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Co-inoculation effects of *Bradyrhizobium japonicum* and *Azospirillum* sp. on competitive nodulation and rhizosphere eubacterial community structures of soybean under rhizobia-established soil conditions

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Bradyrhizobial inoculants used for soybean seed inoculation to maximize the benefit of N₂-fixation should include bradyrhizobial strain with high N₂-fixation rates and ability to compete with the indigenous rhizobial populations. In this study, co-inoculation of plant growth promoting rhizobacteria (PGPR) *Azospirillum* sp. with either of *Bradyrhizobium japonicum* CB 1809 or USDA 110 increased shoot and root dry weight of soybean over non-inoculated control under pot condition with no indigenous soybean nodulating bradyrhizobia. Moreover, competition for nodulation and the effects on rhizosphere soil eubacterial community structures by using single or co-inoculation of *B. japonicum* and *Azospirillum* sp. under rhizobia-established Myanmar and Thailand soils were investigated. By inoculation of *gus*-marked USDA 110 singly or its co-inoculation gave 93.21 to 94.75% and 74.21 to 100% in nodule occupancy, and 23.50 to 41.95% and 50.37 to 73.24% promotion in biomass dry weight over non-inoculated control in Myanmar and Thailand soil samples, respectively. Each of all the tested inoculum levels, that is 10⁶, 10⁷ and 10⁸ cfu/ml of *Azospirillum* sp. enhanced nodulation in combination with USDA 110 with a corresponding increase in 73.8, 62.25 and 95.34%; and 51.52, 62.38 and 79.46% over non-inoculated control, respectively in Myanmar and Thailand soil, respectively. In addition, soybean rhizosphere soil eubacterial community structures were not shifted by bacterial inoculation. Therefore, *Azospirillum* sp. could be used in co-inoculant production with *B. japonicum* for soybean.

Key words: *Bradyrhizobium*, plant growth promoting rhizobacteria (PGPR), soybean, co-inoculation, competition, rhizosphere eubacterial community structure.

INTRODUCTION

Maximum benefit of N₂-fixation by soybean often requires the inclusion of selected strains of *Bradyrhizobium* in seed inoculants. The main criterion used in selection of *Bradyrhizobium* strains for legume inoculation is the ability to form an effective symbiosis with the hosts for

which the inoculant is recommended. However, inoculation may not always lead to improved nodulation or enhanced N₂-fixation because of the presence of indigenous rhizobia which are more competitive than the inoculant strain (Roughley et al., 1976). Both competitive-

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ness and symbiotic effectiveness were independent traits (Castro et al., 2000), therefore, the *Rizobium* strain selected for inoculants should not only have high N₂-fixation rates, but also be able to compete with the indigenous rhizobia populations (Vlassak and Venderleyden, 1997).

Nowadays, plant growth promoting rhizobacteria (PGPR) play an important role as they have several mechanisms to promote the plant growth (Glick, 1995). *Azospirillum* is one of the PGPR and considered as a *Rhizobium* helper by stimulating nodulation, nodule function, and possibly plant metabolism (Andreeva et al., 1993). Effects of *Azospirillum* inoculation are mainly attributed to improved root development and enhanced water and mineral uptake. Secretion of plant growth promoting substances, mainly indole-3-acetic acid (IAA), is strongly associated with the positive response by the plant (Spaepen et al., 2009). Phytohormones produced by *Azospirillum* promoted epidermal-cell differentiation in root hairs that increased the number of potential sites for rhizobial infection (Yahalom et al., 1991) leading to forming more nodules (Andreeva et al., 1993). *Azospirillum brasilense* Az39 and *Bradyrhizobium japonicum* E 109 inoculated singly or in combination have the capacity to promote seed germination and early seedling growth in soybean and corn (Cassan et al., 2009). Moreover, dual inoculation of soybean with *B. japonicum* and *A. brasilense* gave a significantly higher proportion of nodules attached to the main root, and increased number of the most active nodules, and increased 23% of nitrogen content of soybean plants over *B. japonicum* single inoculated plant (Groppa et al., 1998).

Currently, *B. japonicum* strains CB 1809 and USDA 110 are being used in "rhizobial inoculant production" for soybean in Myanmar and Thailand, respectively. However, in both countries, there were no reports on promotion effects on soybean through co-inoculation with *B. japonicum* and any PGPR, and there are no literatures on the study of rhizosphere soil microbial community structures in any leguminous plants with respect to rhizobial inoculations. In this study, *Azospirillum* sp., one of the effective PGPR which was being commercially used in PGPR inoculant production by Suranaree University of Technology (SUT), Thailand (Teaumroong et al., 2009), was selected for co-inoculation with *B. japonicum*. Moreover, it is needed to study the changes of microbial community caused by inoculation of rhizobial inoculants as their potential ecological risks on microbial diversity should not be neglected. Therefore, this study was aimed to evaluate the co-inoculation effect of *B. japonicum* and *Azospirillum* sp. on soybean nodulation and plant growth under no indigenous soybean nodulating bradyrhizobia soil conditions and to detect the competitive nodulation occupancy of co-inoculated *B. japonicum* strain USDA 110 and *Azospirillum* sp. on soybean as well as to observe the changes of rhizosphere soil bacterial community structures.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions

Two *B. japonicum* strains of CB 1809 and USDA 110 those currently used in rhizobial inoculants production for soybean at the Department of Agricultural Research (DAR), Myanmar and Thailand were cultured in yeast extract manitol (YEM) media (Vincent, 1970) and *Azospirillum* sp. that was from the School of Biotechnology Laboratory, Suranaree University of Technology (SUT), Thailand was cultured in Nutrient broth. Those cultures were maintained by periodic transfer and stored in the refrigerator for further studies.

Soil samples collection and analysis

The soil samples for preliminary pot experiment with minimum or absence of indigenous soybean nodulating bradyrhizobia were collected from the field of Muang District, Nakhon Ratchasima, Thailand (14° 52' 10" N and 102° 00' 42.24" E) which has no history of leguminous cultivation. The soil was loamy sand in texture, having a pH 5.25 with 0.42% organic matter content and 4.03 and 34.5 ppm of available P and exchangeable K, respectively.

For nodulation competition study, two soil samples from soybean nodulating bradyrhizobia-established soils were collected from Kyauk Me Agricultural Research Farm (22° 32' 20.93" N and 97° 01' 42.10" E), Department of Agricultural Research, Kyauk Me Township, Myanmar and Farmer's soybean field, Chiang Mai (18° 48' 01.28" N and 98° 39' 59.00" E), Thailand while soybean was grown as a standing crop to maximize the rhizobial and soil bacterial population. Soil samples were kept in clean polyethylene bags and stored at 4°C until used. Soil physicochemical characterization showed that Myanmar soil has pH 4.72 with 2.88% organic matter; and 21.43 and 164.38 ppm of available P and exchangeable K, respectively. In Thailand soil, soil pH was 4.96 with 2.46% organic matter content, and available P and exchangeable K contents were 27.27 and 73.47 ppm, respectively.

Quantification of the number of indigenous soybean nodulating rhizobia

The number of indigenous soybean nodulating rhizobia in experimental soil samples was determined by a modification of the plant infection test using a most probable-number (MPN) technique (Vincent, 1970). 1 ml aliquot of each dilution was inoculated onto pre-sterilized soybean seeds in sterilized growth pouch and grown axenically in light room condition. Two seeds per pouch were grown and four seeds (quadruplicate) were inoculated for each dilution. Non-inoculated control was also included. Plants were grown in growth chamber at 27/20°C light room under 16/8 h light/dark photoperiod, and MPN estimations based on nodulation were determined at three weeks after inoculation.

