CHAPTER II

LITERATURE SURVEY

(Source : Annual Report of the Center of Agricultural Information, 2001)

Figure 2.1 Soybean cultivation areas in Thailand. Soybeans are grown mainly in Chiangmai, Tak, Sukhothai, and Kampaeng pet. Approximately 50,000 – 90,000 *rai* of soybean plantations are found in Phitsanulok. In this research, isolates of bacteria from root nodules of soybeans grown in soils from soybean plantations in 15 subdistricts of Phitsanulok Province will be obtained.

Methods used to distinguish rhizobial strains

It is proposed in this research that a set of DNA fingerprints of each isolate is obtained and combination of identical DNA fingerprints constitute a distinct strain. With the advent of polymerase chain reaction (PCR) by Mullis & Faloona in 1987, it is possible to obtain DNA fingerprints via a technique called Random Amplified Polymorphic DNA-PCR (RAPD-PCR). One advantage of RAPD-PCR is there is no need to know the sequence of the genome in order to design primers. In addition one primer is used in the amplification of DNA fragments. The primer may either be arbitrary or sequence-directed (Welsh and McClelland, 1990 ; Williams et al., 1990 ; Young and Cheng, 1998). Several types of PCR primers have been used in PCR fingerprinting of rhizobia. In 1992 de Bruijn obtained distinct DNA fingerprints of several strains of *Rhizobium meliloti, R. leguminosarum, R. loti* and *Bradyrhizobium japonicum* by using two sets of forward and reverse primers the first of which annealed to the Repetitive Extragenic Palindromic (REP) sequences. REP sequences were originally found adjacent to 16 out of 61 *E. coli* operons examined (Stern et al., 1984). REP sequences consisted of approximately 35 bp with the conserved palindromic inverted repeats underlined as follows :

GCCGGAT G . C GGCGT .. G .. ACGCC TT ATCCGGC CTAC

The second set of primers annealed to the Enterobacterial Repetitive Intergenic Consensus (ERIC) sequences. The ERIC sequence is 126 bp consensus sequence found in transcribed regions of enterobacteria (Hulton et al., 1991). Sequences of primers ERIC 1R and ERIC 2 are as follows :

ERIC 1R 5' ATGTAAGCTCCTGGGGATTCAC 3'
ERIC 2 5' AAGTAAGTGACTGGGGTGAGCG 3'

In 1993 Judd et al. used the two sets of PCR primers REPIR-1 and REP2-1 ; ERICIR and ERIC2 to obtain REP and ERIC PCR fingerprints of 11, 12 and 1 strains of *Bradyrhizobium japonicum* in serogroups 123, 127 and 129 respectively. The results

In 1993 Judd et al. used the two sets of PCR primers REPIR-1 and REP2-1 ; ERICIR and ERIC2 to obtain REP and ERIC PCR fingerprints of 11, 12 and 1 strains of *Bradyrhizobium japonicum* in serogroups 123, 127 and 129 respectively. The results showed that REP and ERIC PCR techniques could be used to distinguish between genetically and phenotypically near-identical *B. japonicum* strains in serogroups 123, 127, and 129.

Most research has concentrated on the use of the highly variable ITS region as one criterion in discerning species (Willems et al., 2001). However, In 1995 Richardson et al. used PCR in conjunction with two arbitrary primers (RPO4 and RPO5) of 10 nucleotides length and a sequence-specific primer (RPO1) of 20 nucleotides length to obtain RAPD-PCR DNA fingerprints which differentiated strains in a diverse collection of *Rhizobium meliloti* , *R. leguminosarum* bv *trifolii* and *R.l.* bv. *viciae*. The sequence of RPO1 was conserved in the promoter of the *nif HDK* operon of *Rhizobium trifolii* strains Rt 329, Rt RS1 and *R. meliloti* RmP1 (Schofield and Watson,1985). In the following experiments, RPO1 was chosen as a primer for RAPD-PCR fingerprinting because it was likely that the presence of PCR product(s) indicated the presence of *nifH* which encoded an α subunit of the enzyme nitrogenase. Therefore, if a bacterial isolate is found to contain RPO1-PCR product(s), it is likely the isolate contains the enzyme nitrogenase for nitrogen fixation.

