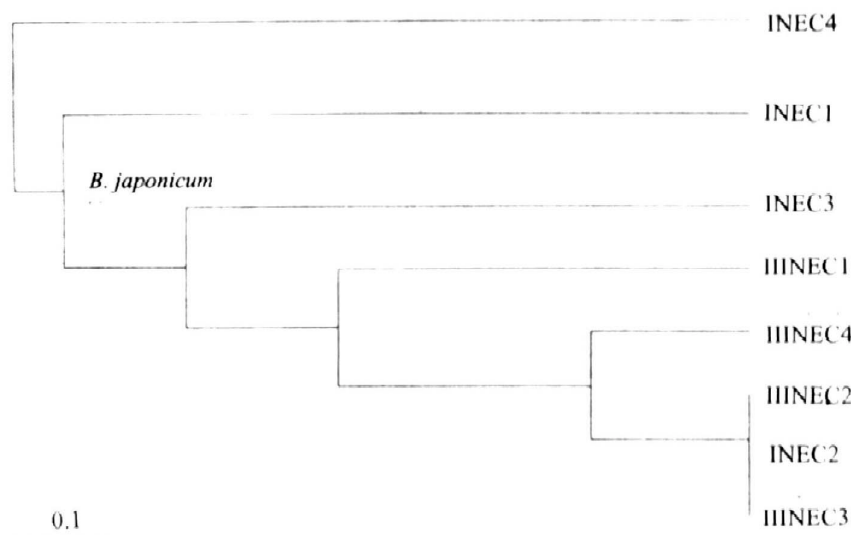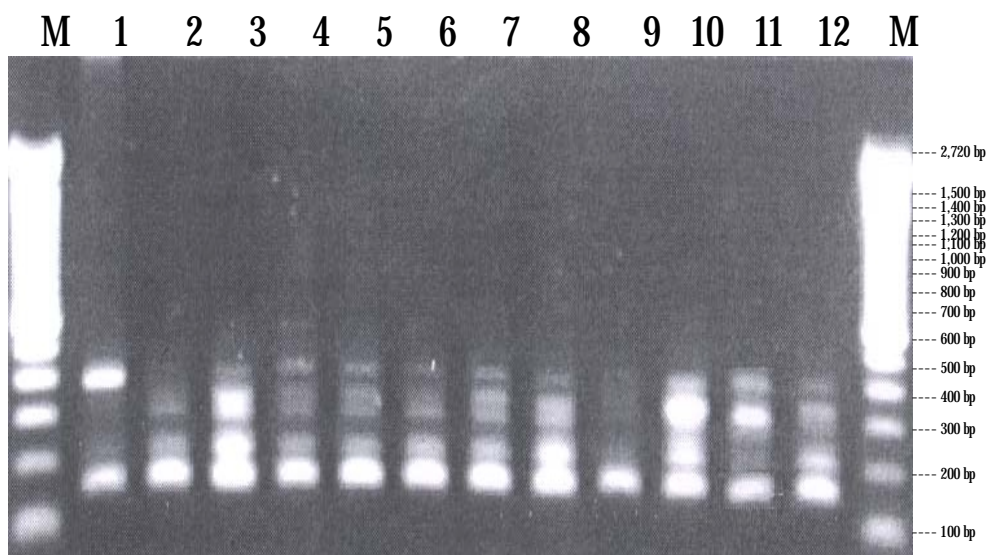

Figure 40 PCR-RAPD patterns of rhizobial isolates from rice in rotation with other crops in winter season
M, 100 bp Ladder marker; Lane 1, IIICCR1; Lane 2, IIICCR2; Lane 3, IIICCR3; Lane 4, IIICCR4; Lane 5, IIINCR1; Lane 6, IIINCR2; Lane 7, IIINCR3; Lane 8, IIINCR4; Lane 9, IIINECR1; Lane 10, IIINECR2; Lane 11, IIINECR3 and Lane 12, IIINECR4.

Figure 41. Phylogenetic tree of rhizobial isolate compared between rainy season and dry season in the Central region, rice in rotation with other crops.
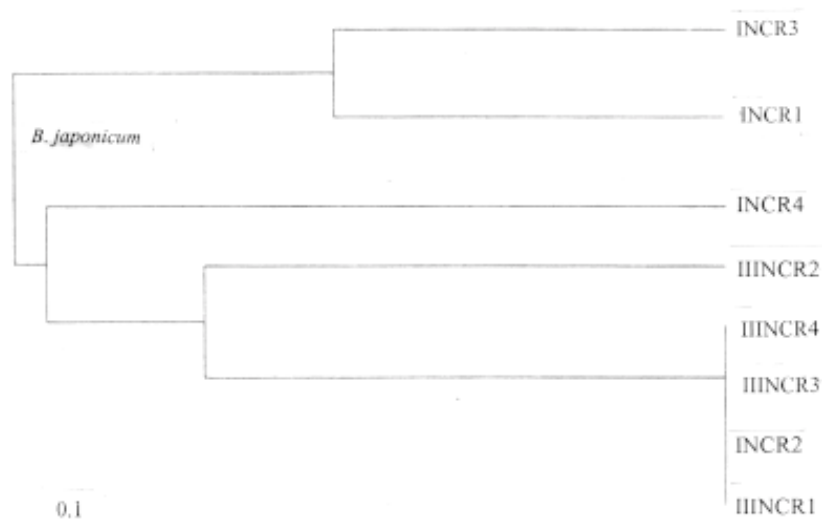

Figure 42. Phylogenetic tree of rhizobial isolate compared between rainy season and dry season in the North region, rice in rotation with other crops.

IIINECR1

B. japonicum

IIINECR2

IIINECR3

INECR1

INECR2

INECR3

INECR4

IIINECR4

0.1

Figure 43 Phylogenetic tree of rhizobial isolate compared between rainy season and dry season in the North Eastern region, rice in rotation with other crops.

3.6.7 PCR-RAPD of rhizobia from uncultivated area

The PCR patterns from this area was compared among 3 regions from the rainy season found that there were many closely strains as lanes 1 and 3, lanes 6 and 7 and lanes 9 and 12 as shown in Fig. 44 while PCR patterns from rhizobia dry season showed closely strains as lanes 1 and 4, lanes 5 and 7 and lanes 11 and 12 (Fig. 45). The phylogenetic tree in the Central, found that the similar strains as IIICF1 and IIICF4 in dry season were similar to strain ICF2 in rainy season that could be assumed as dominant native strain (Fig. 46). The phylogenetic tree in the North found the similar strains as INF1 and INF 2 in rainy season and strains IIINF1, IIINF2 and IIINF3 in dry season that could implied that less rhizobial diversity than other. (Fig. 47). The last phylogenetic tree as shown in Fig. 48 that showed closely related among rhizobia group and found the dominant native strains as INF2 which similar to strains IIINEF3 and IIINEF4 in the new season.
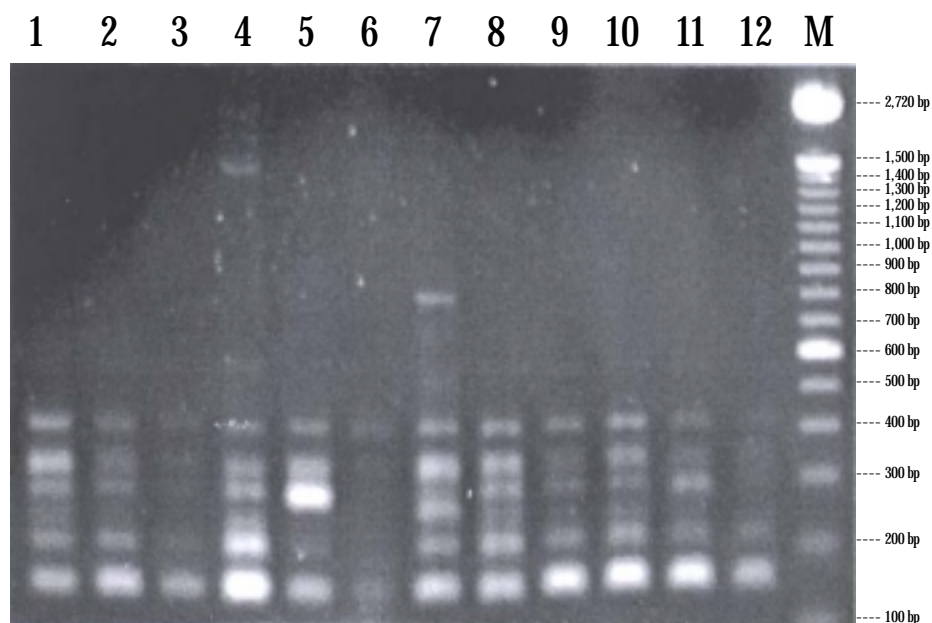
Figure 44 PCR-RAPD patterns of rhizobial isolates from uncultivated area in rainy season
Lane 1, ICF1; Lane 2, ICF2; Lane 3, ICF3; Lane 4, ICF4; Lane 5, INF1; Lane 6, INF2;
Lane 7, INF3; Lane 8, INF4; Lane 9, INEF1; Lane 10, INEF2; Lane 11, INEF3, Lane 12,
INEF4 and M, 100 bp Ladder marker.

Figure 45 PCR-RAPD patterns of rhizobial isolates from uncultivated area in dry season
M, 100 bp Ladder marker; Lane 1, IIICF1; Lane 2, IIICF2; Lane 3, IIICF3; Lane 4,
IIICF4; Lane 5, IIINF1; Lane 6, IIINF2; Lane 7, IIINF3; Lane 8, IIINF4; Lane 9,
IIINEF1; Lane 10, IIINEF2; Lane 11, IIINEF and Lane 12, IIINEF4.
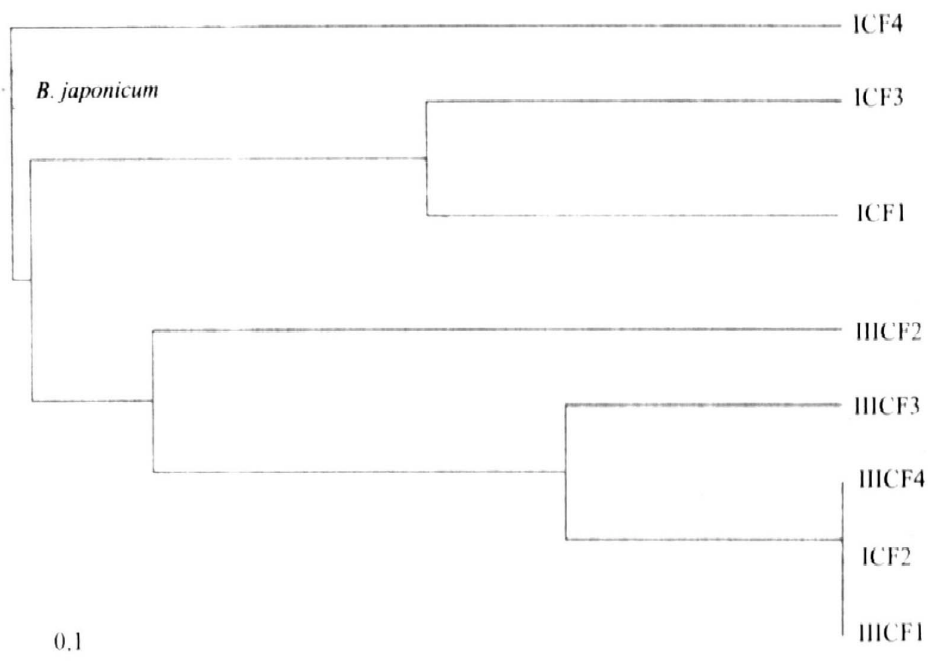
Figure 46 Phylogenetic tree of rhizobial isolates compared between rainy season and dry season in the Central region, uncultivated area.
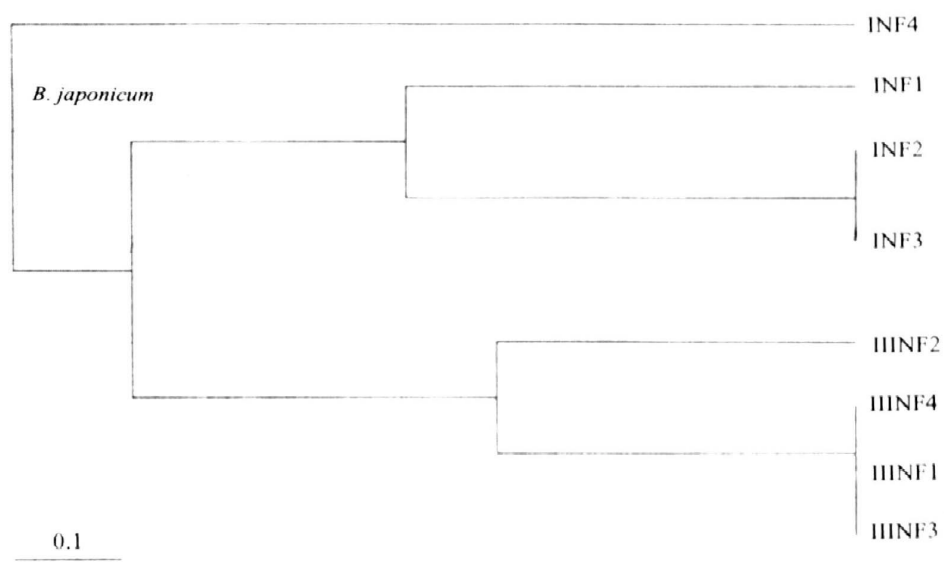