Co-inoculation effects of *B. japonicum* and *Azospirillum* sp. on soybean under indigenous soybean nodulating rhizobia non-established soil

A preliminary pot experiment was conducted during June to July, 2011 to evaluate the co-inoculation effects of *B. japonicum* CB 1809 and USDA 110, and *Azospirillum* sp. on soybean. 9 kg of soils were put into the pot (20 cm diameter x 20 cm height). Ten (10) pre-sterilized and pre-germinated soybean seeds (*Glycine max*, Chiang Mai 60) were sown in each pot and 1 ml of the bacterial broth culture (10⁸ colony forming unit (cfu)/ml) was inoculated onto each seed according to the treatments. For single inoculation, the seeds were inoculated separately with 10⁸ cfu/ml of *Azospirillum* sp., CB

1809, and USDA 110. For co-inoculation, seeds were inoculated by 1:1 ratio of either of CB 1809 or USDA 110 with *Azospirillum* sp. Non-inoculated control was also included. The pots were laid out in a completely randomized design (CRD) with three replications. The plants were watered by tap water whenever necessary and regular agricultural practices were done except pesticide spraying. Plants were sampled at 45 DAI and the nodule number, nodule dry weight, and shoot and root dry weights were recorded. Statistical significance was determined by analysis of variance (ANOVA) and means were compared by the Duncan's multiple range test ($p \leq 0.05$) (Duncan, 1955).

Rep-PCR amplification

The bacterial DNA were extracted from *B. japonicum* CB 1809 and USDA 110, and *Azospirillum* sp. Rep-PCR DNA fingerprint was used to investigate the genetic differences of the *B. japonicum* strains USDA 110 and CB 1809. Rep-PCR fingerprints were obtained by using BOX-AIR primer (5'-CTA CGG CAA GGC GAC GCT GAC G- 3') (Sadowsky et al., 1996). The PCR reaction contained 50 ng of DNA template, 50 pmol of primer, 2.5 mM of dNTP, 1x PCR buffer, and 2.5 U Taq DNA polymerase (Promega, USA) in total volume of 50 μ l. Each PCR was performed with GeneAmpPCR system 9600 (Perkin Elmer, USA). The PCR reaction condition was used as follows: 1 cycle of 95°C for 2 min, 94°C for 30 s, 53°C for 1 min, 35 cycles of 56°C for 8 min and final, 1 cycle of 65°C for 16 min. Products from PCR were separated on 2% agarose gel, stained with ethidium bromide and viewed under ultraviolet (UV) light in gel documentation.

Construction of *gus*-marked *B. japonicum* strains

Two bacterial strains, *Escherichia coli* S17-1 donor strain (harboring plasmid pCAM120, *Trn5* fusion with *gus*-gene) which is resistant to 20 μ g/ml of both Streptomycin and Spectinomycin, and recipient *B. japonicum* strain USDA 110 which is resistant to Gentamycin (20 μ g/ml), were grown to stationary phase in Luria-Bertani broth (LB) (Sambrook et al., 1989) and yeast-extract-mannitol (YEM) broth for overnight and seven days, at 37°C and 28 \pm 2°C, respectively. The method for biparental mating was that of Krause et al. (2002). Blue forming colonies on HEPES-MES HM solid media (Cole and Elkan, 1973) containing Streptomycin (200 μ g/ml), Gentamycin (30 μ g/ml) and X-gluc (5-Bromo-4-chloro-3-indolyl-beta-D-glucoside) (20 μ g/ml) were selected as transconjugants and sub-cultured on yeast-malt extract agar (YMA) medium to check purity and *gus*-stability. Stable blue colonies were then picked up and inoculated into YEM broth with appropriate antibiotics and stored with 50% sterilized glycerol at -70°C until needed. The nodule formation of *gus*-marked *B. japonicum* strains were checked on both siratro (*Macroptilium atropurpureum*) and soybean hosts by using growth pouch method (Vincent, 1970).

Competitive nodulation ability of *B. japonicum* strain by co-inoculation with PGPR in rhizobia-established soils

Pot experiment was conducted to determine the competitive ability of single and/or co-inoculation effects of *B. japonicum* strain USDA 110 with *Azospirillum* sp. on soybean nodulation and rhizosphere eubacterial community structure. The *gus*-marked *B. japonicum* USDA 110, wild type USDA 110, and *Azospirillum* sp. were cultured in YEM broth containing appropriate antibiotics, normal YEM broth and LG (N-free) broth (Hirschi et al., 1991), respectively and shaken on the rotary shaker (180 rpm) at 28 \pm 2°C for seven to 10 days for bradyrhizobia, and two days for *Azospirillum* sp. About 250 g of soil was put into the pre-sterilized modified Leonard's jar and four pre-

sterilized and pre-germinated soybean seeds were grown in each jar. The cultures were centrifuged (4,000 x *g* for 5 min) and washed with 0.85% (w/v) sterilized saline to remove the antibiotic and excess media from the culture media, and the cell pellet was resuspended in 0.85% (w/v) saline. 1 ml of the bacterial broth culture (10⁸ colony forming unit (cfu)/ml) was inoculated onto each seed according to treatments. For the single inoculation, the seedlings were inoculated separately with 10⁸ cfu/ml of *Azospirillum* sp., USDA 110 wild type (wt) and *gus*-marked USDA 110 (tr). For the co-inoculation, 10⁸ cfu/ml of USDA 110 (tr) were mixed in a ratio of 1:1 with three different inoculum levels (10⁶, 10⁷ and 10⁸ cfu/ml) of *Azospirillum* sp. Bulk soil (no planted and non-inoculated control) and non-inoculated controls were also included.

The experiment was conducted as a CRD design with three replications. Plants were grown on a growth shelf at 27/20°C in light room condition under 16/8 h light/dark photoperiod. Additional experiment was set up as the same treatments; however, the vermiculite was used as growth media instead of soils under sterilized conditions. At 30 DAI, soybean plants were carefully uprooted from the jars from both sterilized and non-sterilized experiments; roots were gently washed with water not to remove the root hairs and nodules, and the nodulation competitiveness of inoculated bradyrhizobial strain was detected by *gus*-staining method. Nodule numbers per plant were counted and nodule dry weight per plant (mg) and biomass dry weight per plant (mg) were determined after oven dried at 70°C for 48 h. Total root length (m) for each fresh root samples was measured by scanning for three times with "Comair Root Measurement Scanner" (Commonwealth Aircraft Crop Ltd., Melbourne, Australia).

Detection of *gus*-activity inside soybean root nodules

For the detection of inoculated *gus*-marked bradyrhizobia, root nodules from each treatment from non-sterilized soil experiments were cut in half. The nodules were immersed in a microtiter plate containing the *gus*-assay solution [20 mg/ml 40 μ l X-Gluc in N, N-Dimethylformamide, 20 mg SDS, 2 ml Methanol, 1 M sodium phosphate buffer (0.2 ml) and distilled water 7.76 ml], in vacuum for 2 h before incubated for overnight at 28°C. Nodule formation by the inoculated transconjugant *B. japonicum* USDA 110 was compared with those by normal *B. japonicum* USDA 110 (wt) and competitiveness was compared by non-inoculated control. Nodulation occupancy was calculated by percent nodulation formed by *gus*-marked USDA 110. Results were statistically analyzed by analysis of variance (ANOVA) and least significant different (LSD) test was applied at 0.05 level of significant. Root nodules from sterilized conditions were also stained by *gus*-buffer to calibrate the *gus*-activity expression in the soybean nodules.