Mathis & McMillin (1996) successfully used CRL-7 which was an arbitrary GC rich primer in the DNA fingerprinting of *B. japonicum*. CRL-7 was used in the following experiments because it was postulated that a higher number of CRL-7 PCR products implied a large number of GC rich areas in DNA molecules. Hence, the higher the number of CRL-7 PCR products the more heat tolerant DNA molecules. Thus, strains with a large number of CRL-7 PCR products might be more heat tolerant. CRL-7 PCR fingerprints of soybean rhizobia will contribute significant information to the development of heat-tolerant, high-nitrogen fixing biofertilizers.

Methods used in grouping of rhizobial strains

Once individual strains of soybean rhizobia are identified, there are several methods which have been used to group rhizobial strains .These include restriction fragment length polymorphism (RFLP) of 16S rDNA, symbiotic genes (*nifHDK*, *nodD*) (Laguerre et al., 1996) and constitutively expressed genes including *hemA*, *glnA*, and *ntrC* for strain grouping in *Rhizobium galegae* strains. (Kaijalainen and Lindstrom, 1989). In 1989, Kaijalainen and Lindstrom obtained RFLP results when DNA of four rhizobial strains which nodulated the legume *Galega orientalis* (isolated from USSR and Finland) seven rhizobial strains which nodulated *G. officinalis* (isolated from USA, New Zealand, and the United Kingdom) were digested with *Bam*H1, *Eco*R1 and *Hin*dIII separately. Southern blotting and hybridization with heterogenous probes (sequences carrying common *nod* genes of *Rhizobium meliloti*, *nifHDK* genes of *R. meliloti*) revealed at least one common hybridizing fragment was found when the probes contained symbiotic gene sequence. Use of relative proportion of conserved hybridizing fragment compared with all hybridizing fragments of a strain pair to calculate percent sequence divergences showed the *R. galegae* strains could be grouped into two groups according to the two host plants as shown in Figure 2.2

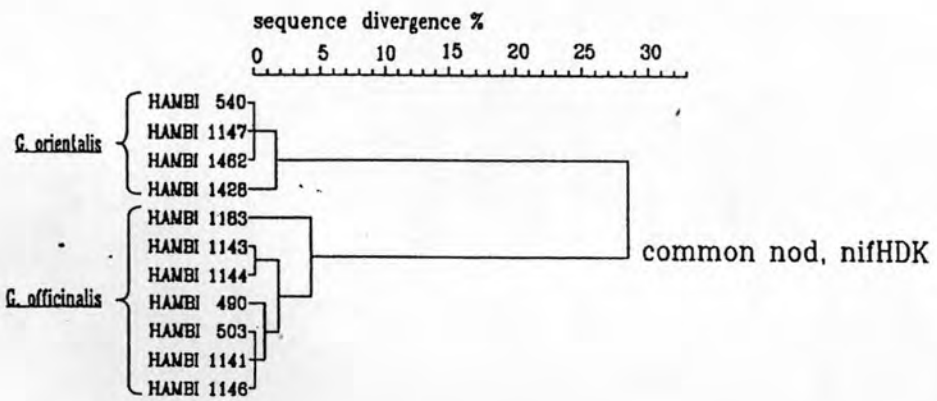


Figure 2.2 Dendrogram of the *R. galegae* strains derived from the combined data of different probe groups (Kaijalainen and Lindstrom, 1989).

Research on isolation and characterization of soybean rhizobia in Thailand has been limited (Yokoyama et al., 1996 ; Nuntagij et al., 1997) In 1998 Teaumroong and Boonkerd used RAPD primer, 5' GGAAGTCGCC 3' to obtain fingerprints of 18 *B. japonicum* isolates.

Multiplex PCR

In 2002 Videira et al. employed two sets of primers as follows to differentiate *S. fredii* and *B. japonicum* in two separate PCR reactions :

Primer		
nolXWBTUV locus		
Forward primer *nolBT*	tgggcaagcgacgctgccgg	260 bp
Reverse primer	ggcgagaaataggcgccgt	
RSα		
Forward SAL1	agcgggcgcggatagttctgttt	900 bp
Reverse SAL1	ggctcggctctgtcgttgtatgc	