Figure 47. Phylogenetic tree of rhizobial isolates compared between rainy season and dry season in the North region, uncultivated area.
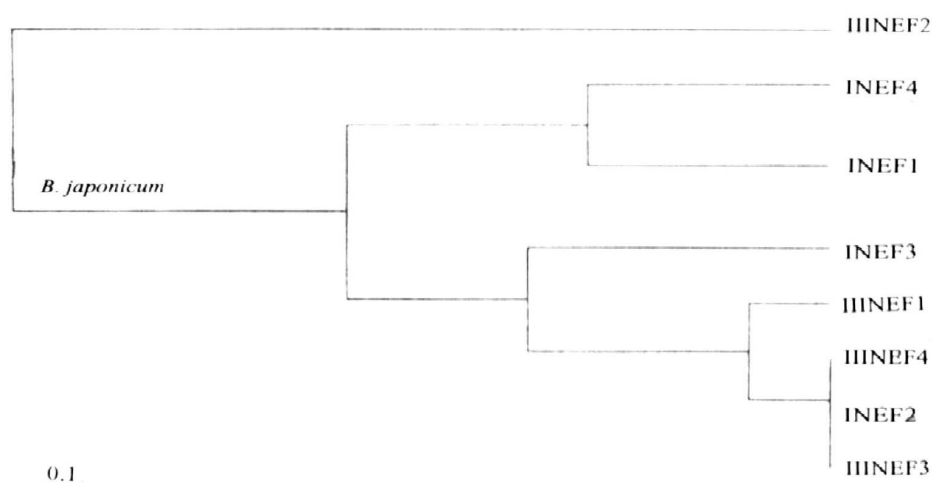
Figure 48 Phylogenetic tree of rhizobial isolates compared between rainy season and dry season in the North Eastern region, uncultivated area.

3.6.8 PCR-RAPD of rhizobia from undisturb forest

The PCR patterns from this area found that almost rhizobia in the rainy season were closely related among as in lanes 5 and lane 6 (Fig. 49A) while PCR patterns in dry season indicated high diversity among rhizobial strains (Fig. 49B). The phylogenetic tree from the Central was shown strains ICA1 was closely related with strain IIICA2 (Fig. 50) and the phylogenetic tree in the North in dry season, strains as INA1 and INA2 were closely related in the group (Fig. 51). The last phylogenetic tree in the North Eastern was found similar strains in rainy season as INEA1 and INEA2 (Fig. 52).

Figure 49(A,B). PCR-RAPD patterns of rhizobial isolates from undisturb forest in rainy season (A) and winter season (B)
A) M, 100 bp Ladder marker; Lane 1, ICA1; Lane 2, ICA2; Lane 3, INA1; Lane 4, INA2;Lane5, INEA1 and Lane 6, INEA2. B) M, 100 bp Ladder marker; Lane 1, IIICA1; Lane 2, IIICA2; Lane 3, IIINA1; Lane 4, IIINA2 and Lane 5, IIINEA1.

Figure 50 Phylogenetic tree of rhizobial isolates compared between rainy season and dry season in the Central region, undisturb forest.
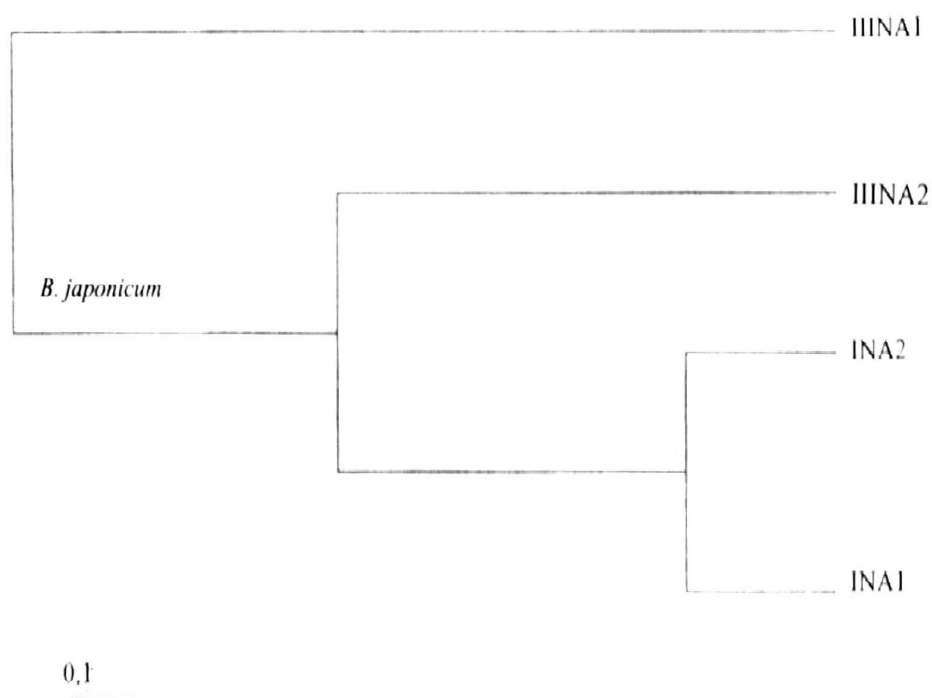
Figure 51. Phylogenetic tree of rhizobial isolates compared between rainy season and dry season in the North region, undisturb forest.
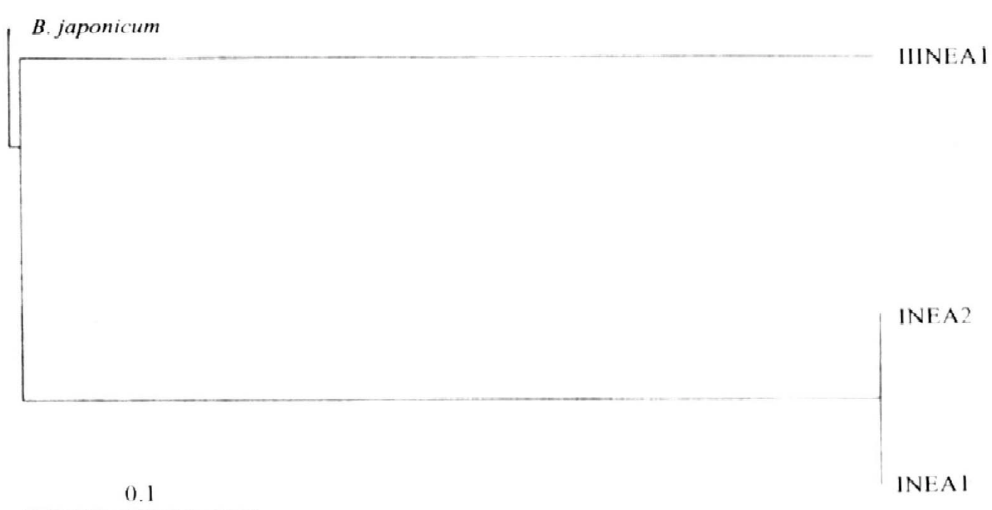
Figure 52. Phylogenetic tree of rhizobial isolates compared between rainy season and dry season in the North Eastern region, undisturb forest.

3.6.9 PCR-RAPD of rhizobia from forest clearance for crop cultivation for 1-2 years

The PCR patterns in this area was found closely related strains as lanes 7 and 8 from dry season and on the other hand, different strains were found in rainy season. This patterns were found high diversity in rainy season (Fig. 53). The phylogenetic tree in the Central found only 2 closely related strains as IIICB1 and IIICB2 in the dry season (Fig. 54) while the phylogenetic tree from the North found only 2 different strains were observed (Fig. 55). The last phylogenetic tree (Fig. 56) showed similar results as other regions.
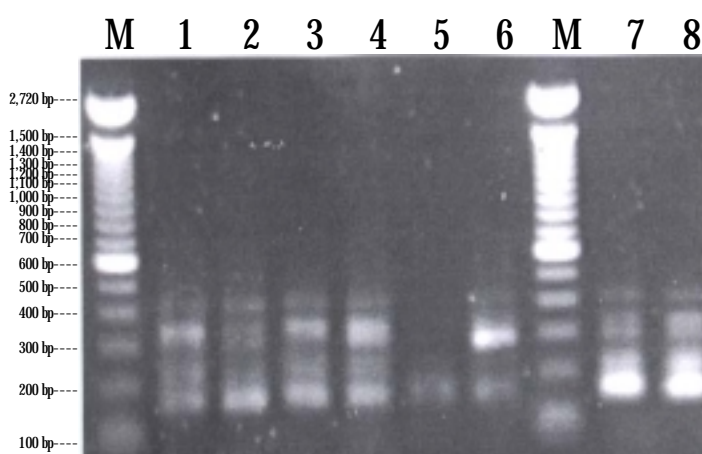
Figure 53 PCR-RAPD patterns of rhizobial isolates from forest clearance for crop cultivation for 1-2 years in rainy season (Lanes 1-6), and in winter season (Lanes 7-8). M, 100 bp Ladder marker; Lane 1, ICB1; Lane 2, ICB2; Lane 3, INB1; Lane 4, INB2; Lane 5, INEB1; Lane 6, INEB2; Lane 7, IIICB1 and Lane 8, IIICB2.
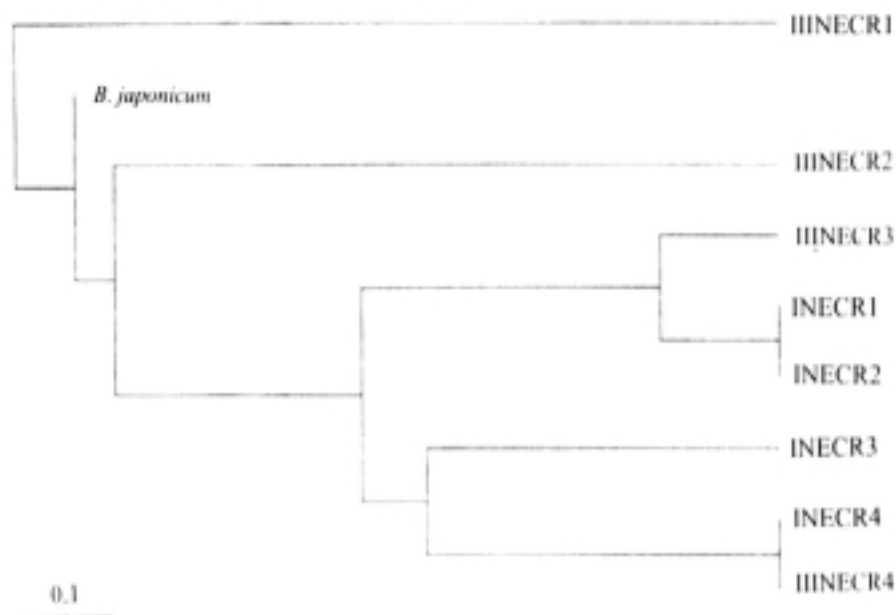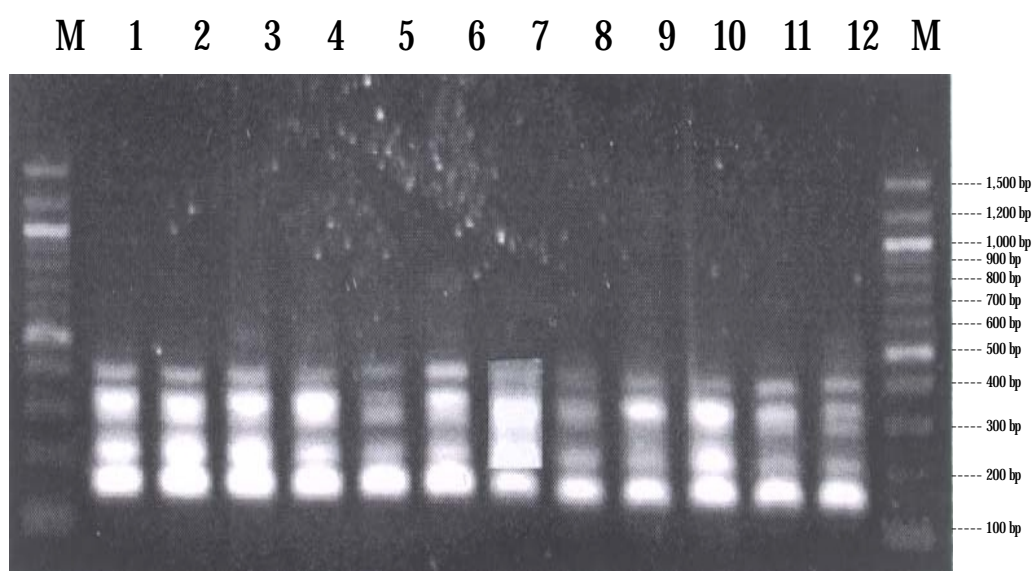

Figure 54 Phylogenetic tree of rhizobial isolates compared between rainy season and dry season in the Central region, forest clearance for crop cultivation for 1-2 years.