Total community DNA extraction and denaturing gradient gel electrophoresis (DGGE) analysis

For soil microbial (eubacterial) community structure analysis by DGGE method, the sampling was done without disturbing the root system and the rhizosphere soil samples were taken at weekly interval for five times including at the day of sowing until one month after inoculation, that is at 0, 1st, 2nd, 3rd and 4th weeks after inoculation. Total genomic DNA of *B. japonicum* strain USDA 110 and *Azospirillum* sp. were extracted (Prakamhang et al., 2009) and kept at -20°C before using as markers for next experiments. Soil rhizosphere microbial DNA from plant samples was directly extracted from 0.5 g rhizosphere soil using with the Ultra Clean soil DNA kit (MO BIO Laboratories, Solana Beach, California, USA), following the manufacturer's instructions. Group-specific PCR-amplification of eubacterial 16S rRNA gene fragments (V6-V8 variable regions of the 16S rRNA gene) which yielded the products

Table 1. Co-inoculation effects of *B. japonicum* (CB 1809 and USDA 110) and selected PGPR on soybean nodulation and plant growth under pot conditions at 45 DAI (June to July, 2011).

Treatment	Nodule number per plant	Nodule dry weight per plant (mg)	Biomass dry weight per plant (mg)	Root dry weight per plant (mg)
Non-inoculated control	0.00±0.00 ^b	0.00±0.00 ^e	812.20±35.84 ^c	217.80±8.43 ^c
<i>Azospirillum</i> sp.	0.00±0.00 ^b	0.00±0.00 ^e	1054.35±111.27 ^c	202.48±6.43 ^c
CB 1809	84.50±4.21 ^a	237.37±27.98 ^b	4598.30±387.70 ^{ab}	611.60±53.98 ^{ab}
USDA 110	78.75±19.20 ^a	248.03±29.38 ^{ab}	3873.68±327.09 ^b	506.30±24.03 ^b
CB 1809 + <i>Azospirillum</i> sp.	112.50±10.90 ^a	264.25±30.54 ^{ab}	4892.85±305.24 ^a	640.63±59.35 ^a
USDA 110+ <i>Azospirillum</i> sp.	90.50±7.82 ^a	346.90±34.35 ^a	5289.80±666.61 ^a	712.33±46.97 ^a

Values followed by the same letter within the same columns are not significantly different by Duncan's multiple range test ($P \leq 0.05$).

of approximately 400 base pairs (Heuer et al., 1997) was done followed by using universal primers F984 GC and R1378. A GC-clamp (Costa et al., 2005) was added to the 5' end of the forward primer. The reaction mixture and PCR conditions were conducted along with the protocol of Piromyou et al. (2011). Aliquots (3 µl) of the amplification products were analyzed first by electrophoresis in 1% agarose gels and quantified using a 1 kb ladder marker and PCR products were stored at -20°C before DGGE analysis.

The PCR products of inoculated bacteria and those of soil bacterial community were subjected separately to DGGE analysis. DGGE was performed using a Dcode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA). About 45 µl of PCR products were loaded onto 6% (w/v) polyacrylamide (Acrylamide: Bisacrylamide ratio, 37.5:1 Bio-Rad Laboratories, Inc.), and 1 mm thick (20 x 20 cm) gel in TAE buffer. The polyacrylamide gel was prepared with a linear denaturing gradient ranging from 40 to 70% (urea and formamide). A 100% denaturant consisted of 40% (v/v) formamide and 7M urea. PCR products of the rhizosphere soil eubacterial community were loaded in the middle lanes and those of inoculated bacteria were loaded at both the left and right sides of the sample lanes as "Marker bacteria". DGGE was conducted at a constant voltage of 75 V for 10 min and thereafter 110 V for 18 h and maintained at 60°C. Subsequently, the gel was stained with SYBR Green (3 µl in 15 µl 1x Tris acetate EDTA (TAE) buffer) for 30 min and rinsed for 3 min in running water before photographing.

Sequencing of DGGE bands

The microbial community composition in DGGE gel was analyzed by cloning and partial sequencing of the 16S rRNA genes. Bands of interest in DGGE gels were carefully excised from the UV illuminated acrylamide gels by sterilized pipette tip (10 µl) and DNA was eluted from the excised gel by incubation in 30 µl ddH₂O at 4°C overnight. Eluted DNA (~0.5 µl supernatant) was used as a template DNA for PCR amplification as described above by using with the same primer pair without GC-clamp, F984 and 1378 R for bacterial 16S rRNA genes amplifications. The purified PCR products were ligated into the pGEM®-T Easy Vector System (Promega, USA) and then further transformed into *E. coli* DH5α competent cells, following the manufacturer's protocol. PCR amplification and DNA sequencing was performed by MACROGEN Company (Korea). Sequences were generated and the most closely related sequences were obtained from the NCBI database.

Statistical analyses

The experimental data of nodulation and plant growth parameters were statistically analyzed according to Stell et al. (1980), and

means were compared by Duncan's multiple range test (Duncan, 1955). The cluster analysis and dendrogram generation of DGGE fingerprint profiles, and principle component analysis (PCA) were carried out by the NTSYSpc (2.2, Exeter Software, USA) (Rohlf, 2000). The Shannon index (H') (Shannon and Weaver, 1963) was calculated according to the following equation:

$$H' = -\sum P_i \log P_i$$

Where, P_i is the proportion represented by each DGGE band relative to the total number of bands. The indices obtained were statistically analyzed as described for other univariate data.

RESULTS

Co-inoculation effects of *B. japonicum* and *Azospirillum* sp. on soybean under indigenous soybean nodulating rhizobia non-established soil

No nodule formation was observed in both MPN plant infection count (data not shown) and pot experiment. Increases in number of nodule and nodule dry weight were observed by both co-inoculations although those were not significantly different from bradyrhizobial single inoculation (Table 1). Positive responses on shoot and root dry weights of soybean were obtained by co-inoculation of *Azospirillum* sp. with either of USDA 110 or CB 1809 (Figure 1). Combined inoculation of USDA 110 and *Azospirillum* sp. gave the maximum shoot and root dry weight and that was significantly higher than USDA 110 inoculation alone. Shoot and root growth was increased from 4.77 to 6.51 and from 2.32 to 3.27 times over non-inoculated control, respectively. Although co-inoculation of CB 1809 with *Azospirillum* sp. promoted the nodulation and plant growth, it gave less benefit compared to those of USDA 110 and *Azospirillum* sp. co-inoculation.