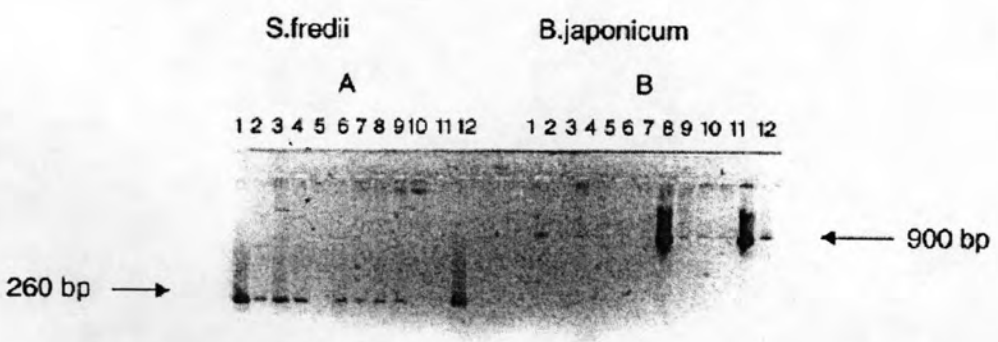


Figure 2.3 Amplification of (A) the 260 bp *nolBT* fragments from nodule extracts isolated from plants inoculated with *S. fredii* or (B) amplification of the 900 bp RSα fragments from nodule extracts isolated from plants inoculated with *B. japonicum*. (Videria et al., 2002).

Videira, Pastorino, et al. could differentiate the fast-growing *S. fredii* from the slow-growing *B. japonicum* in two separate PCR reactions. The 260 bp were fragments found to be specific to *S. fredii* while the 900 bp fragments were found to be specific to *B. japonicum* as shown in Figure 2.3. However, the authors stated that identification through the two PCR reactions was a time-consuming and costly protocol. Therefore, the same group of researchers (Pastorino et al. 2003) redesigned the reverse primer for *nolXWBTUV* locus as follows : Reverse primer *nolBT* : cgctcttgcaactgggtata which, in combination with the forward primer *nolBT* were used in the multiplex amplification of the 730 bp fragment. (Table 2.1 and Figure 2.4).

Table 2.1 Sequences of the 20-mer primers that specifically amplify the *nolXWBTUV* locus of *S. fredii* and the RSα repetitive sequence of *B. japonicum* (Pastorino et al., 2003).

Primer	5'-3' sequence	Fragment	%GC	Tm (°C)
nolXWBTUV locus				
Forward primer*nolBT*	tgggcaagcgacgctgccgg		75	60.0
Reverse primer*nolBT*	cgctcttgcaactgggtata	730 bp	50	57.0
RSα				
Forward SAL1	agcgggcgcggatagttctgttt		61	65.5
Reverse SAR1	ggctcggctctgtcgttgtatgc	900 bp	61	65.0

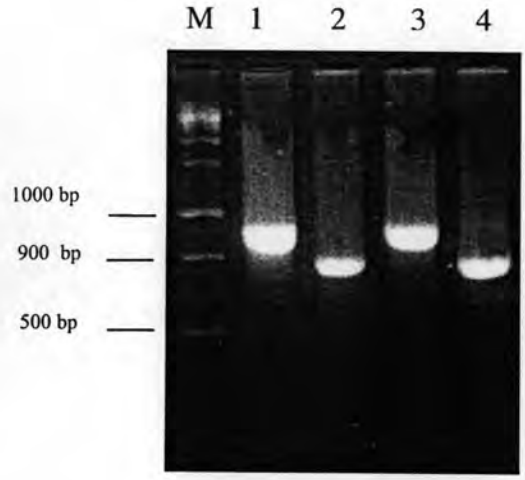


Figure 2.4 PCR products when primers for RSα and/or *nol BT* were used for DNA templates derived from *B. japonicum* or *S. fredii*. Lane M, molecular size marker. Lane 1, reaction with template DNA from *B. japonicum*. Lane2, reaction with template DNA from *S. fredii*. Lanes 3 and 4 multiplex PCR with 2 sets of primers and template DNA from *B. japonicum* (Lane3) and *S. fredii* (Lane4). (Pastorino et al., 2003).

Differences between fast- and slow- growing soybean rhizobia

You et al. (2002) examined bradyrhizobia populations that nodulate yardlong bean and sunnhemp in Guam for genetic diversity and their relatedness to *B. japonicum* and *B. elkanii* reference strains. *B. japonicum*, *nodY* is a 420-bp gene located within the 785-bp region between *nodD1* and *nodABC* (Nieuwkoop et al. 1987). In *B. elkanii*, there is no *nodY* gene but, in its place, there is *nodK* gene which is 402 bp in size (Dobert et al., 1994). *Bradyrhizobium* sp. (*Parasponia*) also contains a *nodK* gene in the same position (Scott 1986). You et al. (2002) had used homology to *nodY* and *nodK* as a method to determine similarity to *B. japonicum* or *B. elkanii*. The *nodY* and *nodK* genes share less than 30% sequence similarity. *B. japonicum* and *B. elkanii* strains were shown to be distinguishable on the basis of their hybridization to *nodY* and *nodK* genes. Therefore, one difference between *B. japonicum* and *B. elkanii* is the former contains *nodY* while the latter contains *nodK* in the corresponding place. *B. liaoningense* was shown to be extremely slow- growing soybean rhizobia (Xu et al., 1995).