Figure 55. Phylogenetic tree of rhizobial isolates in rainy season in the North region, forest clearance for crop cultivation for 1-2 years.
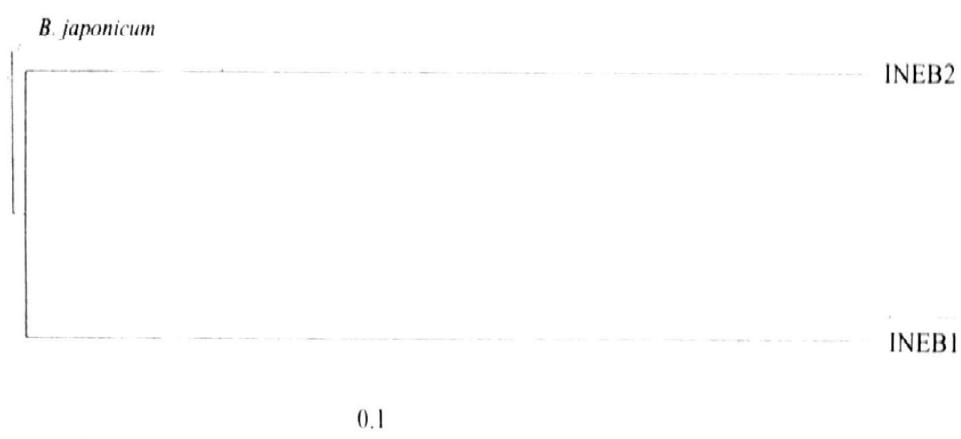
Figure 56 Phylogenetic tree of rhizobial isolates in rainy season in the North Eastern region, forest clearance for crop cultivation for 1-2 years.

3.6.10 PCR-RAPD of rhizobia from forest clearance for crop cultivation for 3 years

The PCR patterns in this area was shown closely strains in both of seasons, the rainy season (Fig. 57A) and dry season (Fig. 57B). The phylogenetic tree from the Central showed close strians as ICC*1 and IIICC*1 in this region (Fig. 58) while the phylogenetic tree in the North found close strains as IIINC*2 and INC*1 (Fig. 59). The phylogenetic tree in the North Eastern found closed strains in the group as INEC*1 and INEC*2 in rainy season (Fig. 60).
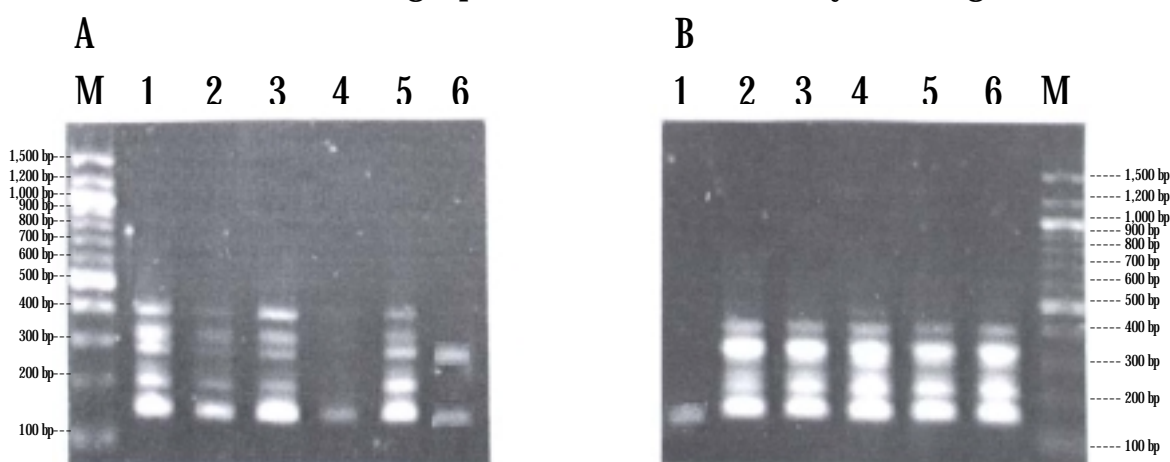
Figure 57 (A,B). PCR-RAPD patterns of rhizobial isolates from forest clearance for crop cultivation for 3 years in rainy season (A) and dry season (B) A) M, 100 bp Ladder marker; Lane 1, ICC*1; Lane 2, ICC*2; Lane 3, INC*1; Lane 4, INC*2; Lane 5, INEC*1 and Lane 6, INEC*2. (B) Lane 1, IIICC*1; Lane 2, IIICC*2; Lane 3, IIINC*1; Lane 4, IIINC*2; Lane 5, IIINEC*1; Lane 6, IIINEC*2 and M, 100 bp Ladder marker.
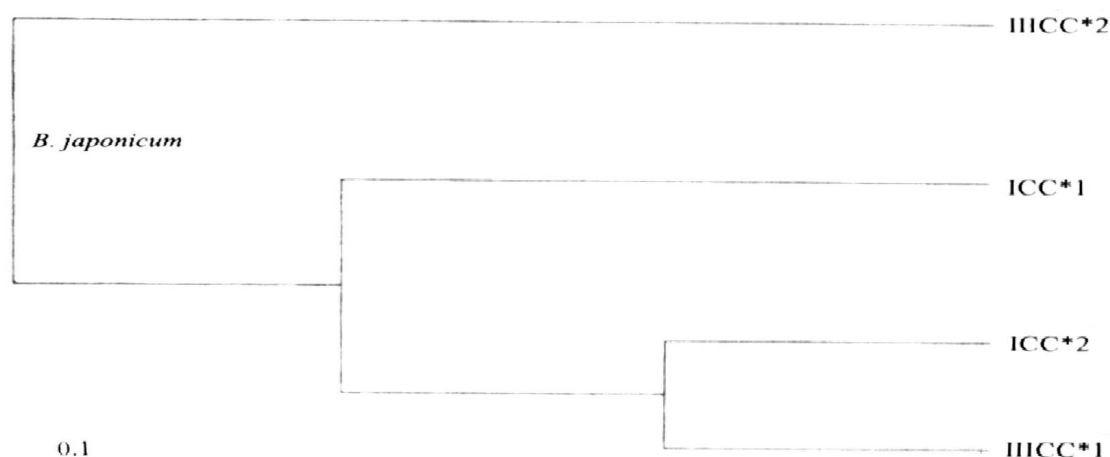
Figure 58 Phylogenetic tree of rhizobial isolates compared between rainy season and dry season in the Central region, forest clearance for crop cultivation for 3 years.
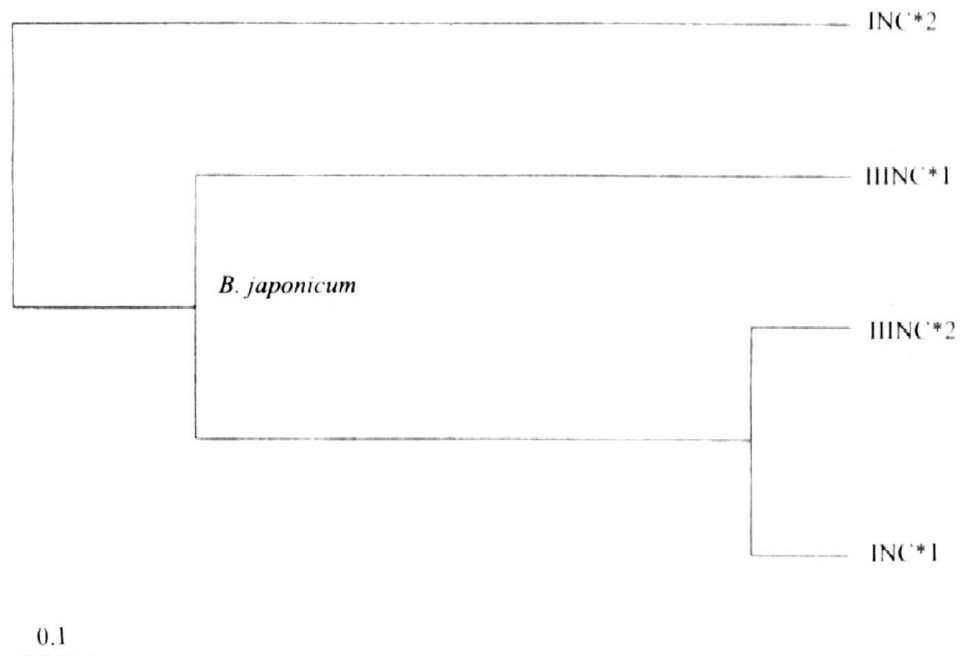
Figure 59 Phylogenetic tree of rhizobial isolates compared between rainy season and dry season in the North region, forest clearance for crop cultivation for 3 years.
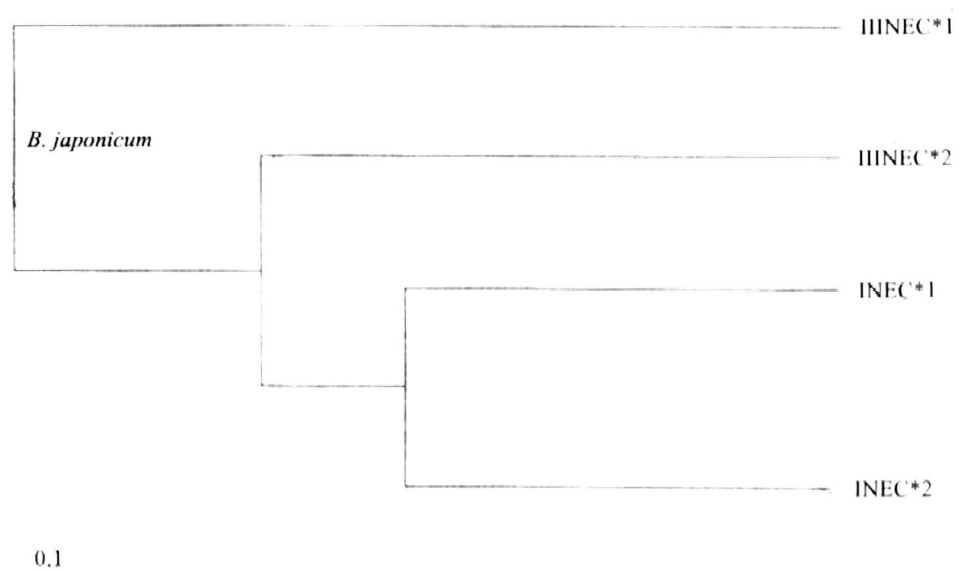
Figure 60 Phylogenetic tree of rhizobial isolates compared between rainy season and dry season in the North Eastern, forest clearance for crop cultivation for 3 years.

