CHAPTER II

LITERATURE SURVEY

2.1 Previous research on soybean rhizobium diversity in Thailand

Soybeans are grown in the northern, upper central, and some parts of the north-eastern parts of Thailand. Figure 2.1 illustrates the percentages of soybean cultivation areas in different provinces of Thailand.

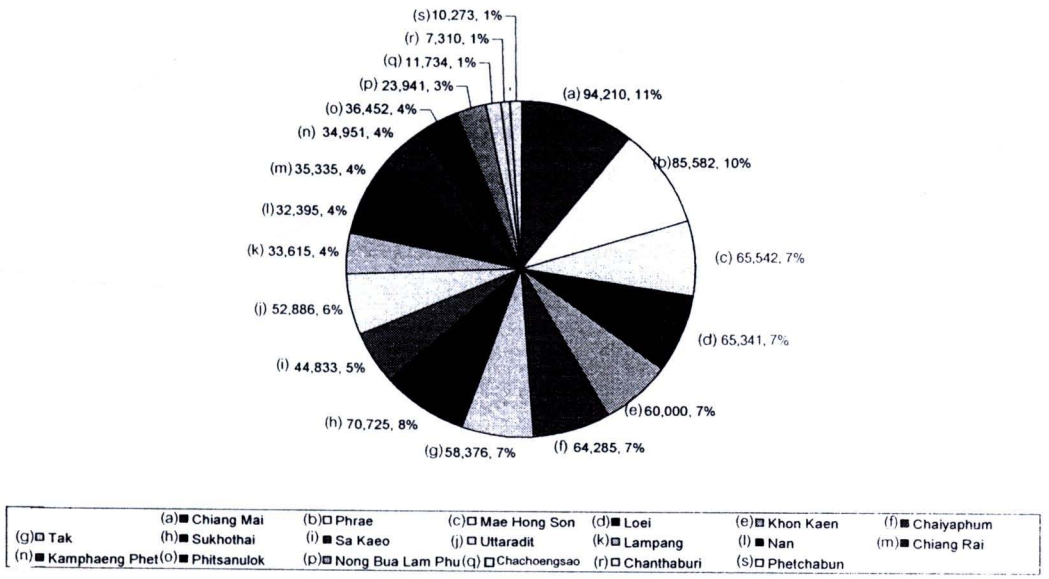


Figure 2.1 Percentages of soybean cultivation areas in different provinces of Thailand in the crop year 2008. Units in *rai* and percent. Data were obtained from the Department of Agriculture in 2009.

Figure 2.1 shows soybean is mostly cultivated in Chiangmai province in the northern part of Thailand covering the area of 94,210 *rai* or 11% of the total area for soybean cultivation. Although the area planted with soybeans in Phitsanulok is about 36,452 *rai* or 4% of the total soybean cultivation area, there is a need to isolate and characterize soybean rhizobia in Phitsanulok province because at present there is no record of soybean rhizobium diversity in the area.

In 1991 Thompson et al. isolated 1,536 bacterial isolates from root nodules of uninoculated soybeans and cowpeas grown in 2 meter long experimental rows in 25

