CHAPTER V

DISCUSSION

5.1 Colony morphology and Bromthymol blue reactions in slow-growing soybean rhizobia

The results obtained for colony morphology, Bromthymol blue reactions and the identification of slow-growing soybean rhizobia as shown in Figures 4.1, 4.2 and Tables 4.5 and 4.16 showed that in most cases, it is possible to predict the type of Bromthymol blue reactions from the colony morphology. Almost all of the identified *Bradyrhizobium elkanii* strains and *B. yuanmingense* strain STB169 with Type I colony morphology (irregular and slimy colonies) secreted alkali product(s) throughout the 10-day incubation period while the slow-growing *B. japonicum* with Type II colony and *B. yuanmingense* strain STB264 secreted alkali product(s) in the first 5-day incubation and secreted acidic product(s) in the last 5-day of incubation. According to Somasegaran and Hoben (1994), the indicator dye Bromthymol blue is green in YMA with pH 6.8. Fast-growing soybean rhizobia secrete acidic product(s), therefore, Bromthymol blue is changed to yellow color. Slow-growing soybean rhizobia turn the color of Bromthymol blue to blue due to the secretion of alkali product(s). Other researchers also reported that fast-growing soybean rhizobia showed an acid Bromthymol blue reaction while slow-growing soybean rhizobia showed an alkali Bromthymol blue reaction (Alberton et al., 2006; Chen et al., 2002; Chen et al., 2004; Hungria et al., 2001). However, in this research , it is demonstrated for the first time that two types of Bromthymol blue reactions were found in slow-growing soybean rhizobia as described above. The experimental results showed that during growth on YMA with Bromthymol blue at the initial pH of 6.8, strains of *B. elkanii* and *B. liaoningense* as well as *B. yuanmingense* strain STB169 secreted alkali product(s) which turned the medium blue throughout the 10-day incubation time while strains of *B. japonicum* and *B. yuanmingense* strain STB264 were found to secrete acidic product(s) during the first 5-day incubation and secrete acidic product(s) during the last 5-day incubation. The results could be interpreted as *B. elkanii*, *B. liaoningense* as well as *B. yuanmingense* strain STB169

could survive under alkali condition with no need for an adaptation to changes of pHs in the surroundings while *B. japonicum* and *B. yuanmingense* strain STB264 preferred an acidic condition. Therefore, when pHs of the surroundings were in the alkali range, *B. japonicum* secreted acidic product(s) to adjust the surrounding pHs to the acidic range. The findings that slow-growing soybean rhizobia could secrete either acidic or alkali products to change pHs of the surroundings were confirmed by the experimental results on responses of *B. elkanii* strain NA7, *B. japonicum* strain S76, *B. liaoningense* strain SK3, and *B. yuanmingense* strain STB264 to the pHs of the medium with and without buffer as shown in Tables 4.6 to 4.15. The results showed that in the absence of buffer, when the initial pHs of the medium were acidic (pH 4.0, 5.0, and 6.0), the four slow-growing soybean rhizobium strains secreted alkali product(s) to turn the values of pHs of the supernatant to more than those of the initial pHs. On the other hand, when initial pHs of the medium were 7.0 or 8.0, cells of *B. liaoningense* strain SK3 and *B. yuanmingense* strain STB264 were found to secrete acidic product(s) to turn pHs of the supernatant to the values of pHs in an acidic range. In terms of an adaptation for growth and survival in soils with different pHs, it would be advantageous for slow-growing soybean rhizobia to be able to change pHs of the surrounding soils to the optimum pH for growth and survival by secreting either acidic or alkali products, depending on pHs of the surroundings.

5.2 Predominance of slow-growing soybean rhizobia in 16 subdistricts of Phitsanulok province and the prevalence of natural variants

The authentication test results on isolated bacteria from root nodules obtained in the experiments indicated that only slow-growing soybean rhizobia were obtained. One reason for the predominance of slow-growing soybean rhizobia was the acidity of the soil samples which were in the range of 4.5-6.5. Suzuki et al. (2008) reported that soybean rhizobia on the Okinawa Islands in Japan were mainly fast-growing soybean rhizobia due to the alkalinity of the soils. In addition, Han et al. (2008) also reported the presence of fast-growing soybean rhizobia as well as the slow-growing *B. liaoningense* in saline alkali soils in Xinjiang, People's Republic of China. The first report on the isolation of fast-growing soybean rhizobium, *Rhizobium fredii* was carried out by Keyser et al. in 1982. Since then other researchers have isolated fast-growing soybean rhizobia

from Hubei province in mainland China (Camacho et al., 2002; Dowdle and Bohlool, 1985; Stephen and Bohlool, 1985), and in Brazil (Hungria et al., 2001).