3.6.11 PCR-RAPD of rhizobia from intensive agriculture production using high rate of pesticides and fertilizers

The PCR products obtained from rhizobia in this area showed some closely related strains in rainy season as in lanes 4 with 6 and Lanes 3 with 5 (Fig. 61A). The phylogenetic tree of rhizobial isolates in the North were very near among group and found similar strains as IND1 in rainy season with IIIND1 in dry season (Fig. 62). This data indicated its establisment over the season isolate in this area. While the phylogenetic tree of rhizobia in the North Eastern region (Fig. 63) found closely related strains in the same area as INED1 and INED3.
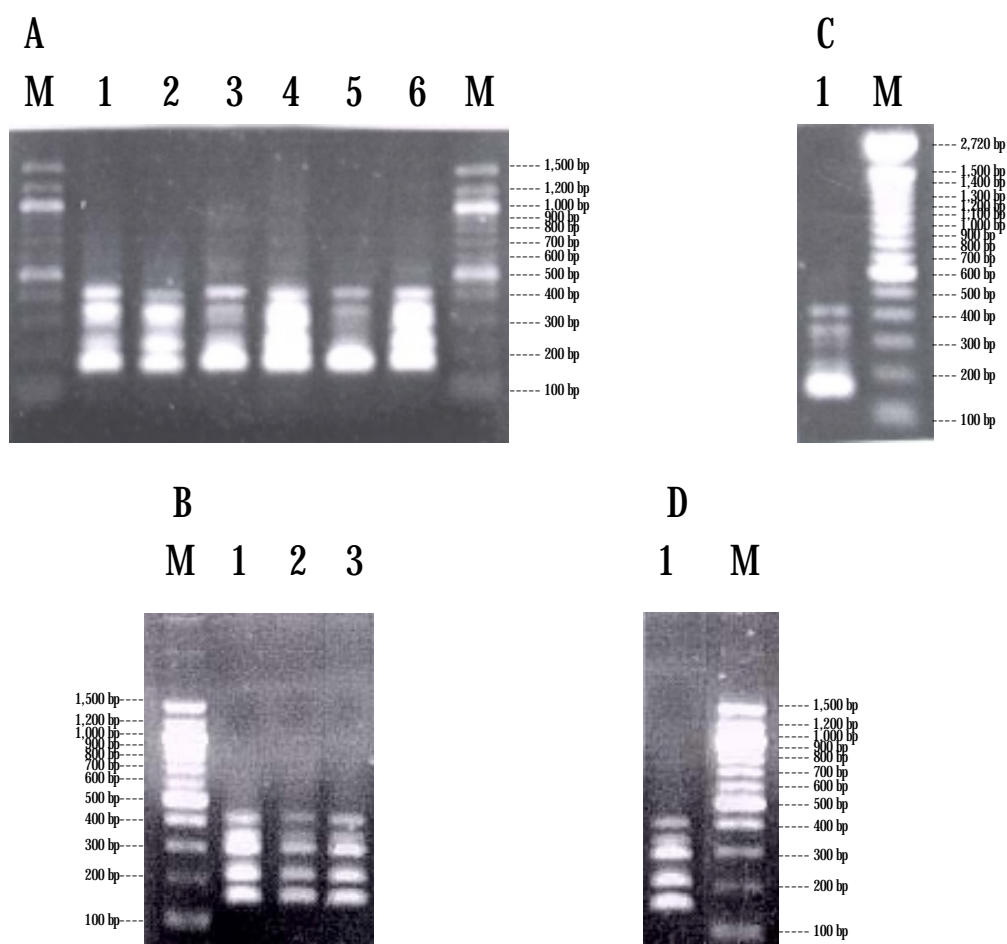
Figure 61. (A, B, C, D). PCR-RAPD patterns of rhizobial isolates from intensive agricultural production using high rate of pesticides and fertilizers in rainy season (A,B) and dry season (C,D). A) M, 100 bp Ladder marker; Lane 1, IND1; Lane 2, IND2; Lane 3, IND3; Lane 4, INED1; Lane 5, INED2 and Lane 6, INED3. B) M, 100 bp Ladder marker; Lane 1, IIIND1; Lane 2, IIIND2 and Lane 3, IIIND3. (C) Lane 1, IIINED1 and M, 100 bp Ladder marker (D) Lane 1, IIINED3 and M, 100 bp Ladder marker.
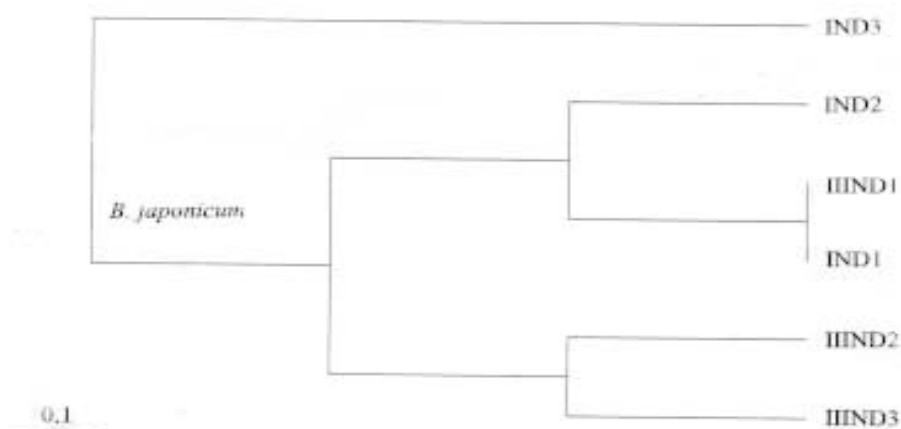
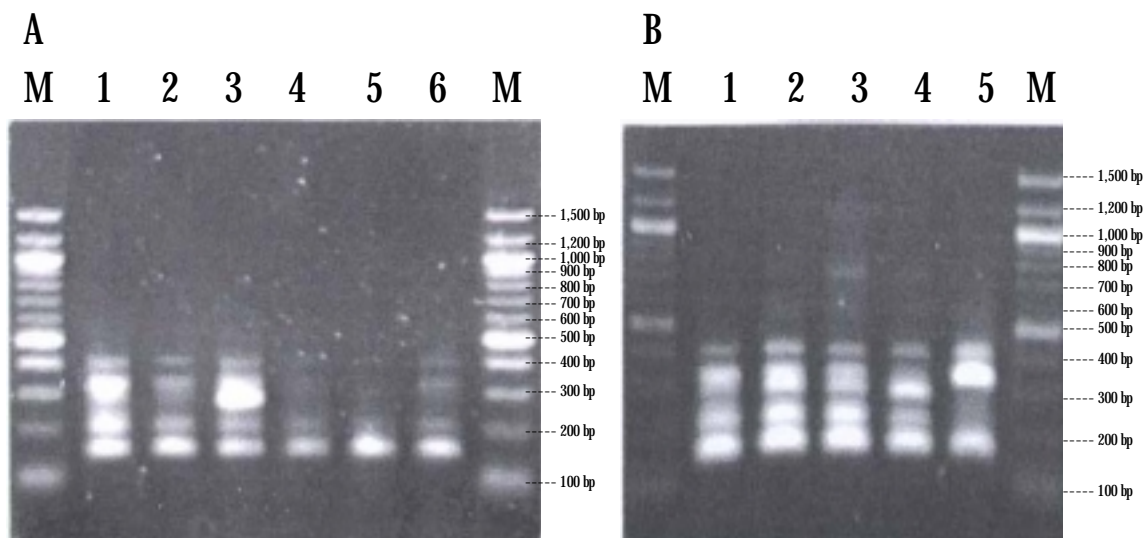**Figure 62.** Phylogenetic tree of rhizobial isolates compared between rainy season and dry season in the North region, intensive agriculture production using high rate of pesticides and fertilizers.
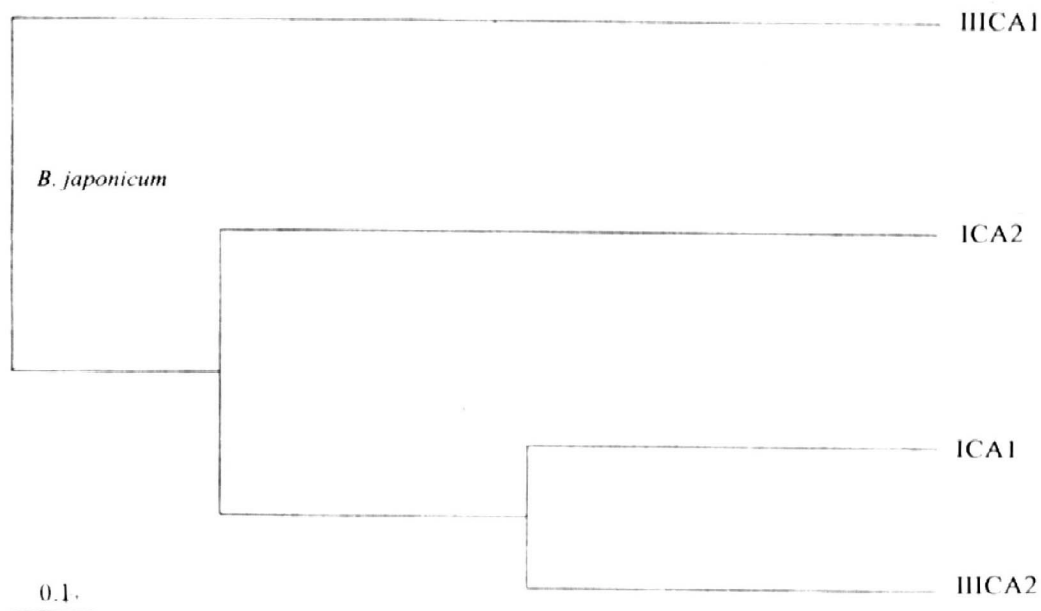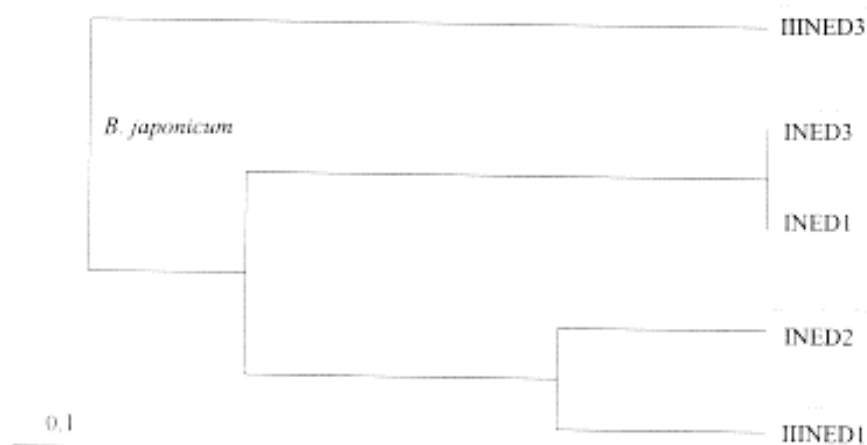

Figure 63. Phylogenetic tree of rhizobial isolates compared between rainy season and dry season in the North Eastern region, intensive agriculture production using high rate of pesticides and fertilizers.

4 DISCUSSIONS

The number of rhizobial population from different soils was observed previously (Mendes and Bottomley, 1998) and they found that soil were complex environments in which it was generally recognized that change in crop system in variably cause changes in soil physical properties that influence for rhizobial population. However, the information from these results were the first to illustrate that the population of rhizobia could be distributed in various soils. The dynamics population and diversity of rhizobia from diversed ecosystems in Thai soil were scarce and very interesting to distinguish. The data showed that rice in rotation with leguminous crop was suitable for rhizobia to maintain sustainably. This was evident by leguminous plant that rotation with rice in this area was mung bean in the Central and North Eastern and soybean in the North region. The report supported this solution by Mendes and Bottomley (1998) who studied distribution of a population of *Rhizobium leguminosarum* bv. trifolii among different size classes of soil agregates and found that the population of nodulation *Rhizobium* organisms would be greater in a legume cover crop soil than either the cereal of follow soil. The area which showed high effect on the population of rhizobia after environment and ecosystem changed was intensive agricultural production using high rate of pesticides and fertilizers area in the Central region. Because this area never cultivated leguminous plant which could maintain rhizobial population in soil. The soil were complex environments in which it was generally recognized that changes in crop systems invariably cause changes in soil physical properties that influence microbial activity. However, to what extent physical properties control the distribution of bacteria and influence their growth activities and turnover. The interest of microbiologist to gain and understanding of how soil management might influence the activities of soil microorganism by modifying soil structural properties.

The biodiversity of rhizobia in this thesis were studied by many methods. The first was some morphological and physiological aspects. It was found from 200 rhizobial strains that there were 99% of slow-grower or *Bradyrhizobium* The slow-growing isolates were more diverse, production IAA and alkaline reaction. This result confirmed invalidity of linking slow growth to alkaline production (Hernandez and Focht, 1984) and was in agreement with similar observation

made on cowpea rhizobia from Nigerian soils (Eaglesham et al., 1987). IAA production by rhizobia from this experiment found that slow-grower could exhibit higher IAA than fast-grower and the work by Fuhrman (1993) and Nuntagij et al. (1997) who studied IAA production in *Bradyrhizobium* strains isolated from soybean nodulate also support this study. Almost *Bradyrhizobium* from this experiment could not produce IAA indicated that rhizobia from Thai soil were not *B. elkani*. The results obtained from this study could be explained that inoculation of *B. japonicum* has still been important for agricultural field in Thailand.

The nodulation and N_2-fixing with index plant (siratro) was selected to characterize rhizobial isolates and found wide range in N_2-fixing efficiency. Lawn and Bushy (1982), reported that host plant was the first important factor but the high efficiency of symbiosis process could occur by joint other factors together. However, the genotypic of plant and rhizobia was shown crucial role with N_2-fixing ability under the effect by environment (Miller and Fernandez, 1987; Holl and La Rue, 1976; Nambia et al., 1981).