sites in soybean-cultivation areas at Hang Dong, San Sai, Mae Rim, Mae Thaeng, Mae Sariang, Mae Hong Son, Khum Yuom, Mae La Noi, Sri Sachanalai, Sawankalok, Sri Samrong, Uttaradit, and Doi Tao. Seeds of local soybean cultivars Black Yodson, San Keuw, SJ2, SJ5, 7842, and the following soybean cultivars from US breeding programs were used: Peking, Improved Pelican, Hardee, Bossier, Bragg, and Forrest. All isolates were stained with conjugated fluorescent dye fluorescin isothiocyanate and polyclonal antisera from each of the following soybean rhizobium strains: THA7, USDA140, USDA24, USDA31, USDA110, USDA122, TAL379, and the following strains isolated by Thompson et al. (1991): 7DX9, 7DX36, 10DX21, 10EX51, and 1C3. All serologically-stained isolates were examined with UV fluorescence microscope. The results indicated most strains belonged to serogroups of local soybean rhizobium strains 1C3 and 7DX9. However, the researchers reported there were too many cross-reactions for the serological method to be used to differentiate different soybean rhizobium strains. Intrinsic antibiotic resistance (IAR) patterns were thus conducted on 116 isolates with 10 $\mu g.ml^{-1}$ of each of the following antibiotics: vancomycin, rifampicin, spectinomycin, and tetracycline, 40 $\mu g.ml^{-1}$ of each of the following antibiotics: streptomycin, kanamycin, erythromycin and 200 $\mu g.ml^{-1}$ of polymixin. Nearly all (93%-100%) of the isolates were found to be resistant to spectinomycin, vancomycin, tetracycline, and polymixin. Therefore, these four antibiotics were not useful in the differentiation of different strains. However, 83%, 69%, 68%, and 17% of the isolates were found to be resistant to kanamycin, streptomycin, rifampicin, and erythromycin, respectively. Therefore, qualitative differences in the resistance and susceptibility to these last four antibiotics were used to separate isolates into various groups for testing effectiveness in nodulation and in nitrogen fixation. Thompson et al. (1991) found that out of the 356 rhizobium isolates tested on 6 soybeans cv. Black Yodson. SJ5, Improved Pelican, Bossier, Peking and Hardee, only 5.6% of the isolates yielded less than 20 nodules per plant and that most of the isolates significantly increased ($p< 0.001$) nitrogen yield of plants over that obtained for the uninoculated control. The results indicated most of the soybean rhizobium isolates obtained by Thompson et al. (1991) from soybean-cultivation areas in the northern part of Thailand were effective in nodulation and in nitrogen fixation. However, Thompson et al. (1991) did not employ any method to group

identical soybean rhizobium isolates into the same strains. In addition, the authors did not identify any of the soybean isolates.

Most of the other previous research on the diversity of soybean rhizobia in Thailand was either incomplete or did not employ many strains of soybean rhizobia in the research. In 1997, Nuntagij et al. partially characterized soybean rhizobia isolated from soybean cultivation areas in Thailand. The authors did not report the sites of soybean cultivation areas where 5 strains were isolated. The 5 strains were tentatively identified as *Bradyrhizobium japonicum* strains THA2, THA5, THA6, THA7, and THA211 based on their inability to produce IAA (3-Indole Acetic Acid). In addition, 5 reference strains were used in the research: *Bradyrhizobium japonicum* strains TAL102, USDA136b, SM5, USDA76 and 61A101C. The 10 strains were characterized based on their IAA production, growth on YMA at pH 3.0-9.0, and growth at 17^0C, 28^0C, 37^0C, and 42^0C. Research was also conducted on the effects of pH 4.5 or 6.8 on activities of 19 enzymes using the APIZYM-kit (API system, France). Strains THA2, THA7, THA211 were found to be acid-tolerant at pH 3.0. The optimum range of pH for the 5 isolated strains were found to be pH 6.0-8.0. All the 5 strains except THA7 were found to grow at 42^0C. pH 4.5 and 6.8 were found to have different effects on the 19 enzymes of the 5 strains. An interesting result was the report on RAPD-PCR fingerprints of the 10 strains using each of primers 1-6 purchased from Pharmacia Biotech (Uppsala, Sweden). Diversity One Software (New York, USA) was used to construct a dendrogram from the similarity of the RAPD fingerprint profiles. The dendrogram revealed three clusters with the first cluster consisting of reference strains USDA136b, USDA76, 61A101C, and SM5 which were temperate strains. The Thai strain THA211 was found to belong to the same cluster as the temperate strains. The Thai strains THA2, THA5, and THA7 which were found to be acid-tolerant were found to cluster in the second cluster while the Thai strains THA6 and THA102 were found to belong to the third cluster. The researchers did not comprehensively identify all the 5 isolated strains obtained in their study. In addition, no information was given on the locations of soybean cultivation fields in Thailand where the 5 soybean rhizobium strains were isolated.

In 1998, Teaumroong and Boonkerd employed primers REP, ERIC, and RAPD to obtain PCR-DNA fingerprints of 18 strains of *Bradyrhizobium japonicum*. The

sequence of RAPD primer was 5'GGAAGTCGCC3'. Sequences of REP abd ERIC were shown in Figure 2.2.