This dissertation is the first report on the record of *B. yuanmingense* in Thailand. Previously there were two published papers on the isolation and characterization of *B. yuanmingense* strain that nodulated soybean (Appunu et al., 2009) and *B. yuanmingense* strain that did not nodulate soybean but nodulated legume species of the genus *Lespedeza* (Yao et al., 2002). In this dissertation, two strains of *B. yuanmingense*, namely, strains STB169 and STB264 were isolated. The two strains were found to have different colony morphology and BTB reactions and shown in the Appendices C and D. Future taxonomic work on all the 76 slow-growing soybean rhizobium STB strains will be carried out by Multilocus Sequencing Analysis (MLSA) (Gevers et. al., 2005). In addition, this dissertation presented two lines of evidence for the detection of natural variants of slow-growing soybean rhizobia for the first time in Thailand. The first evidence was 16S rDNA dendrograms (Figures 4.20, a-c) grouped the 12 STB strains (STB8, STB119, STB120, STB147, STB173, STB176, STB179, STB185, STB220, STB245, STB283, and STB327) and the 6 STB strains (STB30, STB54, STB67, STB96, STB250, and STB310) into the same species of *B. elkanii* and *B. japonicum*, respectively. The second line of evidence showed the STB strains which were grouped into the same species had different DNA fingerprints as shown in Figure 4.19a for the 12 STB strains of *B. elkanii* and the 6 STB strains of *B. japonicum* (Figure 4.19b). Figure 4.19 showed the above-mentioned STB strains had different DNA fingerprints. Previously, natural variants in slow-growing soybean rhizobia were reported from Brazil where natural variants of *B. japonicum* SEMIA 566 strain used in Brazilian commercial inoculants from 1966 to 1978 were found (Barcellos et al., 2007). In fact, all the reference strains used in the construction of *nodY* dendrograms were quoted from the paper by Barcellos et al. (2007) with the expectation that some of the Thai natural variants might have close phylogenetic relationship with the Brazilian natural variants. However, the *nodY* dendrograms showed all the Brazilian natural variants were found in the same cluster. It was not surprising to find the Brazilian natural variants in the same cluster because they were all arose from genetic adaptations to the soil

environments in Brazil and by possibly by lateral gene transfer (Boucher et al.,2003; Wright, 2004).

5.3 Multilocus Sequencing Analysis (MLSA) in the identification and determination of phylogenetic relationship in natural variants of slow-growing soybean rhizobia STB strains

The average size of isolated 16S rDNAs of the 20 slow-growing STB strains around 1450 bp were in the same range as those reported by Binde et al. (2009) and Menna et al. (2006). The dendrograms constructed with sequences of *nodY* of the 20 slow-growing soybean rhizobium STB strains yielded less satisfactory results because the two *B. yuanmingense* strains STB169 and STB264 could not be identified by *nodY* sequences. The results agreed with other researchers who reported the limitations of using homology to sequences deposited in GenBank database for the identification purpose. If less numbers of sequences of genes of interest are deposited in the GenBank database, the chance of being able to identify a particular species is reduced. In addition, sequences of only one gene, 16S rDNA, cannot be used to resolve differences amongst natural variants of the 12 *B. elkanii* and 6 *B. japonicum* STB strains observed in PCR-DNA fingerprints (Figure 4.19). Moreover, phenotypic differences in the ability to use/not use 95 carbon and nitrogen sources determined by the Biolog™ tests of the 20 STB strains could not be used to resolve differences in the 12 and 6 natural variants of *B. elkanii* and *B. japonicum*. Therefore, there is a trend towards identification and phylogenetic relationship determination by the Multilocas Sequence Analysis (MLSA) which was first introduced by Gevers et al. (2005) .In 2008 Vinuesa et al. employed Multilocus Sequence Analysis to identify soybean rhizobia.