Antibiotic sensitivity of rhizobial strains has been intensively used for general characterization (Beynon and Josey, 1980; Rupela et al., 1982; Eaglesham, 1987; Mueller et al., 1988; Date and Hurse, 1991). In this study found that the resistant of rhizobial isolates to antibiotics varied in the number and type of antibiotics. This observation agreed with results of other studies that showed slow-growing rhizobia to be less sensitive to antibiotic (Elkan, 1992). Mueller et al. (1988) observed that sensitivity of rhizobia might be low for those antibiotics that might be routinely produced by other soil organisms. This results showed high diversification in rhizobia group and also corresponded to the results previously reported by Teaumroong et al. (1996) who studied on 38 strains of *Bradyrhizobium* sp. and *B. japonicum* for mung bean, groundnut and soybean.

The host-independent cross-inoculation group system for classifying rhizobia was selected to determine possibility of some interested genera. However, this technique has been subjected to much criticism because it is not a taxonomy one it is the best practical system currently available (Burton, 1965).

Classification of rhizobia is becoming increasingly complex because of new findings, e.g. some soybeans are now known to be nodulated by a distinct group of fast-growing acid-production rhizobia. A new system of classification has been formulated to classify rhizobia.

This new system recognizes only two genera for the Rhizobiaceae. All fast-growing acid-producing rhizobia now fall under the new genus *Rhizobium* and all slow-growing alkali-producing rhizobia under the new genus *Bradyrhizobium* Also, under this system, *R. trifolii, R. phaseoli,* and *R. leguminosarum* are combined as one species, designated as *Rhizobium leguminosarum,* comprising three biovars *(trifolii, phaseoli* and *viceae).* *R. meliloti* remains as before and *R. loti* has been assigned to the fast-growing *Lotus* rhizobia. Genetically related to *R. loti* are rhizobia from *Lotus corniculatus, Lotus tenuis, Cicer arietinum, Leucaena leucocephala, Sophora microphylla* etc. The soybean rhizobia are now *R. japonicum* (fast-growing and acid producing) and *B. japonicum* (slow-growing and alkali-producing). Rhizobia from *Vigna, Arachis, Desmodium, Macroptilium, Stylosanthes* etc. are still unclassified, but grouped as *Bradyrhizobium* sp. The non-legume *Parasponia* (called *Trema* previously) is also nodulated by *Bradyrhizobium* sp. Besides *Leucaena,* whose rhizobia are now *R. loti,* there are othe legumes *(Sesbania, Neptunia, Calliandra, Acacia)* which are nodulated by fast-growing acid-producing rhizobia and the taxomic status of these organisms may by resolved in the future (Somasegaran et al 1982).

Rhizobia from different areas were isloated to analyze the genetic diversity that using PCR-RAPD technique. The UPGMA method was used to analyze the RAPD variation within rhizobial population. The result obtained from this study showed the majority of genetic diversity of rhizobial isolates was closely related between each area and region. The dominant native strain was found in the areas which showed high population number as foot hill of mountain and rice in rotation with other crops. The data supported the first objective of this study. The development of randomly amplified polymorphic DNA (RAPD) provided a new tool for investigating genetic polymorphisms in many different organisms, including bacteria (Cancilla et al, 1992; Fani et al, 1993; Jayrao et al, 1992) and recently this method has been used for *Rhizobium* identification and *Bradyrhizobium* genetic analyses. The PCR-RAPD technique which selected to study genetic diversity of rhizobial population that support the findings of De Bruijn (1992), that PCR with a suitable primer could be applied for molecular genetic characterization of rhizobia. Nishi et al. (1996) and Lung et al. (1994) reported of *Bradyrhizobium* strains, thus this RAPD technique could be used for classification of *Bradyrhizobium* group, the majority population of rhizobial isolates in this study.

The phylogenetic tree constructed from PCR-RAPD patterns could separate rhizobia into 3 groups were the similar strains, dominant native strains and other different strains. The biodiversity of rhizobial isolates from this study found that some areas as highest mountain, undisturb forest and forest clearance for crop cultivation for 3 years showed high diversity among rhizobia group. These data indicated the influence by undisturb ecosystems to rhizobial diversity in natural areas. Only forest clearance for crop cultivation for 3 years area showed high effect by agricultural system. The other areas which found similar strains and dominant native strains could elucidate that the influence by agricultural system was a crucial factor to control rhizobial diversity in soil especially, the rotation by leguminous plant.

The dominant native strains of rhizobial isolates from this study were displayed in Table 13.

Table 13 Dominant native strains of rhizobia from different soils in Thailand

Dominant native strains	Areas/Regions	Host plant	Antibiotic groups
1. $INEM_1$-1, $IIINEM_1$-4	HM/NE	cowpea	g 19 (T. 5A), g 3 (T. 6A)
2. ICM_3-4, $IIICM_3$-3	FM/C	cowpea	g 9 (T. 5A), g 4 (T. 6A)
3. INM_3-2, $IIINM_3$-3	FM/N	cowpea	g 40 (T. 5A), g 16 (T. 6A)
4. $INEM_3$-3, $IIINEM_3$-2	FM/NE	cowpea	g 1 (T. 5A), g 47 (T. 6A)
5. ICC4, IIICC3	FC/C	cowpea	g 12 (T. 5A), g 12 (T. 6A)
6. INR2, IIINR2	RC/N	cowpea	g 12 (T. 5A), g 38 (T. 6A)
7. ICCR4, IIICCR2	RRC/C	cowpea	g 10 (T. 5A), g 14 (T. 6A)
8. INCR2, IIINCR1	RRC/N	cowpea	g 8 (T. 5A), g 6 (T. 6A)
9. INECR4, IIINECR4	RRC/NE	cowpea	g 23 (T. 5A), g 7 (T. 6A)
10. INEF2, IIINEF3	UC/C	cowpea	g 23 (T. 5A), g 53 (T. 6A)

HM = Highest mountain   FM = Foot hill of mountain   FC = Field crop cultivation
RC = Rice cultivation   RRC = Rice in rotation with other crops
UC = Uncultivated   C = Central   N = North   NE = North Eastern

5. CONCLUSION

The objectives of this reserch were to investigate the suitable environment and ecosystem for rhizobia to maintain sustainably, the effect on population of rhizobia after environment and ecosystem changed and biodiversity of rhizobia under diversed ecosystems. To reach these objectives, soil samples were collected from various ecosystems in the Central, the North and the North Eastern region to isolate rhizobia. To distinguish rhizobial isolates from diversed ecosystems different methods such as 1) MPN plant-infection method for rhizobial enumeration 2) isolation and characterization of rhizobia 3) nodulation and N_2-fixing ability with index plant (Siratro) 4) antibiotic resistant profiles 5) host-dependent cross-inoculation group and 6) PCR-RAPD amplification and phylogenetic tree were employed.

The result obtained from these studies indicated that rhizobial persistence and their population dynamic somewhat depend on the type of crops in particular area. It was found that rice in rotation with other legumes could maintain rhizobial population better than other areas. The effect by environment and ecosystem changed on decreasing rhizobial population found in intensive agricultural production using rate of pesticides and fertilizers in the Central region. The decreasing in rhizobial population in this area was mainly due to the leguminous plant had never been cultivated which areas also supported this phenomenon.

The biodiversity of rhizobial isolates were analysed by different means. The rhizobial isolates were characterized on the basis of acid-base production and colony forming time. Most of them were most likely belong to genus *Bradyrhizobium* By using antibiotic resistant profiles, most of the strains performed highly resistant to trimetroprim and highly susceptible to kanamycin that was one of the characteristic of *Bradyrhizobium* By using host-dependent cross-inoclation group found that about 88% of rhizobia could nodulate with cowpea, the common host plant of *Bradyrhizobium* sp. and about 12% could nodulate with soybean, the host plant of *B. japonicum* and *B. elkani*. However, about 2 % of rhizobia could nodulate only root of *Sesbania rostata*. The information from PCR-RAPD and phylogenetic tree could explain the diversification of rhizobia in each ecosystem and found that most of rhizobial strains from Thai soil have close relation among group. The areas where mostly found dominant native rhizobial strains which able to persist across every season were foot hill of mountain and rice in rotation with other crops areas.

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APPENDIX A

Table A1. Isolation and characterization rhizobia in rainy reason.

Isolate	Single coloy (days)	Type of colony	Reaction in YMA+BTB media	IAA production
ICM_1-1	5	Medium wet	Alkaline	+
ICM_1-3	8	Small dry	Alkaline	-
ICM_1-4	5	Medium dry	Alkaline	-
ICM_2-1	2	Large wet	Acid	+
ICM_2-2	5	Small dry	Alkaline	+
ICM_2-3	6	Small wet	Alkaline	-
ICM_2-4	6	Small dry	Alkaline	-
ICM_3-1	5	Medium wet	Alkaline	-
ICM_3-2	5	Medium wet	Alkaline	-
ICM_3-3	5	Medium wet	Alkaline	-
ICM_3-4	6	Medium dry	Alkaline	+
ICC1	5	Medium wet	Alkaline	-
ICC2	5	Medium wet	Alkaline	-
ICC3	5	Medium dry	Alkaline	-
ICC4	7	Small dry	Alkaline	-
ICR1	5	Medium wet	Alkaline	+
ICR2	5	Medium wet	Alkaline	-
ICR4	7	Small dry	Alkaline	-
ICCR1	5	Medium wet	Alkaline	-
ICCR2	5	Medium wet	Alkaline	-
ICCR3	6	Medium dry	Alkaline	+
ICCR4	5	Medium dry	Alkaline	+
ICF1	6	Small dry	Alkaline	+
ICF2	5	Medium wet	Alkaline	-
ICF3	6	Small dry	Alkaline	-
ICF4	6	Medium dry	Alkaline	+
ICA1	6	Small dry	Alkaline	-
ICA2	6	Small wet	Alkaline	+
ICB1	5	Medium wet	Alkaline	-
ICB2	5	Medium wet	Alkaline	-
ICC^1	6	Small dry	Alkaline	+
ICC^2	5	Medium wet	Alkaline	-
INM_1-1	5	Medium dry	Alkaline	+
INM_1-2	6	Medium dry	Alkaline	+
INM_1-4	5	Medium dry	Alkaline	+
INM_2-1	5	Medium wet	Alkaline	+
INM_2-2	6	Small dry	Alkaline	+
INM_2-3	5	Medium wet	Alkaline	-
INM_2-4	5	Medium wet	Alkaline	-

Table 1A. (continued)

Isolate	Single coloy (days)	Type of colony	Reaction in YMA+BTB media	IAA production
INM_3-1	5	Medium wet	Alkaline	-
INM_3-2	5	Medium wet	Alkaline	-
INM_3-3	6	Medium dry	Alkaline	+
INM_3-4	5	Medium wet	Alkaline	+
INC1	5	Medium wet	Alkaline	-
INC2	5	Medium dry	Alkaline	-
INC3	6	Medium dry	Alkaline	+
INC4	6	Small dry	Alkaline	+
INR1	5	Medium wet	Alkaline	-
INR2	6	Small wet	Alkaline	-
INR4	5	Medium wet	Alkaline	-
INCR1	7	Small dry	Alkaline	+
INCR2	6	Small dry	Alkaline	+
INCR3	5	Medium dry	Alkaline	+
INCR4	8	Small dry	Alkaline	-
INF1	6	Small dry	Alkaline	-
INF2	6	Medium dry	Alkaline	-
INF3	6	Medium dry	Alkaline	-
INF4	8	Small dry	Alkaline	-
INA1	5	Medium wet	Alkaline	-
INA2	5	Medium dry	Alkaline	+
INB1	5	Medium dry	Alkaline	+
INB2	6	Small dry	Alkaline	+
INC^*1	5	Medium wet	Alkaline	+
INC^*2	5	Medium dry	Alkaline	+
IND1	7	Small wet	Alkaline	+
IND2	5	Medium dry	Alkaline	+
IND3	5	Medium dry	Alkaline	+
$INEM_1$-1	6	Small dry	Alkaline	+
$INEM_1$-2	5	Medium dry	Alkaline	-
$INEM_1$-3	8	Small dry	Alkaline	-
$INEM_1$-4	5	Medium dry	Alkaline	-
$INEM_2$-1	5	Medium dry	Alkaline	-
$INEM_2$-2	6	Small wet	Alkaline	+
$INEM_2$-3	5	Medium dry	Alkaline	+
$INEM_2$-4	8	Small dry	Alkaline	-
$INEM_3$-1	7	Small dry	Alkaline	-
$INEM_3$-2	8	Small dry	Alkaline	-
$INEM_3$-3	7	Small dry	Alkaline	-

Table 1A. (continued)