Rep-PCR amplification and genetic marking of *B. japonicum* strain

Comparison between the BOX-PCR fingerprints of two bradyrhizobia, CB 1809 and USDA 110 showed dif-



Figure 1. Co-inoculation effects of *B. japonicum* and *Azospirillum* sp. on soybean plant growth under indigenous soybean nodulating rhizobia non-established soil. **A.** Non-inoculated control. **B.** *Azospirillum* sp. inoculation alone. **C.** USDA 110 inoculation alone. **D.** Co-inoculation of USDA 110 and *Azospirillum* sp., **E.** CB 1809 inoculation alone. **F.** Co-inoculation of CB 1809 and *Azospirillum* sp.

ferences in banding patterns (Figure SF 1). The previous results from pot experiment revealed that *Azospirillum* sp. co-inoculated with *B. japonicum* strain USDA 110 gave higher shoot and root dry weight than with CB 1809. Therefore, only USDA 110 was selected for further studies.

Competition for nodule occupancy analysis in rhizobia-established Myanmar and Thailand soils

Plant infection test used to assess the presence of indigenous soybean-nodulating bradyrhizobial populations in tested soil samples showed that both Myanmar and Thailand soils had indigenous soybean rhizobial population (3.1×10^6 and 1.7×10^5 cells/g dry soil, respectively). Under sterilized conditions, USDA 110 (tr) gave 100% nodule occupancy on soybean. Moreover, soybean inoculated with USDA 110 (tr) produced similar nodule number and biomass compared to those of the unmarked USDA 110 (wt) strain in both sterilized growth media and un-sterilized soil conditions (Tables 2, 3 and 4).

Under sterilized growth media conditions, significant differences in nodulation were observed among the treatments. Co-inoculation of USDA 110 (tr) with *Azospirillum* sp. (10^7 cfu/ml) gave significantly highest nodule number (Table 2). Maximum and significantly

highest biomass dry weight was given by co-inoculation of USDA 110 (tr) and *Azospirillum* sp. (10^8 cfu/ml).

All of the inoculation treatments increased in nodule number and nodule dry weight compared to non-inoculated control in both Myanmar and Thailand soils (Tables 3 and 4). Although when soybean seeds were inoculated singly with PGPR strain *Azospirillum* sp., the plants showed different responses on growth, root development, and increased the number of nodules compared to non-inoculated control in both tested soils. The root length measured by using "Comair Root Measurement Scanner" clearly showed the positive response to the inoculation of *Azospirillum* sp. In rhizobia-established Myanmar soil, co-inoculation of *B. japonicum* strain USDA 110 (tr) with *Azospirillum* sp. in 10^8 cfu/ml gave maximum nodule formation and it was significantly different compared to the non-inoculated control. Combined inoculation of USDA 110 (tr) with *Azospirillum* sp. (10^8) gave the maximum enhancement of soybean nodulation and plant growth followed by 10^6 and/or 10^7 cfu/ml of *Azospirillum* sp. In rhizobia-established Thailand soil, co-inoculation of USDA 110 (tr) with different tested inoculum levels of *Azospirillum* sp. (10^6 - 10^8 cfu/ml) gave significantly higher nodule formation and biomass dry weight compared to those of non-inoculated control.

Each of all tested inoculum levels that is 10^6 , 10^7 , and 10^8 cfu/ml of *Azospirillum* sp. enhanced nodulation in com-

Table 2. Single and co-inoculation of *B. japonicum* and *Azospirillum* sp. on soybean nodulation in sterilized growth media. *Azospirillum* sp. inoculation on soybean in rhizobia-established Myanmar soil (30 DAI).

Treatment	Nodule number per plant	Nodule dry weight per plant (mg)	Biomass dry weight per plant (mg)
Control	0.00±0.00 ^d	0.00±0.00 ^c	241.30±53.70 ^d
<i>Azospirillum</i> sp. (10 ⁸)	0.00±0.00 ^d	0.00±0.00 ^c	249.52±19.68 ^d
USDA 110 wt (10 ⁸)	12.67±2.09 ^c	30.75±1.28 ^{ab}	517.20±34.64 ^{bc}
USDA 110 tr (10 ⁸)	18.33±1.45 ^c	27.65±0.80 ^b	449.15±18.14 ^c
USDA 110 tr (10 ⁸)+ <i>Azospirillum</i> sp. (10 ⁶)	32.00±2.08 ^b	27.03±3.68 ^b	494.13±91.38 ^{bc}
USDA 110 tr (10 ⁸)+ <i>Azospirillum</i> sp. (10 ⁷)	44.83±4.91 ^a	39.40±5.38 ^a	662.38±87.50 ^c
USDA 110 tr (10 ⁸)+ <i>Azospirillum</i> sp. (10 ⁸)	32.50±1.32 ^b	42.08±1.52 ^a	873.92±49.84 ^a

Values followed by the same letter within the same columns are not significantly different by Duncan's multiple range test ($P \leq 0.05$).

ination with *B. japonicum* USDA 110 with a corresponding increase in 73.80, 62.25 and 95.34%; and 51.52, 62.38 and 79.46% over non-inoculated control in Myanmar and Thailand soil, respectively. Overall, the results obtained in the present study clearly indicate that all of the tested inoculum levels of PGPR *Azospirillum* sp. influenced the biomass development and nodulation when co-inoculated with *B. japonicum* USDA 110 (10⁸ cfu/ml). In terms of nodulation occupancy in rhizobia-established Myanmar soil, 93.21 to 94.75% of the nodules were occupied by *gus*-marked *B. japonicum* USDA 110 when inoculated singly or combination with and percent occupancies were not significantly different among them. However, in rhizobia-established Thailand soil, significant differences in competitive abilities of *gus*-marked *B. japonicum* were observed in a range of 74.21 to 100% nodule occupation when co-inoculated with different inoculum levels of *Azospirillum* sp. In addition, co-inoculations gave 23.50 to 41.95% and 50.37 to 73.24% biomass dry weight over non-inoculated control in rhizobia-established Myanmar and Thailand soil, respectively.

DGGE analysis (rhizobia-established Myanmar soil and Thailand soil samples)

There were 22 to 39 bands observed in 16S rRNA eubacterial community profiles of Myanmar soil generated by DGGE analysis and it was clearly classified into two main groups with 68% similarities (Figure SF 2) by cluster analysis. The first cluster included ~73% similarity of week zero bulk soil sample, 1st, and 3rd week samples, and the second cluster included the 2nd and 4th week samples with ~75% similarity. Except from the 4th week samples, bulk soil samples from other sampling times were clearly separated from inoculated and non-inoculated samples. In the case of Thailand soil, 12 to 20 bands were observed in DGGE fingerprints of different sampling times which were 76 to 100% similarities and grouped into two main clusters with 76% similarity, that is

the first cluster included week 0, 1st, 2nd and 3rd week samples with 80% similarity and the second included only 4th week samples (Figure SF 3). The presence of 100% similarity among the DGGE patterns of different treatments indicated that the eubacterial community structures were not significantly shifted by bacterial inoculation.

Principal component analysis (PCA) separated the DGGE profiles of both Myanmar and Thailand soil samples into four groups. It was gradually and continuously changed from the 1st week to last week sampling in Myanmar soil samples (Figure 2). However, in the case of Thailand soil samples, there were two groups: those were not clearly separated between 1st week and 3rd week samples (Figure 3).

Prominent DGGE bands were excised for nucleotide sequence determination. Four bands were presented in nearly all profiles in Myanmar soil samples were *Bacillus cecembensis* (JX. 290163), *Azotobacter nigricans* (JX. 290160), *Bradyrhizobium elkanii* (JX. 290163) and *Burkholderia* sp. (JX. 290164) had 99, 98, 100 and 95% similarity, respectively. In Thailand soil samples, two prominent bands were sequenced to be *Bradyrhizobium* sp. (NR. 0417851) and *Nitrospira moscoviensis* (JX. 290162) with 100 and 99% similarity, respectively.

