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Enhanced soybean biomass by co-inoculation of *Bradyrhizobium japonicum* and plant growth promoting rhizobacteria and its effects on microbial community structures

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Co-inoculation of rhizobia with plant growth promoting rhizobacteria (PGPR) plays an important role in both promotion of nodulation and plant growth of leguminous plants. In this study, rhizobacteria were screened for co-inoculation with *Bradyrhizobium japonicum* on soybean for their capacity to promote the nodulation under aseptic condition. The obtained rhizobacteria were further screened in soybean-nodulating bradyrhizobia-free soils to evaluate their co-inoculation effects on soybean nodulation, plant growth and rhizosphere soil microbial community structures. Under pot conditions, co-inoculation of either *B. japonicum* CB 1809 or USDA 110 with *Azospirillum* sp. gave more benefits in nodulation and plant growth than *Bacillus solisalsi* did. Under field conditions, *Azospirillum* sp. co-inoculation with either *B. japonicum* CB 1809 or USDA 110 gave 32.23 and 16.85% nodulation, and 26.51 and 18.83% nodule dry weight increased over single inoculation of CB 1809 and USDA 110, respectively. In addition, each co-inoculation significantly increased 23.65 and 34.92% seed yield over single inoculation of CB 1809 and USDA 110, respectively, and three to six times seed yield over non-inoculated control. Denaturing gradient gel electrophoresis (DGGE) and principle component analysis (PCA) results revealed that soybean rhizosphere eubacterial community structures in both pot and field experiments were shifted by plant growth stages but not by bacterial inoculation. In contrast, neither inoculation of tested bacteria nor plant growth stages shifted the rhizosphere soil fungal community structures.

Key words: Soybean, *Bradyrhizobium japonicum*, plant growth promoting rhizobacteria (PGPR), co-inoculation, nodulation, rhizosphere soil microbial community structures.

INTRODUCTION

Bradyrhizobium japonicum forms a symbiotic relationship with soybean (*Glycine max*) and gives an increase in nodulation which leads to increases in plant fresh weight, seed protein and seed yield. Soybean plant in symbiosis with *B. japonicum* can fix up to 200 kg N ha^{-1} $year^{-1}$ (Smith and Hume, 1987). However, not all the rhizobial inoculation gives positive response to nodulation because a variety of biotic or abiotic factors affects nodulation of

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plants. There were many approaches which tried to overcome this problem. Among them, co-inoculation of rhizobia with proper plant growth promoting rhizobacteria (PGPR) is one of the popular methods. Co-inoculation studies with PGPR and *Rhizobium*/*Bradyrhizobium* spp. have been shown to increase root and shoot biomass, nodule dry matter, nitrogenase activity, N_2-fixation and grain yield in legumes (Elkoca et al., 2008). For instance, inoculation with mixed culture of *B. japonicum* containing either *Azotobacter vinelandii* or *Azospirillum brasilense* gave increased yields in soybean (Crossman and Hill, 1987; Herschkovitz et al., 2005). Improvement in crop production of groundnut and mungbean due to *Rhizobium* and *Azotobacter* inoculation has been reported by Sethi and Adhikary (2009). *Pseudomonas fluorescens* showed the best compatiblility with *B. japonicum* among tested beneficial microorganisms (Belkar and Gate, 2012). Anandaraj and Leema (2010) reported that bacterization of green gram with the composite inoculants of *Rhizobium* sp., *Pseudomonas fluorescens* and *Bacillus megaterium* were highly beneficial in enhancing the plant growth and yield of green gram besides effecting a reduction in the cost of inorganic fertilizers. Moreover, co-inoculation of phosphate solubilizing bacteria (PSB) *Pseudomonas* sp. and *B. japonicum* (TAL 379) significantly increased nodulation, plant total N, P uptake, seed yield and yield components of soybean over negative control and chemical fertilizers (Argaw, 2012).

Although, the inoculation of plants with PGPR may occur naturally, it is mainly an artificial agricultural procedure. To commercialize PGPR, 'effective strategies' for initial selection and screening of rhizobacterial isolates are required (Nelson, 2004) because exploitation of PGPR as biocontrol or biofertilizer inoculants has been hampered by inconsistent results at the field scale (Mark et al., 2006). Moreover, soil is considered to be the richest environment, with a high diversity of microorganisms (Fierer and Jackson, 2006), and PGPR that have been added to soil or seeds to improve plant growth and/or health will also modify the composition of the resident bacterial community of the rhizosphere.