Isolate	Single coloy (days)	Type of colony	Reaction in YMA+BTB media	IAA production
INEM$_3$-4	5	Medium wet	Alkaline	-
INEC1	6	Small dry	Alkaline	-
INEC2	5	Medium wet	Alkaline	-
INEC3	5	Medium wet	Alkaline	-
INEC4	5	Medium wet	Alkaline	-
INER1	5	Medium wet	Alkaline	-
INER2	5	Medium wet	Alkaline	-
INER3	5	Medium wet	Alkaline	-
INER4	5	Medium wet	Alkaline	-
INECR1	6	Small wet	Alkaline	-
INECR2	4	Medium wet	Alkaline	-
INECR3	5	Small wet	Alkaline	-
INECR4	5	Medium dry	Alkaline	-
INEF1	5	Medium dry	Alkaline	-
INEF2	5	Medium wet	Alkaline	-
INEF3	5	Medium wet	Alkaline	-
INEF4	5	Medium wet	Alkaline	+
INEA1	6	Medium dry	Alkaline	+
INEA2	6	Small dry	Alkaline	-
INEB1	5	Medium dry	Alkaline	-
INEB2	5	Medium dry	Alkaline	+
INEC^1	8	Small dry	Alkaline	-
INEC^2	6	Medium dry	Alkaline	+
INED1	5	Medium wet	Alkaline	-
INED2	5	Medium wet	Alkaline	-
INED3	5	Medium dry	Alkaline	+

Table 2A. Isolation and characterization rhizobia in dry season

Isolate	Single coloy (days)	Type of colony	Reaction in YMA+BTB media	IAA production
IIICM$_1$-1	6	Small wet	Alkaline	+
IIICM$_1$-2	8	Small dry	Alkaline	-
IIICM$_1$-3	7	Small dry	Alkaline	-
IIICM$_1$-4	8	Small dry	Alkaline	-
IIICM$_2$-1	7	Small dry	Alkaline	-
IIICM$_2$-2	7	Small dry	Alkaline	-
IIICM$_2$-3	5	Medium wet	Alkaline	-
IIICM$_2$-4	7	Small dry	Alkaline	+
IIICM$_3$-1	6	Medium dry	Alkaline	-
IIICM$_3$-2	6	Medium wet	Alkaline	-
IIICM$_3$-3	5	Medium wet	Alkaline	-
IIICM$_3$-4	5	Medium wet	Alkaline	-
IIICC1	5	Medium wet	Alkaline	-
IIICC2	6	Small wet	Alkaline	-
IIICC3	7	Small dry	Alkaline	-
IIICC4	5	Medium dry	Alkaline	-
IIICR1	7	Small wet	Alkaline	+
IIICR2	6	Small wet	Alkaline	+
IIICR3	5	Small wet	Alkaline	-
IIICR4	5	Medium dry	Alkaline	-
IIICCR1	6	Small dry	Alkaline	-
IIICCR2	6	Small dry	Alkaline	+
IIICCR3	5	Medium wet	Alkaline	+
IIICCR4	6	Small dry	Alkaline	-
IIICF1	5	Medium wet	Alkaline	-
IIICF2	5	Medium dry	Alkaline	-
IIICF3	6	Small dry	Alkaline	-
IIICF4	5	Medium dry	Alkaline	-
IIICA1	5	Medium dry	Alkaline	-
IIICA2	5	Medium wet	Alkaline	-
IIICB1	5	Medium wet	Alkaline	-
IIICB2	5	Medium wet	Alkaline	-
IIICC^1	5	Medium dry	Alkaline	+
IIICC^2	5	Medium dry	Alkaline	+
IIINM$_2$-1	6	Medium dry	Alkaline	-
IIINM$_2$-2	7	Small dry	Alkaline	-
IIINM$_2$-3	5	Medium dry	Alkaline	+
IIINM$_2$-4	5	Medium wet	Alkaline	-

Table 2A. (continued)

Isolate	Single coloy (days)	Type of colony	Reaction in YMA+BTB media	IAA production
IIINM$_3$-1	5	Medium wet	Alkaline	-
IIINM$_3$-2	6	Medium dry	Alkaline	+
IIINM$_3$-3	5	Medium dry	Alkaline	-
IIINM$_3$-4	6	Small wet	Alkaline	-
IIINC1	6	Medium dry	Alkaline	+
IIINC2	5	Medium dry	Alkaline	-
IIINC3	5	Medium dry	Alkaline	+
IIINC4	5	Medium dry	Alkaline	-
IIINR1	5	Medium wet	Alkaline	+
IIINR2	5	Medium wet	Alkaline	-
IIINR3	7	Small dry	Alkaline	-
IIINR4	5	Medium wet	Alkaline	-
IIINCR1	6	Small dry	Alkaline	+
IIINCR2	6	Medium dry	Alkaline	+
IIINCR3	5	Medium wet	Alkaline	-
IIINCR4	5	Medium wet	Alkaline	-
IIINF1	5	Medium wet	Alkaline	-
IIINF2	7	Medium wet	Alkaline	-
IIINF3	6	Medium dry	Alkaline	-
IIINF4	6	Medium dry	Alkaline	-
IIINA1	5	Medium dry	Alkaline	-
IIINA2	6	Small dry	Alkaline	-
IIINC*1	6	Small dry	Alkaline	-
IIINC*2	5	Medium wet	Alkaline	-
IIIND1	4	Medium wet	Alkaline	-
IIIND2	6	Small wet	Alkaline	-
IIIND3	6	Small dry	Alkaline	-
IIINEM$_1$-1	7	Small dry	Alkaline	-
IIINEM$_1$-2	8	Small dry	Alkaline	-
IIINEM$_1$-3	6	Medium dry	Alkaline	+
IIINEM$_1$-4	6	Small dry	Alkaline	+
IIINEM$_2$-2	7	Small dry	Alkaline	-
IIINEM$_2$-3	8	Small dry	Alkaline	+
IIINEM$_2$-4	8	Small dry	Alkaline	+
IIINEM$_3$-1	6	Medium dry	Alkaline	-
IIINEM$_3$-2	6	Small dry	Alkaline	+
IIINEM$_3$-3	6	Medium wet	Alkaline	+
IIINEM$_3$-4	6	Small wet	Alkaline	-
IIINEC1	5	Medium wet	Alkaline	+
IIINEC2	6	Medium wet	Alkaline	-
IIINEC3	6	Medium dry	Alkaline	-

Table 2A. (continued)

Isolate	Single coloy (days)	Type of colony	Reaction in YMA+BTB media	IAA production
IIINEC4	5	Medium wet	Alkaline	-
IIINER1	6	Small wet	Alkaline	+
IIINER3	5	Medium wet	Alkaline	-
IIINER4	5	Mesium wet	Alkaline	+
IIINECR1	5	Medium dry	Alkaline	+
IIINECR2	5	Medium wet	Alkaline	-
IIINECR3	5	Medium dry	Alkaline	+
IIINECR4	5	Medium dry	Alkaline	-
IIINEF1	7	Small wet	Alkaline	+
IIINEF2	5	Medium wet	Alkaline	+
IIINEF3	6	Small wet	Alkaline	-
IIINEF4	6	Medium dry	Alkaline	-
IIINEA1	6	Medium dry	Alkaline	-
IIINEC^1	5	Medium wet	Alkaline	-
IIINEC^2	5	Medium dry	Alkaline	-
IIINED1	5	Medium wet	Alkaline	-
IIINED3	6	Small dry	Alkaline	-

Table 3A. Nodulation and N_2-fixation efficeincy of rhizobia with index plant (Siratro) in rainy season.

Isolate	Weigh of dry plants (g/plant)	ARA mmol C_2H_4/g nod/h	Amount of nodules (nodules/plant)	Weigh of nodules g/plant
ICM_1-1	0.80	0.35	47	0.58
ICM_1-4	0.16	14.25	28	0.04
ICM_2-1	0.44	1.10	56	0.23
ICM_2-2	ND^-	ND	ND	ND
ICM_2-3	ND	ND	ND	ND
ICM_2-4	0.37	0.06	82	0.21
ICM_3-1	0.42	0.29	42	0.30
ICM_3-2	0.55	0.64	47	0.32
ICM_3-3	0.37	0.09	58	0.31
ICM_3-4	0.41	0.17	42	0.32
ICC1	0.28	19.17	53	0.10
ICC2	0.15	7.56	53	0.04
ICC3	0.19	42.14	36	0.02
ICC4	0.08	0.02	23	0.08
ICR1	0.34	0.48	41	0.22
ICR2	0.34	4.57	65	0.24
ICR4	0.32	0.88	47	0.18
ICCR1	0.03	15.76	8	0.02
ICCR2	56.00	0.47	54	0.43
ICCR3	0.12	0.33	33	0.12
ICCR4	0.24	10.51	36	0.13
ICF1	ND	ND	ND	ND
ICF2	0.23	0.45	88	0.34
ICF3	0.25	0.99	36	0.20
ICF4	0.42	0.17	52	0.33
ICA1	0.39	1.96	35	0.21
ICA2	0.23	0.15	50	0.67
ICB1	0.20	0.31	66	0.21
ICB2	0.24	1.54	57	0.23
ICC^1	0.23	0.08	46	0.12
ICC^2	0.23	3.85	40	0.25
INM_1-1	0.18	47.19	46	0.03
INM_1-2	0.02	0.01	13	0.03
INM_1-4	0.39	2.44	50	0.23
INM_2-1	0.19	15.53	33	0.07
INM_2-2	0.41	0.42	44	0.29
INM_2-3	0.49	13.16	62	0.46

Table 3A. (continued)

Isolate	Weigh of dry plants (g/plant)	ARA mmol C_2H_4/g nod/h	Amount of nodules (nodules/plant)	Weigh of nodules g/plant
INM_2-4	0.22	2.54	67	0.17
INM_3-1	0.40	1.58	39	0.25
INM_3-2	0.28	0.10	63	0.29
INM_3-3	0.17	3.74	38	0.06
INM_3-4	0.30	0.53	95	0.43
INC1	0.67	0.16	46	0.38
INC2	0.56	1.55	45	0.33
INC3	0.20	3.35	15	0.21
INC4	0.13	18.25	28	0.10
INR1	0.19	0.03	32	0.17
INR2	0.41	0.04	78	0.41
INR4	0.37	2.20	68	0.27
INCR1	0.18	0.56	32	0.17
INCR2	0.26	4.65	53	0.03
INCR3	0.18	41.76	27	0.03
INCR4	0.12	1.67	27	0.11
INF1	ND	ND	ND	ND
INF2	0.16	0.22	39	0.27
INF3	0.39	3.16	37	0.22
INF4	0.30	0.75	36	0.22
INA1	0.22	19.27	42	0.10
INA2	ND	ND	ND	ND
INB1	0.10	19.00	40	0.02
INB2	0.23	22.17	41	0.08
INC^1	0.19	4.38	15	0.12
INC^2	0.17	26.71	27	0.01
IND1	0.16	0.53	31	0.13
IND2	0.20	0.45	54	0.06
IND3	0.01	0.48	27	0.15
$INEM_1$-1	0.18	258.68	31	0.13
$INEM_1$-2	0.24	14.09	50	0.18
$INEM_1$-3	0.14	0.99	37	0.15
$INEM_1$-4	ND	ND	ND	ND
$INEM_2$-1	0.33	14.09	36	0.21
$INEM_2$-2	0.06	0.03	45	0.09
$INEM_2$-3	ND	ND	ND	ND
$INEM_2$-4	0.26	6.34	39	0.07
$INEM_3$-1	0.20	3.13	58	0.10

Table 3A. (continued)

Isolate	Weigh of dry plants (g/plant)	ARA mmol C_2H_4/g nod/h	Amount of nodules (nodules/plant)	Weigh of nodules g/plant
$INEM_3$-2	0.24	1.90	64	0.15
$INEM_3$-3	0.22	0.10	62	0.17
$INEM_3$-4	0.25	10.69	71	0.21
INEC1	0.25	2.88	70	0.03
INEC2	0.39	0.86	54	0.30
INEC3	0.26	0.07	37	0.14
INEC4	0.13	0.42	42	0.31
INER1	0.27	4.33	43	0.09
INER2	0.25	1.25	37	0.22
INER3	0.14	0.57	61	0.14
INER4	0.06	2.03	32	0.10
INECR1	0.22	0.30	56	0.30
INECR2	0.61	0.18	64	0.44
INECR3	0.20	1.80	57	0.17
INECR4	0.27	3.20	44	0.14
INEF1	0.51	0.30	41	0.35
INEF2	0.42	0.66	44	0.37
INEF3	0.48	0.15	88	0.44
INEF4	0.63	1.24	29	0.45
INEA1	0.19	42.73	31	0.03
INEA2	0.16	0.87	33	0.16
INEB1	0.31	0.31	45	0.33
INEB2	0.42	2.24	66	0.29
INEC^1	0.56	0.50	61	0.51
INEC^2	0.24	29.83	67	0.04
INED1	0.28	1.55	34	0.18
INED2	0.24	19.93	64	0.07
INED3	0.16	0.50	26	0.24

1/ ND = Not Detect

Table 4A. Nodulation and N_2-fixation efficeincy of rhizobia with index plant (Siratro) in dry season.