DISCUSSION

In this study, it was indicated that increasing nodule number, nodule dry weight, and root dry weight given by single or co-inoculation either of *B. japonicum* strain CB 1809 or USDA 110 with *Azospirillum* sp. support plant growth of soybean. However, USDA 110 was different from CB 1809 when compared by Rep-PCR. CB 1809 produced better nodulation but less plant growth compared to USDA 110 and its co-inoculation with *Azospirillum* sp. Therefore, USDA 110 was selected to be a promising strain for further co-inoculation studies with *Azospirillum* sp. on competition for nodulation against indigenous bradyrhizobia because more consistent re-

Table 3. Competitive ability, nodulation efficiency, and plant growth enhancement of *gus*-marked *B. japonicum* strain USDA 110 and *Azospirillum* sp. inoculation on soybean in rhizobia-established Myanmar soil (30 DA).

Treatment	Nodule number per plant	Nodule occupancy by <i>gus</i> -marked USDA 110 (%)	Nodule dry weight (mg)	Plant height per plant (cm)	Biomass dry weight per plant (mg)	Root length per plant (m)
Control	19.31±3.60 ^b	-	28.90±7.11 ^b	30.33±0.75 ^d	568.20±41.69 ^c	15.85±1.74 ^b
<i>Azospirillum</i> sp. (10 ⁸)	25.67±5.78 ^{ab}	-	31.82±7.73 ^b	29.75±0.36 ^d	593.22±29.27 ^{bc}	29.90±2.94 ^{ab}
USDA 110 wt (10 ⁸)	30.21±3.63 ^{ab}	-	41.36±5.11 ^{ab}	32.50±0.73 ^{cd}	701.70±35.62 ^{ab}	24.28±1.00 ^{ab}
USDA 110 tr (10 ⁸)	34.58±2.52 ^{ab}	94.50±3.67 ^a	33.71±3.33 ^b	30.25±0.70 ^d	705.12±39.26 ^{ab}	29.58±7.70 ^{ab}
USDA 110 tr (10 ⁸)+ <i>Azospirillum</i> sp. (10 ⁶)	29.11±3.36 ^{ab}	96.31±1.87 ^a	44.93±5.25 ^{ab}	36.58±1.14 ^{ab}	755.28±61.45 ^a	38.67±7.68 ^a
USDA 110 tr (10 ⁸)+ <i>Azospirillum</i> sp. (10 ⁷)	31.33±3.51 ^{ab}	93.21±3.92 ^a	39.80±4.68 ^{ab}	34.25±0.76 ^{bc}	748.16±40.18 ^a	32.38±4.71 ^a
USDA 110 tr (10 ⁸)+ <i>Azospirillum</i> sp. (10 ⁸)	37.72±6.67 ^a	94.75±2.24 ^a	58.00±6.98 ^a	38.83±2.34 ^a	806.58±30.32 ^a	32.43±2.12 ^a

Values followed by the same letter within the same columns are not significantly different by Duncan's multiple range test ($P \leq 0.05$).

Table 4. Competitive ability, nodulation efficiency, and plant growth enhancement of *gus*-marked *B. japonicum* strain USDA 110 and *Azospirillum* sp. inoculation on soybean in rhizobia-established Thailand soil (30 DA).

Treatment	Nodule number per plant	Nodule occupancy by <i>gus</i> -marked USDA 110 (%)	Nodule dry weight (mg)	Plant height per plant (cm)	Biomass dry weight per plant (mg)	Root length per plant (m)
Control	20.79±4.10 ^c	-	26.15±5.68 ^b	29.30±1.86 ^c	502.85±24.55 ^b	14.25±1.18 ^c
<i>Azospirillum</i> sp. (10 ⁸)	25.28±2.67 ^{bc}	-	32.05±2.82 ^{ab}	35.25±2.09 ^b	690.89±39.60 ^a	17.18±3.41 ^{bc}
USDA 110 wt (10 ⁸)	31.87±3.41 ^{ab}	-	33.02±2.35 ^{ab}	37.75±2.22 ^{ab}	761.82±43.08 ^a	21.03±1.90 ^a
USDA 110 tr (10 ⁸)	28.71±3.69 ^{abc}	86.77±3.46 ^{ab}	37.68±2.22 ^a	35.67±2.36 ^b	756.14±86.09 ^a	15.63±1.09 ^{bc}
USDA 110 tr (10 ⁸)+ <i>Azospirillum</i> sp. (10 ⁶)	31.50±3.48 ^{ab}	74.21±4.60 ^b	34.68±3.14 ^{ab}	43.08±1.91 ^a	805.92±75.16 ^a	16.45±0.34 ^{bc}
USDA 110 tr (10 ⁸)+ <i>Azospirillum</i> sp. (10 ⁷)	33.75±3.48 ^{ab}	100.00±0.00 ^a	33.02±4.24 ^{ab}	43.08±1.65 ^a	836.03±67.43 ^a	19.88±2.15 ^{abc}
USDA 110 tr (10 ⁸)+ <i>Azospirillum</i> sp. (10 ⁸)	37.31±4.64 ^a	95.38±2.75 ^{ab}	42.83±2.56 ^a	40.17±1.66 ^{ab}	871.13±56.98 ^a	24.56±0.61 ^a

Values followed by the same letter within the same columns are not significantly different by Duncan's multiple range test ($P \leq 0.05$).

sults are necessary for the commercial development of inoculants with *Azospirillum* (Fages, 1994).

Generally, indigenous soybean nodulating rhizobia were established in most of the soybean growing fields either with effective or ineffective N₂-fixation ability. Plant infection test from soil samples of Myanmar and Thailand soybean growing fields revealed the presence of high popula-

tions of indigenous soybean-nodulating bradyrhizobia. Shamseldin and Werner (2004) mentioned that in major soybean crop regions, most of the indigenous rhizobial strains ineffective in symbiosis are prioritized over the inoculation strains because of their competitiveness for population and adaptation to the environment.

Inoculation of soybean with rhizobial inoculants is a common practice in most of the soybean grow-

ing areas in Myanmar, but only a few percents of rhizobial inoculants for soybean are being produced by Department of Agricultural Research (DAR). However, since 10 years ago, there was not much information on competitive nodulation of inoculated *B. japonicum* strains against indigenous soybean rhizobia on field grown soybean in Myanmar.

To evaluate the competition for nodulation of

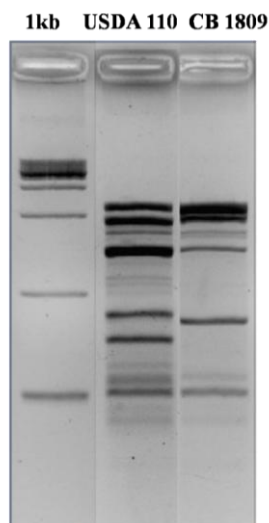


Figure SF 1. Comparison of Rep-PCR products of USDA 110 and CB 1809 with 1 kb ladder marker.