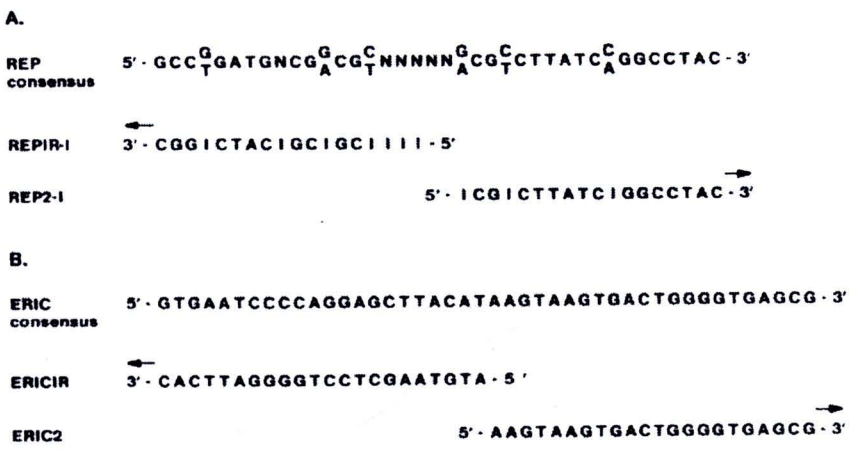


Figure 2.2 Nucleotide sequences of the REP and ERIC primers. (A) REP consensus sequence and nucleotide sequences of the two REP primers (REP1R-I and REP2-I), positioned relative to the REP consensus sequence. The I's denoted inosines. (B) ERIC consensus sequence and nucleotide sequences of the two ERIC primers (ERICIR and ERIC2), positioned relative to the ERIC consensus sequence. The arrows denoted the direction of *Taq* polymerase extension (de Bruijn, 1992).

Six of the 18 strains were *Bradyrhizobium japonicum* USDA strains (USDA8-0, USDA8-T, USDA35, USDA94, USDA117, and USDA136). Five of the strains were the same strains as used by Nuntagij (1997), namely, THA2, THA5, THA7, TAL102(USDA110), and TAL211. Therefore Teaumroong and Boonkerd (1998) reported on new 7 *Bradyrhizobium japonicum* strains. As with Nuntakij (1997), the authors did not provide details on where in Thailand the soybean nodules used for the isolation of the soybean rhizobia were obtained. The authors also did not elaborate on how the 7 strains were identified as *B. japonicum*. Combined banding patterns of the fingerprints obtained from using each of the set of primers (REP, ERIC, and RAPD) were used in the construction of a dendrogram with Primer Version 3.1B program. Figure 2.3 showed the dendrogram.

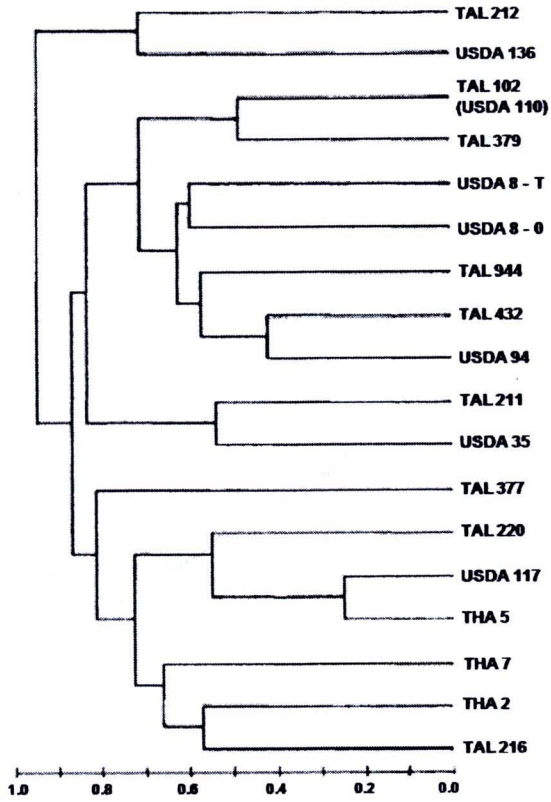


Figure 2.3 Dendrogram constructed by using combined banding patterns of the fingerprints obtained from using each of the set of primers (REP, ERIC, and RAPD) (Teaumroong and Boonkerd, 1998).