Isolate	Weigh of dry plants (g/plant)	ARA mmol C_2H_4/g nod/h	Amount of nodules (nodules/plant)	Weigh of nodules g/plant
IIICM$_1$-1	0.11	3.49	69	0.14
IIICM$_1$-2	0.50	1.00	40	0.19
IIICM$_1$-3	ND⁻	ND	ND	ND
IIICM$_1$-4	0.17	1.82	53	0.24
IIICM$_2$-1	0.15	10.88	48	0.06
IIICM$_2$-2	0.15	0.46	28	0.16
IIICM$_2$-3	0.12	7.89	59	0.11
IIICM$_2$-4	0.21	2.60	52	0.17
IIICM$_3$-1	0.17	1.82	53	0.24
IIICM$_3$-2	0.10	1.58	11	0.25
IIICM$_3$-3	0.19	4.47	39	0.15
IIICM$_3$-4	0.20	2.28	52	0.15
IIICC1	0.16	1.85	49	0.16
IIICC2	0.26	2.37	55	0.42
IIICC3	0.24	2.43	40	0.30
IIICC4	ND	ND	ND	ND
IIICR1	0.15	65.80	40	0.01
IIICR2	0.12	3.07	40	0.16
IIICR3	0.08	0.88	51	0.11
IIICR4	0.10	1.45	53	0.12
IIICCR1	0.11	2.98	60	0.09
IIICCR2	0.23	1.00	55	0.16
IIICCR3	0.17	4.84	30	0.06
IIICCR4	0.17	1.13	34	0.15
IIICF1	0.14	0.99	34	0.29
IIICF2	ND	ND	ND	ND
IIICF3	0.29	3.14	51	0.15
IIICF4	0.26	2.96	79	0.27
IIICA1	0.18	29.24	29	0.06
IIICA2	0.23	14.58	60	0.06
IIICB1	0.12	2.66	51	0.12
IIICB2	0.09	14.28	48	0.06
IIICC^1	0.29	48.06	84	0.01
IIICC^2	0.12	14.22	43	0.10
IIINM$_2$-1	0.19	3.10	42	0.11
IIINM$_2$-2	0.27	0.58	62	0.22

Table 4A. (continued)

Isolate	Weigh of dry plants (g/plant)	ARA mmol C_2H_4/g nod/h	Amount of nodules (nodules/plant)	Weigh of nodules g/plant
IIINM$_2$-3	0.19	2.31	42	0.14
IIINM$_2$-4	0.18	7.64	35	0.12
IIINM$_3$-1	0.26	8.99	46	0.16
IIINM$_3$-2	0.30	3.54	35	0.09
IIINM$_3$-3	0.21	0.91	45	0.18
IIINM$_3$-4	0.22	29.06	31	0.02
IIINC1	0.18	1.45	53	0.14
IIINC2	0.13	2.66	49	0.12
IIINC3	0.17	2.81	38	0.13
IIINC4	ND	ND	ND	ND
IIINR1	0.22	3.10	47	0.16
IIINR2	0.21	7.57	50	0.12
IIINR3	0.17	2.54	48	0.14
IIINR4	0.14	4.55	15	0.10
IIINCR1	0.10	3.97	61	0.16
IIINCR2	0.20	4.56	31	0.09
IIINCR3	ND	ND	ND	ND
IIINCR4	0.26	0.24	32	0.18
IIINF1	0.08	3.37	31	0.08
IIINF2	0.15	9.57	32	0.09
IIINF3	0.29	2.40	26	0.19
IIINF4	0.21	5.27	38	0.14
IIINA1	ND	ND	ND	ND
IIINA2	0.19	4.71	38	0.26
IIINC^1	0.23	0.39	26	0.15
IIINC^2	0.19	1.04	30	0.16
IIINEM$_1$-1	0.15	8.32	37	0.11
IIINEM$_1$-2	0.11	8.48	40	0.08
IIINEM$_1$-3	0.25	1.13	33	0.16
IIINEM$_1$-4	0.26	0.39	61	0.12
IIINEM$_2$-2	ND	ND	ND	ND
IIINEM$_2$-3	0.19	4.26	41	0.10
IIINEM$_2$-4	0.13	0.18	46	0.23
IIINEM$_3$-1	0.14	1.65	39	0.21
IIINEM$_3$-2	0.13	1.08	37	0.13
IIINEM$_3$-3	0.03	0.01	16	0.08
IIINEM$_3$-4	0.04	2.60	25	0.06
IIINEC1	0.55	5.19	40	0.25

Table 4A. (continued)

Isolate	Weight of dry plants (g/plant)	ARA mmol C_2H_4/g nod/h	Amount of nodules (nodules/plant)	Weight of nodules g/plant
IIINEC2	0.03	0.16	8	0.02
IIINEC3	0.24	13.75	13	0.17
IIINEC4	0.30	0.95	26	0.20
IIINER1	0.21	3.42	39	0.20
IIINER3	0.28	0.08	40	0.23
IIINER4	0.32	9.39	35	0.20
IIINECR1	0.31	13.66	45	0.14
IIINECR2	0.19	3.17	30	0.14
IIINECR3	0.46	0.02	58	0.18
IIINECR4	0.24	159.66	30	0.12
IIINEF1	0.02	2.02	35	0.01
IIINEF2	0.42	3.42	69	0.29
IIINEF3	0.13	2.46	29	0.16
IIINEF4	0.36	0.19	45	0.22
IIINEA1	0.09	3.00	29	0.04
IIINEC*1	ND	ND	ND	ND
IIINEC*2	0.14	13.54	35	0.04
IIINED1	0.28	4.67	52	0.08

1/ ND = Not Detect

Table 5A. Antibiotic resistant profiles of rhizobial isolates in rainy season.

group	Antibioltic resistant profiles								Rhizobial isolates
	Car	Chl	Ery	Nal	Kan	Tmp	Tet	Str	
1	r	S_m	S_m	S_m	s	r	r	S_m	INEM₁-2, INEM₁-3, INEM₂-4, INEM₃-1, INEM₃-2, INEM₃-3, INEA1, INEC-2
2	r	S_m	S_m	S_m	S_m	r	S_m	S_m	ICC-1, IND1, IND3, INEB2
3	S_m	S_m	S_m	r	r	r	S_m	S_m	ICB1, ICC-2, INEM₂-2, INER2
4	r	S_m	S_m	S_m	s	r	r	S_m	ICM₃-1, INM₁-1, INB1
5	r	r	S_m	r	S_m	S_m	S_m	s	INF1, INA2, INEM₁-4
6	r	S_m	s	r	S_m	r	r	S_m	ICCR2, ICF4, INEF4
7	S_m	S_m	S_m	S_m	S_m	S_m	S_m	S_m	ICC1, ICC2, IND2
8	S_m	S_m	S_m	S_m	S_m	r	S_m	S_m	ICM₁-4, INCR2
9	S_m	S_m	S_m	S_m	S_m	r	r	s	ICM₃-4, INF4
10	S_m	S_m	S_m	S_m	s	r	S_m	S_m	ICC3, ICCR4
11	S_m	S_m	s	r	S_m	r	r	s	ICR1, ICF1
12	s	S_m	s	S_m	S_m	S_m	r	s	ICC4, INR2
13	S_m	S_m	s	S_m	S_m	r	s	S_m	ICR4, INA1
14	S_m	S_m	S_m	r	S_m	S_m	S_m	S_m	ICCR1, INF2
15	s	S_m	S_m	r	S_m	r	S_m	s	ICF2, ICA1
16	r	S_m	S_m	r	S_m	r	S_m	S_m	ICB2, INC-1
17	S_m	S_m	S_m	S_m	s	S_m	r	S_m	INM₁-2, INM₃-4
18	s	s	s	S_m	S_m	r	r	S_m	INM₁-4, INM₂-4
19	S_m	S_m	S_m	S_m	S_m	S_m	r	S_m	INC1, INEM₁-1
20	S_m	S_m	S_m	r	S_m	r	S_m	S_m	INC2, INED1
21	S_m	S_m	s	r	r	r	r	s	INER1, INER3
22	s	S_m	s	S_m	S_m	r	S_m	s	INECR1, INECR2
23	r	S_m	s	r	S_m	r	S_m	s	INECR4, INEF2
24	r	S_m	s	r	r	r	S_m	S_m	INEF3, INED2
25	r	S_m	s	S_m	s	r	r	s	ICM₁-1
26	S_m	s	S_m	s	s	S_m	S_m	s	ICM₁-3
27	r	S_m	S_m	r	S_m	r	S_m	s	ICM₂-1
28	r	S_m	S_m	S_m	s	r	r	s	ICM₂-2
29	S_m	S_m	S_m	S_m	s	r	r	S_m	ICM₂-3
30	r	S_m	s	r	S_m	r	S_m	S_m	ICM₂-4
31	s	S_m	r	r	S_m	S_m	S_m	S_m	ICM₃-2
32	s	S_m	S_m	r	r	r	S_m	S_m	ICM₃-3
33	r	S_m	s	r	S_m	r	r	s	ICR2
34	r	S_m	S_m	r	S_m	r	S_m	r	ICCR3
35	S_m	S_m	s	S_m	S_m	S_m	s	S_m	ICA2
36	r	S_m	S_m	S_m	S_m	r	S_m	r	INM₂-1
37	S_m	S_m	S_m	r	S_m	S_m	r	S_m	INM₂-2

Table 5A. (continued)

group	Antibioltic resistant profiles								Rhizobial isolates
	Car	Chl	Ery	Nal	Kan	Tmp	Tet	Str	
38	S_m	S_m	s	r	S_m	S_m	r	S_m	INM$_2$-3
39	s	S_m	s	r	S_m	r	r	s	INM$_3$-1
40	s	S_m	s	S_m	S_m	S_m	S_m	s	INM$_3$-2
41	S_m	S_m	S_m	r	S_m	r	r	s	INM$_3$-3
42	r	S_m	S_m	r	S_m	r	r	S_m	INC3
43	S_m	r	S_m	r	S_m	S_m	S_m	r	INC4
44	s	S_m	s	S_m	S_m	r	r	S_m	INR1
45	S_m	S_m	s	r	S_m	r	S_m	S_m	INR4
46	s	S_m	s	r	S_m	r	S_m	S_m	INCR1
47	s	S_m	S_m	S_m	S_m	r	r	r	INCR3
48	S_m	S_m	S_m	S_m	S_m	s	s	s	INCR4
49	s	s	s	S_m	S_m	S_m	r	r	INF3
50	S_m	S_m	S_m	r	S_m	r	S_m	r	INB2
51	S_m	r	S_m	S_m	s	r	r	S_m	INC*1
52	s	S_m	S_m	s	S_m	S_m	r	s	INEM$_2$-1
53	r	r	S_m	S_m	S_m	S_m	r	S_m	INEM$_2$-3
54	S_m	s	S_m	S_m	r	r	S_m	s	INEM$_3$-4
55	s	s	s	S_m	S_m	r	S_m	s	INEC1
56	S_m	S_m	s	r	r	r	r	S_m	INEC2
57	r	S_m	S_m	r	r	r	S_m	S_m	INEC3
58	r	S_m	s	r	r	r	r	S_m	INEC4
59	S_m	S_m	S_m	S_m	S_m	r	r	S_m	INER4
60	r	r	S_m	r	S_m	r	S_m	S_m	INER3
61	S_m	S_m	s	r	S_m	r	r	r	INEF1
62	S_m	r	S_m	S_m	s	S_m	r	s	INEA2
63	r	r	S_m	S_m	S_m	S_m	S_m	S_m	INEB1
64	S_m	r	S_m	r	S	S_m	S_m	S_m	INEC*1
65	r	r	S_m	S_m	S_m	r	r	r	INED3
66	s	s	s	r	s	r	s	s	*R. leguminosarum*
67	s	s	s	S_m	s	r	s	s	*R. cerciri*
68	s	s	s	r	s	S_m	s	r	*R. elti*
69	s	s	s	r	s	S_m	s	S_m	*R. Huakii*
70	r	s	s	S_m	s	r	s	s	*R. tritolii*
71	r	s	s	r	s	r	s	s	*R. tropici*
72	s	r	s	r	s	r	r	r	*B. japonicum*

Table 6A. Antibiotic resistant profiles of rhizobial isolates in dry season.