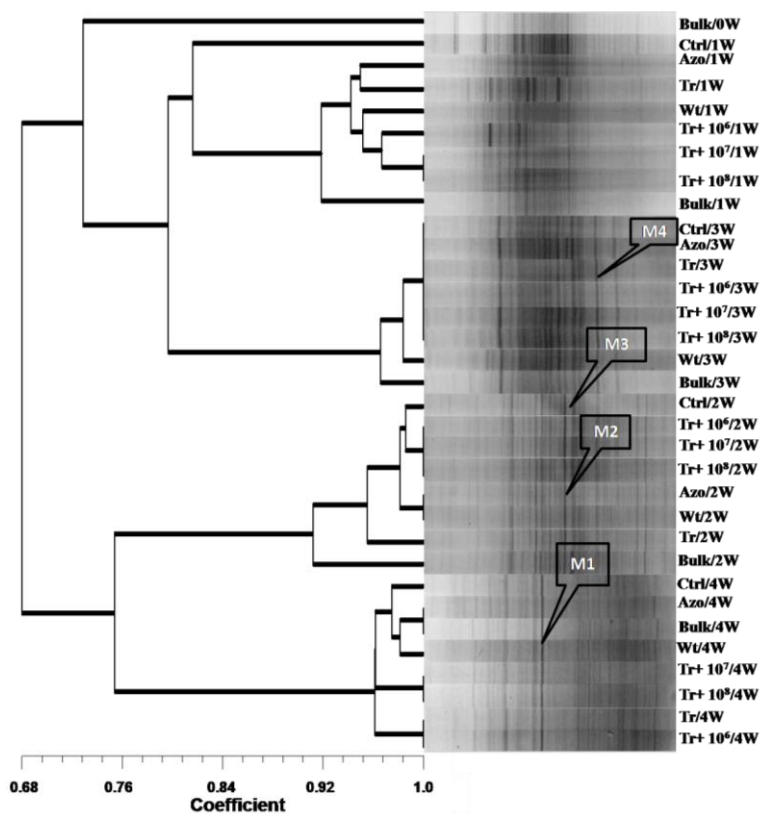


Figure SF 2. Cluster analysis of eubacterial community structures of partial 16S rRNA PCR-DGGE fingerprints of different soybean rhizosphere samples after inoculation with different bacterial inocula: bulk, bulk soil; Ctrl, control; Azo, *Azospirillum* sp.; Wt, USDA 110 wild type; Tr, *gus*-marked USDA 110; Tr + 10^6 - 10^8 , co-inoculation of *gus*-marked USDA 110 (10^8) with different inoculum levels of *Azospirillum* sp. (10^6 - 10^8) at different sampling times (0, 1st, 2nd, 3rd, and 4th weeks after inoculation) under in rhizobia-established Myanmar soil. Labels on fingerprints were subjected to sequence for analysis.

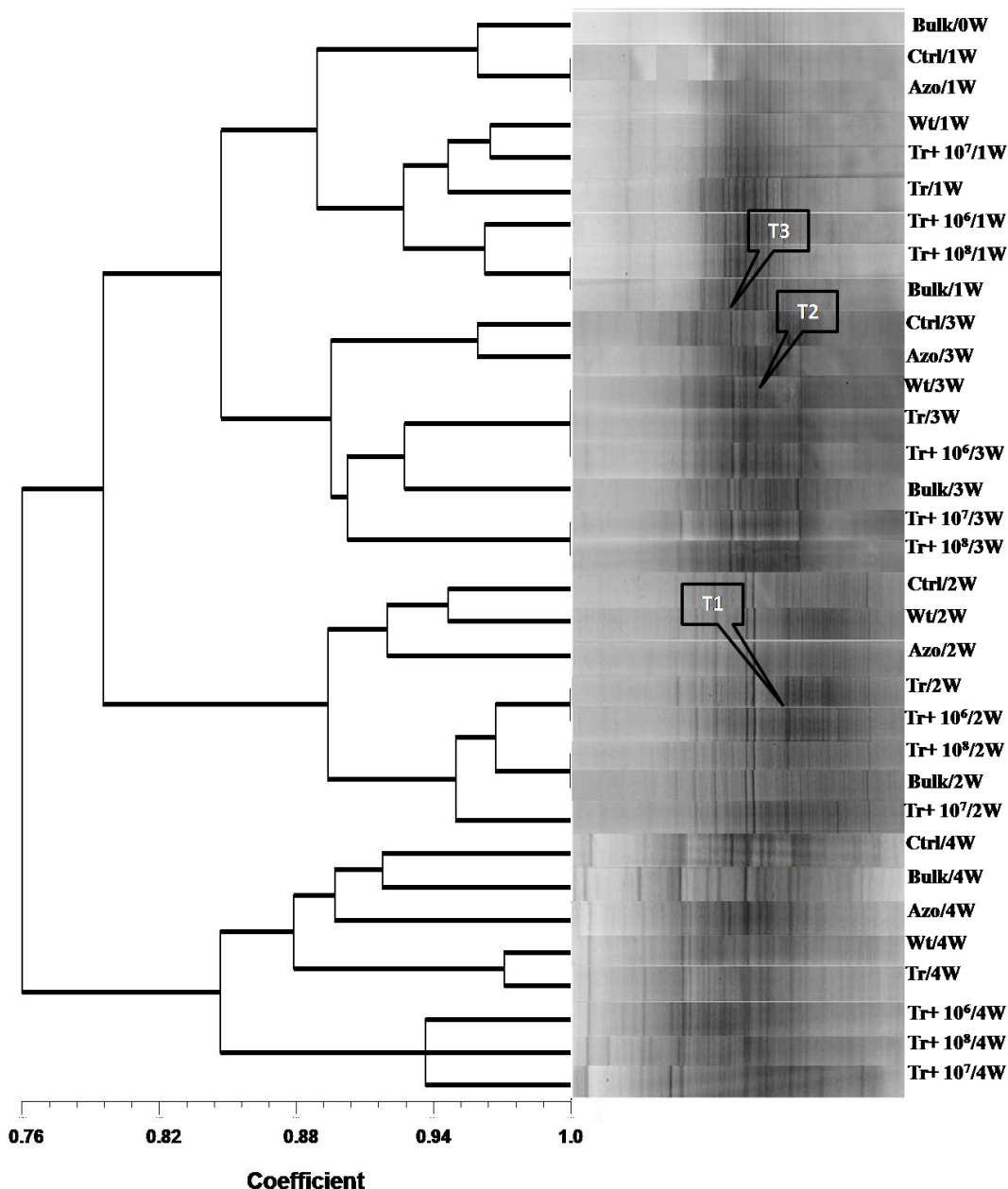


Figure SF 3. Cluster analysis of eubacterial community structures of partial 16S rRNA PCR- DGGE fingerprints of different soybean rhizosphere samples after inoculation with different bacterial inocula: bulk, bulk soil; Ctrl, control; Azo, *Azospirillum* sp.; Wt, USDA 110 wild type; Tr, *gus*-marked USDA 110; Tr + 10^6 - 10^8 , co-inoculation of *gus*-marked USDA 110 (10^8) with different inoculum levels of *Azospirillum* sp. (10^6 - 10^8) at different sampling times (0, 1st, 2nd, 3rd, and 4th weeks after inoculation) under in rhizobia-established Thailand soil. Labels on fingerprints were subjected to sequence for analysis.