5.4 Further research on the collection of soybean rhizobia obtained

The genetic diversity of the 121 strains of slow-growing soybean rhizobia as shown in Figures 4.25 (a,b) is an example to indicate that Thailand has a vast collection of soybean rhizobium strains which could be tested for their suitability for use in the production of inoculants for field trials. In 2009, Chansa-ngavej applied for a Thai patent on the selection method for soybean rhizobia that could be used to produce soybean rhizobium biofertilizers which could be kept at room temperature. The method required

the selection of strains that grew well at 25^{0}C and 30^{0}C which were used to represent soil temperatures in the northern, upper central, and some parts of the north-eastern part of Thailand where soybeans are grown. The selected strains should not increase in numbers when grown at 37^{0}C and 40^{0}C which were chosen as representation of room temperatures where soybean rhizobium biofertilizers are kept during storage and transportation. The results of growth curves obtained when cells of the 5 selected strains of slow-growing soybean rhizobia were grown at 25^{0}C, 30^{0}C, 37^{0}C, and 40^{0}C as shown in Figure 4.24 showed the strains STB30, STB96, STB120, STB220, and STB264 did not meet the criteria set up for the selection of soybean rhizobia that could be used in the production and field testings of soybean rhizobium biofertilizers that could be kept at room temperature. The rationale of maintaining no growth when grown at 37^{0}C and 40^{0}C is to keep the numbers of cells constant at the original minimum10^8 CFU/ml as stipulated in the Royal Gazette for the standard quality of biofertilizers. If the number of rhizobium cells in the biofertilizers is more than 10^8 CFU/ml, there may be inhibition of nodulation gene expression by the quorum sensing mechanism (Loh et al., 2001, 2002a, b, 2003, Sharma et al., 2003).

The remaining 116 strains of slow-growing rhizobia obtained in this dissertation could be further used in the determination of growth at different temperatures to find desirable strains for the production of inoculants for field trials. Other desirable properties of soybean rhizobium strains that need to be determine for the selection for use in the production of biofertilizers include competitive ability to outcompete indigenous soybean rhizobia in root nodulation, nitrogen fixation ability and survival in the fields as well as formulations (Vlassak and Vanderleyden, 1997). The results on slow-growing soybean rhizobia in 16 subdistricts in Phitsanulok province presented in this dissertation are thus a contribution to the study of soybean rhizobium diversity which has a far-reaching effect on the development of soybean rhizobium biofertilizers which would increase income of soybean growers and would contribute to the preservation of the soil environments for sustainable agriculture in some parts of Thailand. However, a lot more research in terms of basic and applied sciences in soybean rhizobium technology still needs to be carried out in Thailand before we reach the same advanced

stage of research and applications in soybean rhizobium technology as found in developed countries where the whole genome of *B. japonicum* has been sequenced (Kaneko et al., 2002) and approximately 51% of the whole genome of *B. japonicum* CPAC 15 have been sequenced (Goday et al., 2008). Finally, a lot of efforts are still needed in the popularization of the use of soybean rhizobium biofertilizers in Thailand (Chanaseni and Kongngoen, 1992) when compared with the use of soybean rhizobium inoculants in other countries (Abaidoo et al., 2007; Aguilar al., 2001; Brutti et al., 1998; Chen et al, 2000, 2004; Hungria et al.,2001; de Jensen et al, 2004; Judd et al, 1993; Minamisawa et al, 1999, Thomas- Oates et al., 2003 and Yanni, 2004).

5.5 Significance of discovering fast-growing bacteria in soybean rhizosphere

Soybean roots secrete flavonoids such as genistein which creates a gradient along which soybean rhizobia move towards the roots to form nodules (Kosslak et al, 1987). Other bacterial populations in soybean rhizosphere have been known to break down these signal flavonoid molecules. The presence of various bacterial populations capable of breaking down the flavonoid molecules could contribute to reduced extent of nodulation of soybeans resulting in lower soybean yields. In this research, isolation and identification by 16S rDNA sequences were obtained for several fast-growing, acid-secreting bacteria belonging to *Agrobacterium tumefaciens* (STB170, Ban Dong) and *Rhizobium tropici* (STB23, Hua Ro and STB97, Mathong). The collection of fast-growing bacteria from the rhizosphere of soybeans provide sets of soybean rhizobia and bacteria in the soybean rhizosphere which form base-line data for further research on dynamics of bacterial populations in soybean rhizosphere as well as study on the impact of rhizospheric bacterial populations on soybean root nodule formation (Taboran and Chansa-ngavej, 2009). In 2010, Udomchotphruet and Chansa-ngavej reported on reversed-phase HPLC conditions which could be used to determine concentrations of genistein. The aim of the research is to detect if fast-growing bacteria isolated from rhizosphere of soybeans could *in vitro* break down genistien, the signal molecule for soybean root nodulation.