The dendrogram indicated 3 clusters at about 80% similarity. Cluster 1 consisted of TAL212 and USDA136. Cluster 2 consisted of two subgroups 2.1 and 2.2 with subgroup 2.1 containing three groups as follows:

Group 2.1.1 consisted of TAL102(USDA110),and TAL379

Group 2.1.2 consisted of USDA8-T and USDA8-0

Group 2.1.3 consisted of TAL944, TAL432, and USDA94

Subgroup 2.2 consisted of TAL211 and USDA35. Cluster 3 comprised two subgroups with TAL377 in subgroup 3.1 and there were two groups in subgroup 3.2. Group 3.2.1 contained TAL220, USDA117, and THA5. Group 3.2.2 contained THA7, THA2, and TAL216. Both Teaumroong and Boonkerd (1998) and Nuntagij et al. (1997) showed the same results that strain TAL211 belonged to a different cluster from strains THA5, THA7, and THA2.

In 1996 Yokoyama et al. obtained RFLP patterns of *nodDYABC* of 123 soybean rhizobia which consisted of 62 strains isolated in Thailand, 46 strains isolated in Japan, and 15 USDA strains. Dendrogram constructed from the RFLP patterns showed 4 clusters with all the Japanese and USDA strains belonged to Clusters 1 and 2 while Clusters 3 and 4 contained only the Thai isolates. In 1999 Ando and Yokoyama worked with 14 strains of Thai isolates of *Bradyrhizobium* sp. (*Glycine max*), 3 strains of Thai isolates of *Bradyrhizobium elkanii* , 8 USDA strains of *Bradyrhizobium japonicum* and 4 USDA strains of *Bradyrhizobium elkanii*. Genomic DNA of these 29 strains were digested with each to the following four restriction enzymes : *Bam*HI, *Hind*III, *Pst*I, and *Eco*RI. DNA fragments obtained were hybridized with 3.5 kb *Bgl*II fragment containing *nifDK*. 16 RFLP patterns obtained were used to construct a dendrogram with the Unweighted Pair Group Method with Arithmatic Averages (UPGMA) method using the PHYLIP software. The results were shown in Figure 2.4

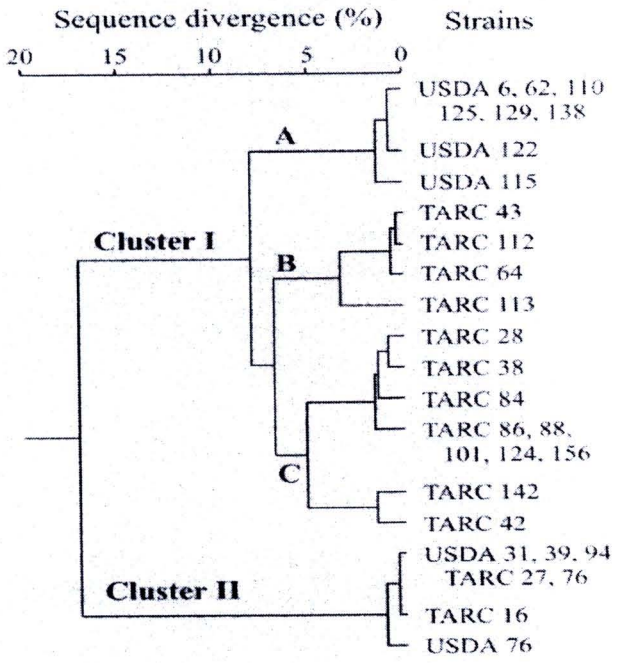


Figure 2.4 A dendrogram of 29 soybean rhizobia strains constructed with UPGMA method using the PHYLIP software (Ando and Yokoyama, 1999).