USDA 110, it was genetically marked by *gus*-marker gene. In this study, 10-100 times of *B. japonicum* was used in a full dose for single inoculation or in a half dose in combination with varied PGPR populations. The results indicate that the *gus*-marker is stably inherited and detected in full percentage (100%) of soybean nodules under sterilized conditions. However, under non-sterilized conditions, nodulation occupancy was lower in Myanmar

and Thailand soils compared to sterilized conditions which indicated the competition of indigenous rhizobia. Based on data from Weaver and Frederick (1974), it can be predicted that an inoculation rate of at least 1000 times the soil rhizobial population must be used in soils if the inoculum rhizobia are to form 50% or more of the nodules. Dowdle and Bohlool (1987) also illustrated high ratios of inoculum : indigenous numbers were required to

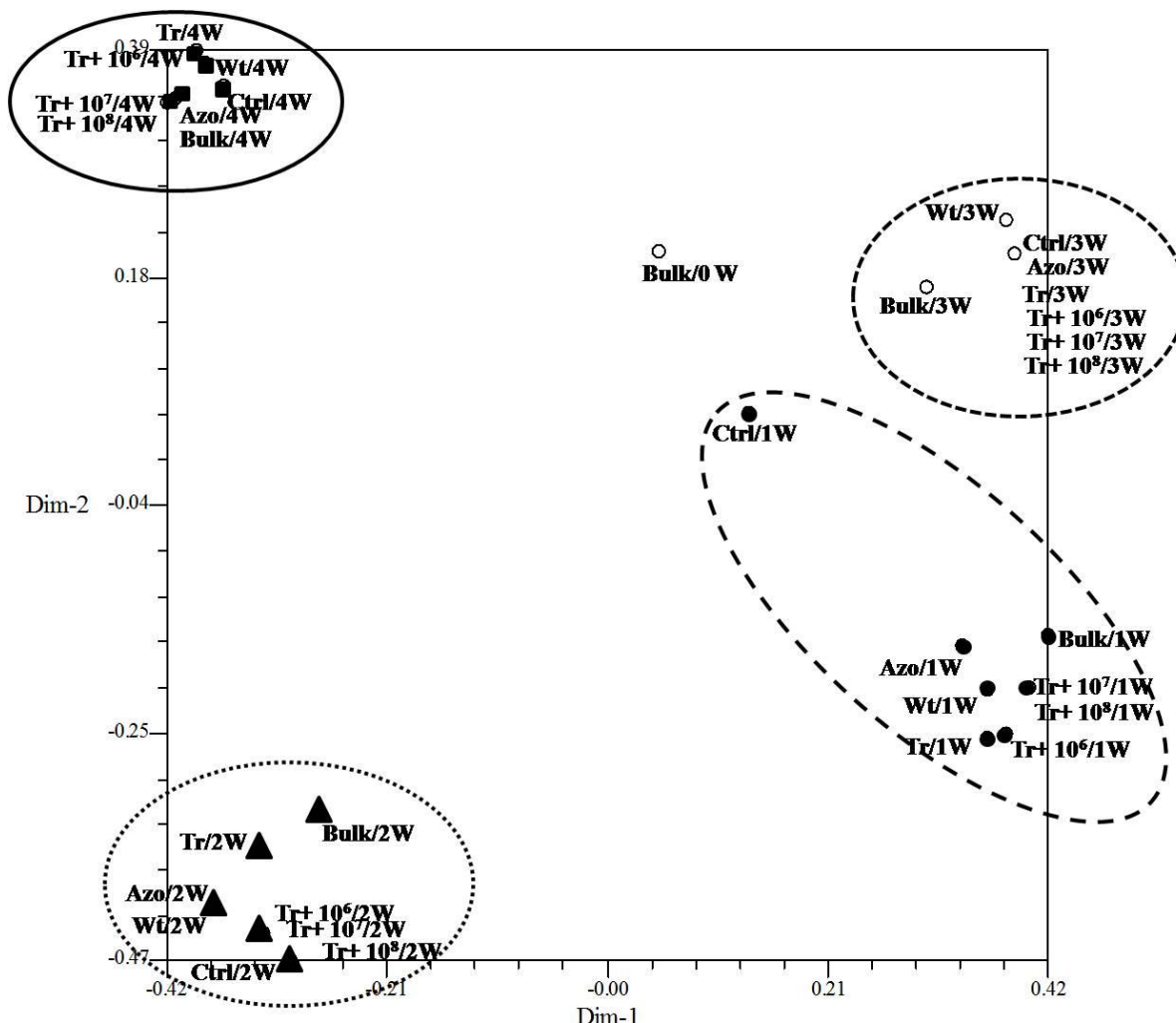


Figure 2. Community analysis derived from two-dimensional plot based on the first two principle coordinates from a principle coordinate analysis (PCA) of partial 16S rRNA banding profiles of soybean rhizosphere soil samples in rhizobia-established Myanmar soil. Letters adjacent to marks indicate the treatments: Bulk, Bulk Soil; Ctrl, Control; Azo, *Azospirillum* sp.; Wt, USDA 110 wild type; Tr, *gus*-marked USDA 110; Tr + 10^6 - 10^8 , co-inoculation of *gus*-marked USDA 110 (10^8) with different inoculum levels of *Azospirillum* sp. (10^6 - 10^8) at different sampling times represented as: 0 Wk, week zero; ●, 1st week; ▲, 2nd week; ○, 3rd week; ■, 4th week, respectively. Different samples formed a cluster which is circled; (---, , - · - · - , and —) shows a trend of 1st, 2nd, 3rd and 4th week, respectively.

displace indigenous rhizobia from nodules.

Strain USDA 110 was the predominant strain and the most competitive strain compared to USDA 138 and 136b in the nodules of all of the soybean varieties and at all of the sites (George et al., 1987). Previously, similar result was observed with high recoveries of USDA 110 (Kosslak and Bohlool, 2000). They found USDA 110 to be highly competitive against USDA 123 in vermiculite and in Hawaiian soils devoid of *B. japonicum*. USDA 110 showed higher nodulation competitiveness than the other strains of bradyrhizobia, THA 5, THA 6 and SEMIA 5019 on three of the five cultivars (Payakapoug et al., 2004). It could suggest that increase in nodule number was favored by increases in root growth that formed new root

hairs. In this study, the results demonstrate that there was a remarkable effect of PGPR *Azospirillum* sp. on enhancement of root development and nodulation by *B. japonicum* strain USDA 110. By single inoculation, although most of the parameters such as nodule number, nodule dry weight, plant height and root length of soybean plants were not significantly different from those of non-inoculated control, highly significant differences were observed in biomass dry weight in both soils.