group	Antibioltic resistant profiles								Rhizobial isolates
	Car	Chl	Ery	Nal	Kan	Tmp	Tet	Str	
1	S_m	S_m	S_m	S_m	S_m	S_m	S_m	S_m	IIICR1, IIINM₂-2, IIINF1, IIINF2, IIINECR2, IIINEF1, IIINFC^1
2	r	S_m	S_m	S_m	S_m	r	S_m	S_m	IIICR2, IIICR3, IIINR1, IIINCR2, IIINEC1, IIINEM₃-3
3	S_m	S_m	S_m	S_m	s	S_m	r	s	IIICM₂-2, IIINM₂-1, IIINM₂-3, IIINEM₁-2, IIINEM₁-4
4	r	S_m	S_m	r	S_m	r	S_m	S_m	IIICM₃-3, IIICNR4, IIINEM₂-3, IIINEM₂-4
5	S_m	S_m	S_m	S_m	S_m	r	S_m	S_m	IIICM₃-4, IIINC2, IIINEC4
6	r	S_m	S_m	S_m	S_m	r	r	r	IIINM₃-2, IIINCR1, IIINER4
7	r	r	S_m	r	S_m	r	r	S_m	IIINF4, IIINECR1, IIINECR4
8	s	s	s	s	s	s	s	s	IIINEM₁-1, IIINEM₁-3, IIINER3
9	r	S_m	S_m	S_m	S_m	r	r	S_m	IIICM₂-1, IIICM₂-4
10	S_m	S_m	S_m	S_m	S_m	r	r	S_m	IIICM₂-3, IIICC1
11	r	s	S_m	S_m	S_m	r	r	S_m	IIICM₃-1, IIINER1
12	S_m	S_m	s	S_m	s	r	S_m	S_m	IIICC2, IIICC3
13	s	S_m	S_m	r	S_m	r	S_m	S_m	IIICR4, IIICCR1
14	r	S_m	s	r	S_m	r	r	S_m	IIICCR2, IIICCR4
15	S_m	S_m	s	r	S_m	r	r	s	IIINM₂-4, IIINF3
16	r	r	S_m	r	s	r	r	s	IIINM₃-1, IIINM₃-3
17	r	S_m	S_m	S_m	s	r	r	S_m	IIINM₃-4, IIINC3
18	r	r	S_m	S_m	S_m	r	r	S_m	IIINCR3, IIINEM₂-2
19	S_m	S_m	S_m	r	S_m	S_m	r	S_m	IIINC^1, IIINC^2
20	S_m	S_m	S_m	r	S_m	r	S_m	r	IIICM₁-1
21	r	S_m	S_m	S_m	S_m	S_m	S_m	S_m	IIICM₁-2
22	s	s	s	s	s	r	s	s	IIICM₁-4
23	S_m	S_m	S_m	r	S_m	r	S_m	S_m	IIICM₃-2
24	s	s	s	r	s	r	r	S_m	IIICC4
25	r	S_m	s	r	r	r	r	r	IIICCR3
26	s	S_m	s	S_m	S_m	r	r	s	IIICF1
27	s	S_m	s	S_m	S_m	r	S_m	s	IIICF2
28	r	S_m	s	S_m	s	r	S_m	S_m	IIICF3
29	S_m	S_m	S_m	S_m	S_m	S_m	r	s	IIICF4
30	S_m	S_m	s	r	S_m	S_m	r	S_m	IIICA1
31	r	r	s	S_m	r	r	r	s	IIICA2
32	S_m	r	s	S_m	S_m	r	S_m	S_m	IIICB1
33	r	r	s	r	S_m	r	r	S_m	IIICB2
34	r	r	S_m	r	s	r	r	S_m	IIICC^1
35	S_m	r	s	S_m	S_m	r	S_m	S_m	IIICC^2

Table 6A. (continued)

group	Antibioltic resistant profiles								Rhizobial isolates
	Car	Chl	Ery	Nal	Kan	Tmp	Tet	Str	
36	s	s	s	S_m	s	S_m	s	s	IIINC1
37	r	S_m	S_m	S_m	s	r	S_m	S_m	IIINC4
38	r	r	S_m	r	s	r	S_m	s	IIINR2
39	S_m	S_m	s	s	s	s	s	s	IIINR3
40	s	r	S_m	r	S_m	r	S_m	s	IIINR4
41	s	S_m	s	S_m	s	r	r	s	IIINA1
42	S_m	S_m	S_m	r	s	S_m	S_m	S_m	IIINA2
43	S_m	S_m	S_m	r	S_m	r	r	S_m	IIIND1
44	S_m	S_m	r	S_m	s	S_m	S_m	s	IIIND2
45	S_m	S_m	S_m	r	s	r	S_m	r	IIIND1
46	r	r	r	S_m	s	S_m	r	s	IIINEM$_3$-1
47	r	r	S_m	S_m	S_m	S_m	r	S_m	IIINEM$_3$-2
48	s	r	s	r	s	r	s	s	IIINEM$_3$-4
49	s	r	s	s	s	s	s	s	IIINEC2
50	S_m	s	s	S_m	s	S_m	s	s	IIINEC3
51	S_m	r	S_m	r	s	r	r	S_m	IIINECR3
52	S_m	S_m	S_m	S_m	S_m	S_m	r	r	IIINEF2
53	r	S_m	S_m	r	S_m	S_m	r	S_m	IIINEF3
54	S_m	S_m	s	S_m	s	r	S_m	S_m	IIINEF4
55	r	S_m	S_m	r	s	r	S_m	S_m	IIINEA1
56	s	S_m	s	r	s	r	S_m	s	IIINEC^2
57	s	r	s	S_m	s	r	S_m	s	IIINED1
58	S_m	S_m	S_m	S_m	s	S_m	S_m	S_m	IIINED3
59	s	s	s	r	s	r	s	s	*R. leguminosarum*
60	s	s	s	S_m	s	r	s	s	*R. cercin*
61	s	s	s	r	s	S_m	s	r	*R. elti*
62	s	s	s	r	s	S_m	s	S_m	*R. Huakii*
63	r	s	s	S_m	s	r	s	s	*R. tritolii*
64	r	s	s	r	s	r	s	s	*R. tropici*
65	s	r	s	r	s	r	r	r	*B. japonicum*

Table 7A. The host dependent cross-inoculation group of rhizobia in rainy season.

Isolate	Host plant
ICM_1-1	Cowpea
ICM_1-4	Cowpea
ICM_2-1	Cowpea
ICM_2-2	Cowpea
ICM_2-3	Cowpea
ICM_2-4	Cowpea
ICM_3-1	Cowpea
ICM_3-2	Cowpea
ICM_3-3	Cowpea
ICM_3-4	Cowpea
ICC1	Cowpea
ICC2	Cowpea
ICC3	Cowpea
ICC4	Cowpea
ICR1	Cowpea
ICR2	Cowpea
ICR4	Cowpea
ICCR1	Cowpea
ICCR2	Cowpea
ICCR3	Cowpea
ICCR4	Cowpea
ICF1	Cowpea
ICF2	Cowpea
ICF3	Cowpea
ICF4	Cowpea, Soy bean
ICA1	Cowpea
ICA2	Cowpea
ICB1	Cowpea
ICB2	Cowpea
ICC^1	Cowpea
ICC^2	Cowpea
INM_1-1	Cowpea
INM_1-2	Cowpea
INM_1-4	Cowpea
INM_2-1	Cowpea
INM_2-2	Cowpea
INM_2-3	Cowpea, Soy bean
INM_2-4	Cowpea
INM_3-1	Cowpea
INM_3-2	Cowpea

Table 7A. (continued)

Isolate	Host plant
INM_3-3	Cowpea, Soy bean
INM_3-4	Cowpea
INC1	Cowpea
INC2	Cowpea
INC3	Cowpea
INC4	Cowpea
INR1	Cowpea
INR2	Cowpea
INR4	Cowpea
INCR1	Cowpea
INCR2	Cowpea
INCR3	Cowpea
INCR4	Cowpea
INF1	Cowpea
INF2	Cowpea
INF3	Cowpea, Soy bean
INF4	Cowpea
INA1	Cowpea
INA2	Soy bean, *Sesbania rostata*
INB1	Cowpea
INB2	Cowpea
INC^1	Cowpea
INC^2	Cowpea
IND1	Cowpea
IND2	Cowpea
IND3	Cowpea, Soy bean
$INEM_1$-1	Cowpea
$INEM_1$-2	Cowpea
$INEM_1$-3	Cowpea
$INEM_1$-4	Cowpea
$INEM_2$-1	Cowpea
$INEM_2$-2	Cowpea
$INEM_2$-3	Cowpea
$INEM_2$-4	Cowpea
$INEM_3$-1	Cowpea
$INEM_3$-2	Soy bean
$INEM_3$-3	Cowpea
$INEM_3$-4	Cowpea
INEC1	Cowpea

Table 7A. (continued)

Isolate	Host plant
INEC2	Cowpea
INEC3	Cowpea
INEC4	Cowpea
INER1	Cowpea
INER2	Cowpea
INER3	Cowpea
INER4	Cowpea
INECR1	Soy bean
INECR2	Soy bean
INECR3	Cowpea
INECR4	Cowpea
INEF1	Cowpea
INEF2	Cowpea
INEF3	Cowpea
INEF4	Cowpea
INEA1	Cowpea
INEA2	Cowpea
INEB1	Cowpea
INEB2	Cowpea
INEC^1	Cowpea
INEC^2	Cowpea
INED1	Cowpea
INED2	Cowpea
INED3	Cowpea

Table 8A. The host dependent cross-inoculation group of rhizobia in dry season.

Isolate	Host plant
$IIICM_1$-1	Cowpea
$IIICM_1$-2	Cowpea
$IIICM_1$-3	Cowpea
$IIICM_1$-4	Cowpea
$IIICM_2$-1	Cowpea
$IIICM_2$-2	Cowpea
$IIICM_2$-3	Cowpea
$IIICM_2$-4	Cowpea
$IIICM_3$-1	Cowpea
$IIICM_3$-2	Cowpea
$IIICM_3$-3	Cowpea
$IIICM_3$-4	Cowpea, Soy bean
IIICC1	Cowpea
IIICC2	Cowpea
IIICC3	Cowpea
IIICC4	Cowpea
IIICR1	Cowpea
IIICR2	Soy bean
IIICR3	Cowpea
IIICR4	Cowpea
IIICCR1	Cowpea
IIICCR2	Cowpea
IIICCR3	Cowpea
IIICCR4	Cowpea
IIICF1	Cowpea
IIICF2	Cowpea
IIICF3	Soy bean
IIICF4	Cowpea
IIICA1	Cowpea
IIICA2	Cowpea, Soy bean
IIICB1	Cowpea
IIICB2	Cowpea
IIICC^1	Cowpea
IIICC^2	Cowpea
$IIINM_2$-1	Cowpea
$IIINM_2$-2	Cowpea
$IIINM_2$-3	Cowpea, Soy bean
$IIINM_2$-4	Cowpea
$IIINM_3$-1	Cowpea
$IIINM_3$-2	Cowpea, Soy bean

Table 8A. (continued)

Isolate	Host plant
IIINM$_3$-3	Cowpea
IIINM$_3$-4	Cowpea, Soybean
IIINC1	Cowpea
IIINC2	Cowpea
IIINC3	Cowpea
IIINC4	Cowpea
IIINR1	Cowpea
IIINR2	Cowpea
IIINR3	Cowpea
IIINR4	Cowpea
IIINCR1	Cowpea
IIINCR2	Cowpea
IIINCR3	Cowpea
IIINCR4	Cowpea
IIINF1	Cowpea
IIINF2	Cowpea
IIINF3	Cowpea
IIINF4	Cowpea
IIINA1	Cowpea, Soybean
IIINA2	Cowpea, Soybean
IIINC^1	Cowpea
IIINC^2	Cowpea
IIINEM$_1$-1	Cowpea, *Sesbania rostata*
IIINEM$_1$-2	Cowpea
IIINEM$_1$-3	Cowpea
IIINEM$_1$-4	Cowpea
IIINEM$_2$-2	Cowpea, *Sesbania rostata*
IIINEM$_2$-3	Cowpea
IIINEM$_2$-4	Cowpea
IIINEM$_3$-1	Cowpea
IIINEM$_3$-2	Cowpea
IIINEM$_3$-3	Cowpea
IIINEM$_3$-4	Cowpea, Soybean
IIINEC1	Cowpea
IIINEC2	Cowpea
IIINEC3	Cowpea
IIINEC4	Cowpea, Soybean
IIINER1	Cowpea, Soybean
IIINER3	Cowpea
IIINER4	Cowpea, Soybean

Table 8A. (continued)

Isolate	Host plant
IIINECR1	Cowpea
IIINECR2	Cowpea
IIINECR3	Cowpea
IIINECR4	Cowpea
IIINEF1	Cowpea
IIINEF2	Cowpea
IIINEF3	Cowpea
IIINEF4	Cowpea
IIINEA1	Cowpea
IIINEC^1	Cowpea
IIINEC^2	Cowpea
IIINED1	Cowpea

BIBLIOGRAPHY

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