The results as shown in Figure 2.4 showed 2 clusters. Cluster 1 consisted of 3 groups (A, B, and C). Group A contained all the *B. japonicum* USDA strains 6, 62, 110,

115, 122, 125, 129, and 138. Group B contained 4 isolates from Thailand. Group C contained 10 isolates from Thailand. Partial 16S rDNA sequencing of the 246 bp at positions 44-337 showed 7 of the Thai isolates belonging to Groups B and C had identical sequences with the partial 16S rDNA sequences of *B. japonicum* USDA6, 115, and 129. Cluster 2 consisted of the 4 *B. elkanii* USDA strains 31, 39, 76, and 94 and three strains isolated in Thailand. The results as reported by Ando and Yokoyama (1999) indicated that the 14 strains of Thai isolates of *Bradyrhizobium* sp. (*Glycine max*) which were found to belong to Groups B and C formed a distinct group of slow-growing soybean rhizobia which were intermediate between the *B. japonicum* and *B. elkanii* USDA strains used in the study.

In 1999 Yokoyama et al. reported on phenotypic characterization by serological and intrinsic antibiotic resistance properties of 94 strains of soybean rhizobia isolated from Bangkok, Chiangmai, Nakorn Sawan, Saraburi, and Uttaradit. From the results obtained from the ELISA (Enzyme-Linked Immunosorbent Assay) using polyclonal antisera prepared against 15 USDA standard serotype strains of *B. japonicum* (USDA4, 6, 62, 110, 115, 122, 123, 124, 125, 127, and 129) and *B. elkanii* (USDA31, 46, 76, and 94), the 94 strains isolated in Thailand were characterized into 14 different cross-reaction groups corresponding to 12 of the 15 antisera used. The authors reported that the Thai strains showed a high degree of cross-reactivity. In addition, antibiotic resistance tests of the 94 Thai strains were carried out with the following antibiotics: 10 $\mu g.ml^{-1}$each of spectinomycin and streptomycin, 15 $\mu g.ml^{-1}$each of kanamycin and nalidixic acid, 25 $\mu g.ml^{-1}$ of rifampicin, and 50 $\mu g.ml^{-1}$each of neomycin and polymyxin. The Thai soybean rhizobia were found to be resistant to kanamycin, nalidixic acid, neomycin, and polymyxin. The results indicated that the Thai isolates were not closely related to the USDA strains of *B. japonicum* and *B. elkanii.*

Since the Ando and Yokoyama's and Yokoyama et al, 's studies in 1999 there have not been any published articles on the isolation and characterization of soybean rhizobia in Thailand. Research on soybean rhizobia in Thailand after the year 1999 concentrated on the determination of suitable cultivars of soybean as hosts for native rhizobia (Shutsrirung et al. 2002a,b,c). This dissertation is the first serious effort to isolate and characterize soybean rhizobia in Phitsanulok, Thailand , employing

polyphasic taxonomy which includes the characterization of several genetic and phenotypic properties including colony morphology, Bromthymol blue reaction, number and type of flagella, specific growth rates at different temperatures, ability/inability to utilize 95 carbon and nitrogen sources using the Biolog™ test kit, PCR-DNA Fingerprinting , and sequencing of 16S rDNA and *nodY*. Polyphasic taxonomy has been used in bacterial taxonomy since 1996 (Vandamme et a. 1996).

2.2 Soybean rhizobium genetics

Soybean rhizobium genetics is relatively well-documented both in terms of genetics of nodulation (Stacey, 1995) and of nitrogen fixation (Fischer, 1994). Loh and Stacey (2003) reported nodulation genes in *B. japonicum* included *nodD1, nodD2, nodYABC* and *nwsB. 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). Banfalvi et al. (1988) reported that in *B. japonicum*, *nodY* is a 420-bp gene located within the 785-bp region between *nodD1* and *nodABC*. In *B. elkanii*, there is no *nodY* gene but, in its place, there is *nodK* gene which is 402 bp in size. *Bradyrhizobium* sp. (*Parasponia*) also contains a *nodK* gene in the same position (Scott 1986). 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.

The complex between genistein and NodD1 formed in the periplasm acts as a transcriptional activator which binds to the promoter regions of *nodD1* and *nodYABC* which are known as *nodD1*box and *nodY*box respectively. Transcription and translation of *nodA, nodB,* and *nodC* lead to the synthesis of the first three enzymes in the synthesis of Nod factor which is essential for root hair deformation and nodulation process. NodC, N-acetylglucosaminyl transferase catalyses the joining of N-

acetylglucosaminyl units by ß-1,4 glycosidic linkages. NodB, N-deacetylase, catalyses the removal of an acetyl group from the N-acetylglucoaminyl group at the non-reducing end of the Nod factor. NodA, N-acyl transferase, catalyses the transfer of an acyl group (C18:1) to the N-glycosyl unit at the non-reducing end of the Nod factor. Figure 2.5 shows synthesis of nod factors which are produced by soybean rhizobia and whose function (s) on nodulation process is still unknown.