According to investigation on co-inoculation effects, any tested level of *Azospirillum* sp. inoculum with *B. japonicum* can enhance nodulation and plant growth over non-inoculated control in both rhizobia-established soils. Positive dual inoculation effects of *Rhizobium* and

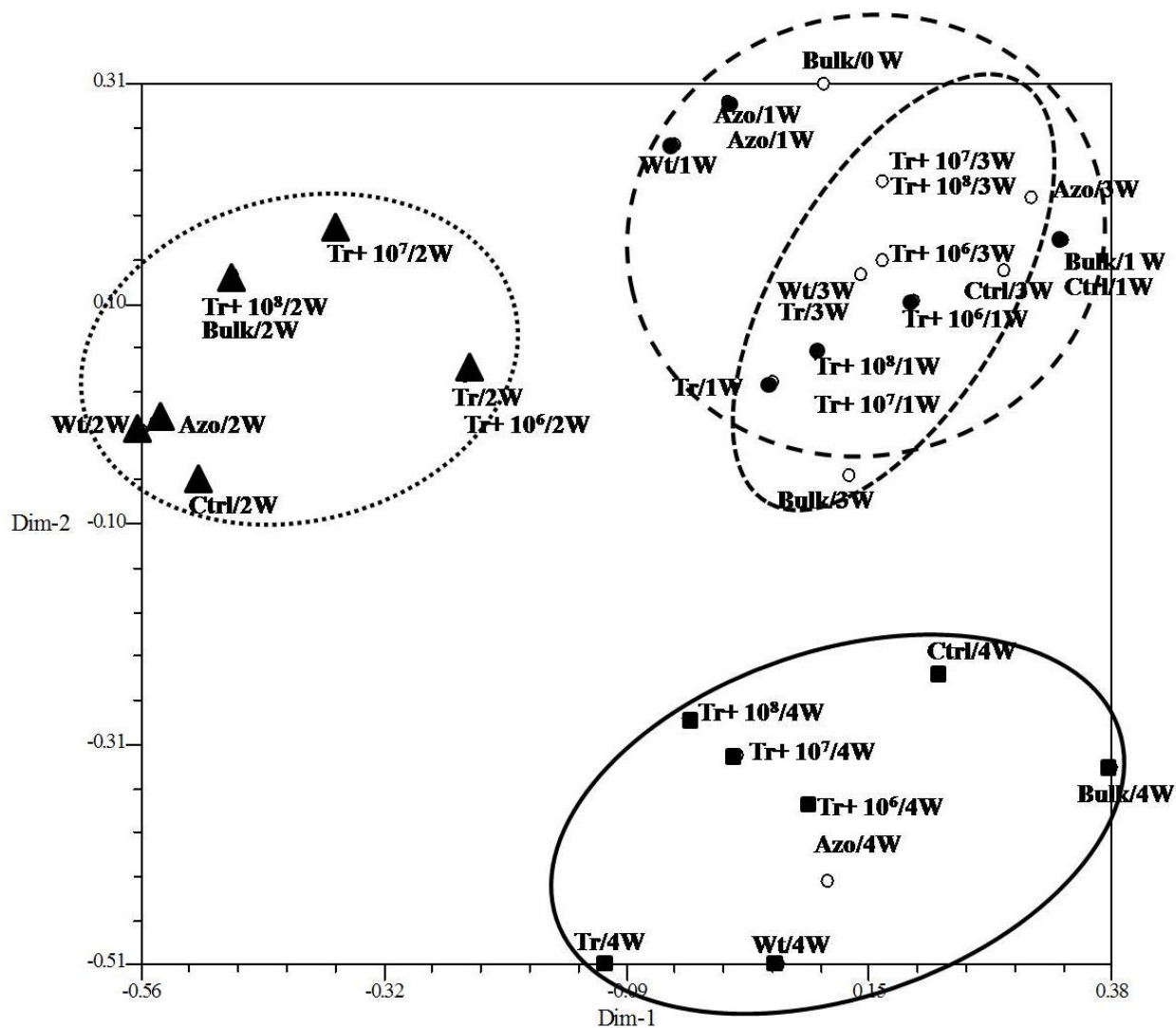


Figure 3. Community analysis derived from two-dimensional plot based on the first two principle coordinates from a principle coordinate analysis (PCA) of partial 16S rRNA banding profiles of soybean rhizosphere soil samples in rhizobia-established Thailand soil. Letters adjacent to marks indicate the treatments: Bulk, Bulk Soil; Ctrl, Control; Azo, *Azospirillum* sp.; Wt, USDA 110 wild type; Tr, *gus*-marked USDA 110; Tr + 10^6 - 10^8 , co-inoculation of *gus*-marked USDA 110 (10^8) with different inoculum levels of *Azospirillum* sp. (10^6 - 10^8) at different sampling times represented as: 0 Wk, week zero; ●, 1st week; ▲, 2nd week; ○, 3rd week; ■, 4th week, respectively. Different samples formed a cluster which is circled; (- - - , , - - - - , and —) shows a trend of 1st, 2nd, 3rd and 4th week, respectively.

Azospirillum in various legume crops were recorded by several authors (Burdman et al., 1996; Iruthayathas et al., 1983; Itzigsohn et al., 1993), and this is attributed to early nodulation, increased number of total and upper nodules (probably due to an increase in the secretion of nod gene inducer signals by roots) and higher nitrogen fixation rates (Burdman et al., 1996). The competition for nodulation is a complex phenomenon depending on soil parameters and genetic traits of both the *Rhizobium* symbiont and the host (Triplett and Sadowsky, 1992).

The numbers of DGGE bands increased with the age of soybean roots for the 1st week and 2nd week rhizosphere

samples, indicating the increase of the bacterial diversity along with the root age at the early stage of soybean growth. Moreover, the band patterns observed in bulk soil samples were even faint, noticeably effects did not occur when the clustering methods were applied, and it was evident that inoculation had no affect on soil eubacterial community structures.

In both soils, the closer related *Bradyrhizobium* genes were detected and this supports the reason of the nodulation occurring on soybean in both non-inoculated control and *Azospirillum* sp. inoculation alone. It was the predominant genus, represented in all samples, in all

sampling time and DGGE profiles. Highly distinct and high intensity bands were detected, and thus suggest that those bacteria colonized the soybean rhizosphere soil of the bacterial community. An associative N₂-fixing bacterium *Azotobacter* was found among the DGGE bands in Myanmar soil, and it can be supposed that is presented as a PGPR because the sampling field areas have been cultivated with rice, maize, and sunflower as alternative crops. Furthermore, *Burkholderia* sp. was detected in Myanmar soil. Shannon's index demonstrated that the species richness was not changed among the treatments (data not shown). Therefore, it can be supposed that changes in eubacterial communities in each sampling time were not affected by inoculation treatments.

Overall, for the DGGE profiles generated from both Myanmar and Thailand soil samples during the planting time, there were no clear differences among the treatments. The DGGE band abundances and PCA results of previously conducted pot and field experiments showed that the eubacterial communities in the soybean rhizosphere changed with the plant growth stage. Thus, it can be assumed that the single bradyrhizobial inoculation or co-inoculation with any tested levels (10⁶-10⁸ cfu/ml) of *Azospirillum* sp. do not shift the soil eubacterial communities, however, the shifting in eubacterial community observed week after week in all treatments can result from plant growth development.

Conclusions

Different inoculum levels of *Azospirillum* sp. and half ml of *B. japonicum* (10⁸ cfu/ml) can enhance and compete with nodulation against indigenous rhizobia better than single inoculation of USDA 110 alone. Therefore, the selected USDA 110 and *Azospirillum* sp. in this research are prominent bacteria that can be applied for co-inoculant formulation for soybean. In addition, prior to large scale production of co-inoculants including *B. japonicum* and *Azospirillum* sp. for soybean, on-farm competition trial is also necessary to determine the competitive ability against native strains in soybean growing areas and their effects on soil microbial community structures in Myanmar. Moreover, new high-yielding soybean cultivars are released yearly. Therefore, the competitiveness of the introduced *B. japonicum* strain to indigenous strains for nodulation should be tested because of their host-specific legume-rhizobium symbiosis.

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