Chemical structures of nod factors of *B. japonicum* also differ from those of *B. elkanii*. Figure 2.5 shows synthesis of nod factors which are produced by soybean rhizobia and whose function (s) on nodulation process is still unknown. The chemical structures of Nod factors of *B. japonicum* strains USDA 110 and USDA 135 and *B. elkanii* strain USDA 61 are shown in Figure 2.6.

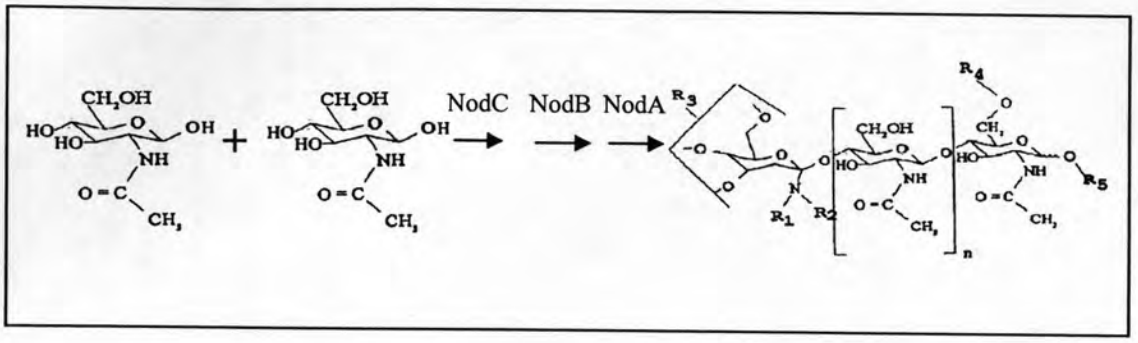
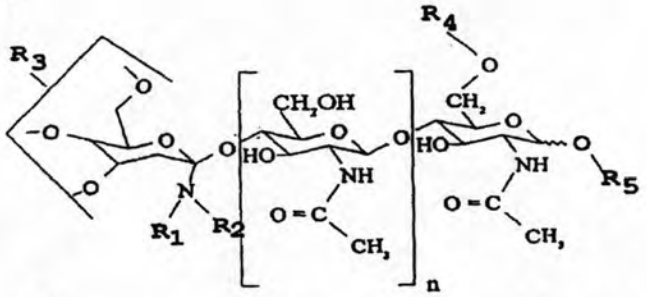


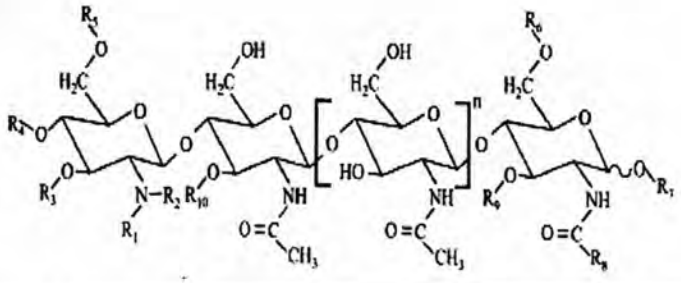
Figure 2.5 Synthesis of Nod factors catalysed by enzymes encoded by *nodC*, *nodB* and *nodA* (Stacey, 1995).