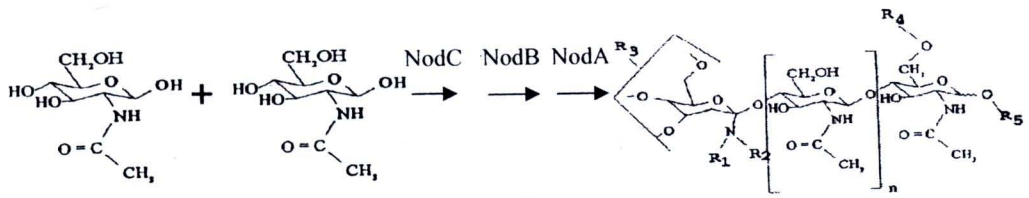
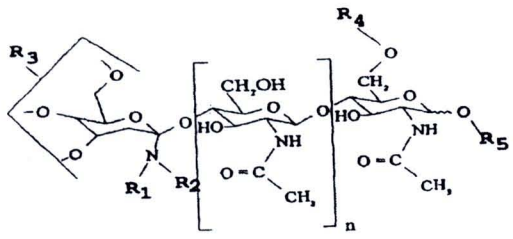


Figure 2.5 Synthesis of Nod factors catalysed by enzymes encoded by *nodC*, *nodB* and *nodA* (Stacey, 1995).

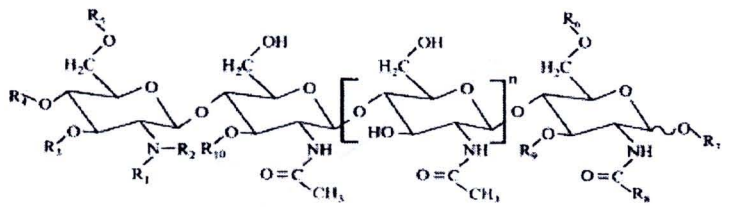
Chemical structures of nod factors of *B. japonicum* differ from those of *B. elkanii*. Nod factor of *Bradyrhizobium japonicum* consists of 5 N-acetylglucosaminyl units with side chains as indicated in Figure 2.5. The chemical structures of Nod factors of *B. japonicum* strains USDA 110 and USDA 135 and *B. elkanii* strain USDA 61 are shown in Figure 2.6.



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(C18:0,MeFuc)	C18:0	H	H	2-O-MeFuc	H	3
NodBj-V(Ac,C18:0,MeFuc)	C18:0	H	Ac	2-O-MeFuc	H	3
NodBj-V(C18:1, MeFuc)	C18:1	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)	C18:1	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)	C18:1	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).

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 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, 2002).

In addition, in 2002 Loh et al. discovered Bradyoxetin in the broth culture of stationary phase cells of *B. japonicum*. In the same year the authors constructed 4 mutants : *B. japonicum* JWS21 (*nwsB* Sm^rSp^r) ; *B. japonicum* JNWS24 (JNWS21 harboring pBGAlac1 with *nolA-lacZ* translational fusion); *B. japonicum* JNWS31 (JNWS21 harboring pZB32 with *nodY-lacZ* translation fusion); *B. japonicum* JNWS41 (JNWS21 harboring pPRJ1248 with *nodD2-lacZ* translational fusion). The mutants were used to demonstrate that at high cell density the expression of *nolA* and *nodD2* increased while that of *nodY* decreased and that *nwsB* was essential for the density-dependent full expression of *B. japonicum nod*, *nolA*, and *nodYABC*. NwsB was

postulated to sense the presence of Bradyoxetin at high cell density which led to the activation of *nolA* and *nodD2* which inhibited the expression of *nodYABC* leading to a decrease in Nod factor synthesis. The results implied that the number of *B. japonicum* cells in a rhizobial biofertilizer should be optimal for optimal expression of nodulation genes *nodYABC* for Nod factor synthesis.