STRAIN	R1	R2	R3	R4	R5	n
***B. japonicum* USDA110**						
NodBj-V(C18:1,MeFuc)	C18:1	H	H	2-O-MeFuc	H	3
***B. japonicum* USDA135**						
NodBj-V(C18:1, MeFuc)	C18:1	H	H	2-O-MeFuc	H	3
NodBj-V(Ac,C18:1,MeFuc)	C18:1	H	Ac	2-O-MeFuc	H	3
NodBj-V(C16:0,MeFuc)	C16:0	H	H	2-O-MeFuc	H	3
NodBj-V(Ac,C16:0,MeFuc)	C16:0	H	Ac	2-O-MeFuc	H	3
NodBj-V(C16:1, MeFuc)	C16:1	H	H	2-O-MeFuc	H	3
***B. elkanii* USDA61**						
NodBe-V(C18:1,MeFuc)	C18:1	H	H	2-O-MeFuc	H	3
NodBe-V(Ac,C18:1,MeFuc)	C18:1	H	Ac	2-O-MeFuc	H	3
NodBe-V(Cb,C18:1,NMe,MeFuc)	C18:1	Me	H	2-O-MeFuc	H	3
NodBe-V(Ac,Cb,C18:1,MeFuc)	C16:1	H	Ac,Cb	2-O-MeFuc	H	3
NodBe-IV(C18:1,MeFuc)	C18:1	H	H	2-O-MeFuc	H	2
NodBe-IV(Cb,C18:1,MeFuc)	C18:1	H	Cb	2-O-MeFuc	H	2
NodBe-IV(C18:1,Fuc,Gro)	C16:1	H	H	Fuc	Gro	2
NodBe-IV(C18:1,NMe,Fuc,Gro)	C18:1	Me	H	Fuc	Gro	2
NodBe-IV(Cb,C18:1,Fuc,Gro)	C18:1	H	Cb	Fuc	Gro	2
NodBe-IV(Cb,C18:1,NMe,Fuc,Gro)	C18:1	Me	Cb	Fuc	Gro	2

Figure 2.6 Summary of the various chito-oligosaccharides nodulation signals produced by *B. japonicum* strains USDA110 and USDA135 and *B. elkanii* strain USDA61. Abbreviations: AC, acetyl; Cb, carbamoyl; 2-0-MeFuc, 2-0-methylfucose; Fuc, fucose; Me, methyl; Gro, glycerol (Stacey et al. 1995).

It is well established that nodulation signals (Lipo-chitooligosaccharides, LCO, Nod factors) produced by *B. japonicum* are pentamers of N-acetylglucosamine with the reducing sugar modified by a 2-*O*-methylfucose and the non- reducing end substituted with an 18:1 fatty acid (Stacey, 1995). Chemical structures of Nod factors of the fast-growing *S. fredii* differ from those of the slow- growers in the fatty acid side chain of the non– reducing end of Nod factors and the number of N- acetylglucosamine units as shown in Figure 2.7.



Rhizobial strain	Nod factor substitutions[a,b]										
	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	R_{10}	n[c]
B. elkanii USDA61	C18:1,C16:0	Me,H	Cb,Ac[f] H	Cb,Ac,H	Cb,Ac,H	2-*O*-MeFuc,Fuc	Gro,H	Me	H	H	1, 2
B. japonicum USDA110	C18:1[g]	H	H	H	H	2-*O*-MeFuc	H	Me	H	H	2
B. japonicum USDA135	C18:1,C16:0,C16:1[h]	H	H	H	Ac,H	2-*O*-MeFuc	H	Me	H	H	2
S. fredii 257DH4	C16:0,C18:1	H	H	H	H	2-*O*-MeFuc	H	Me	H	H	0, 1, 2
S. fredii HH103	C16:0,C16:1 C18:0,C18:1[i]	H	H	H	H	2-*O*-MeFuc,Fuc	H	Me	H	H	0, 1, 2
S. fredii USDA191[e]	C16:0,C16:1 C18:0,C18:1	H	H	H	H	2-*O*-MeFuc,Fuc	H	Me	H	H	0, 1, 2
S. fredii USDA257	C18:1	H	H	H	H	2-*O*-MeFuc,Fuc	H	Me	H	H	0, 1, 2

Figure 2.7 Nod factors structures and their specific substitutions in fast- and slow-growing soybean rhizobia (Gil- Serrano et al., 1997 ; Haeze and Holstera, et al., 2002).

In addition, *B. japonicum* responses to the gradient of flavonoids, Genistein and Daidzein, secreted by soybean roots while the fast- grower *S. fredii* responses to the gradient of soybean flavonoids Daidzein and Coumestrol in the initial step of soybean – rhizobial signal perception (Machado et al., 1998 ; Pueppke et al., 1998). The flavonoids enter the rhizobial periplasm to form complexes with NodD1. NodD1-flavonoid complexes induce expression of *nodD1* and *nod(Y)ABC* operon for the synthesis of Nod factors (Long., 1996 ; Chansa-ngavej, 2005). Since NodD1 proteins of the fast- growing

S. fredii and the slow- growing *B. japonicum* bind to different types of flavonoids, it is expected that *nodD1* of fast- and slow- growing soybean rhizobia may be different. Therefore, in this study, primers for use in multiplex PCR reactions to detect the presence of either fast- and slow- growing soybean rhizobia are designed from sequences of *nodY* and *nodD1*.