The interaction of N_2-fixing bacteria with other bacteria can inhibit or promote their diazotrophic activity (Isopi et al., 1995). In this study, selection of effective PGPR strains which are supposed to be good strains in Thailand soil was conducted with the main purpose of co-inoculating the soybean with bradyrhizobia. In addition, the changes of microbial community structures of soybean rhizosphere by this co-inoculation under soybean-nodulating bradyrhizobia-free soil conditions were also investigated.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions

Two *B. japonicum* strains (CB 1809 and USDA 110) and a total of 200 rhizobacterial isolates including *Azotobacter* sp. and *Azospirillum* sp. were used in this study. CB 1809 was supplied by Department of Agricultural Research (DAR), Myanmar. *B. japonicum* strains USDA 110 and rhizobacterial isolates were sourced from School of Biotechnology Laboratory, Suranaree University of Technology (SUT), Nakhon Ratchasima, Thailand. Rhizobacteria used in this study were isolated from the rhizosphere soil of rice, maize and other vegetables in Thailand (Piromyou et al., 2011) whereas *Azotobacter* sp. and *Azospirillum* sp. were supplied by Department of Agriculture (DOA), Thailand. Bradyrhizobia and rhizobacterial isolates were maintained on Yeast Extract Mannitol agar (YEM) medium (Vincent, 1970) and LG (N-free) medium (Hirschi et al., 1991), respectively by periodically transferring and storing those isolates in the refrigerator for further studies.

In vitro antagonistic test between *B. japonicum* and rhizobacteria

Inhibitory activities of rhizobacteria on *B. japonicum* were determined by spot lawn method, as described by Schillinger and Lucke (1989). *B. japonicum* and rhizobacteria were cultured in YEM and LG broth, and shaken at 180 rpm at room temperature (28 ± 2°C) for seven and two days, respectively. To determine the antagonistic effects of rhizobacteria on bradyrhizobia, 100 µl of each of bradyrhizobial broth cultures (CB 1809 and USDA 110) containing 1×10^8 colony forming unit (cfu) ml^{-1} was separately spread over the surface of duplicate YEM agar plates by using cotton bud and the culture on plates were dried, then plates were incubated at 30°C for two days. Broth cultures (5 µl) of tested rhizobacterial isolates were spotted onto a lawn of bacterial cells (twelve rhizobacterial isolates $plate^{-1}$). The plates were incubated at 30°C for three to four days and examined for signs of clear inhibition zones indicating growth inhibition. Only the rhizobacteria which did not inhibit the growth of tested bradyrhizobia (*Bradyrhizobium* non-inhibitors) were selected for co-inoculation with *B. japonicum* on soybean.

Screening of rhizobacteria for co-inoculation with *B. japonicum* strains

Soybean seeds (*Glycine max*, Chiang Mai 60) obtained from Department of Agriculture (DOA), Thailand were pre-sterilized, pre-germinated and grown into the growth media (vermiculite) under aseptic conditions in sterilized Leonard's Jar (Leonard, 1943) at the rate of 3 seeds jar^{-1}. Each seed was inoculated with 1 ml bacterial culture (10^8 cells ml^{-1}) of *B. japonicum* alone (CB 1809 or USDA 110) or co-inoculated by mixing of selected rhizobacterial and bradyrhizobial cultures in a ratio of 1:1 (v/v). Non-inoculated treatment was also included as a control. Each of 157 rhizobacterial isolates (*Bradyrhizobium* non-inhibitors) were co-inoculated with tested bradyrhizobia. The experiment was laid out in a Completely Randomized Design (CRD) with three replications. Plants were cultivated on a growth shelf at 27/20°C under 16/8 h light/dark photoperiod. The N-free nutrient solution in the lower part was supplemented whenever necessary. At 21 days after inoculation (DAI), two effective rhizobacteria with better nodulation were selected for co-inoculation with bradyrhizobia on soybean under potted field soil conditions.

Characterization of selected bacteria

Acetylene reduction assay (ARA): The selected bradyrhizobia and rhizobacteria were cultured in 5 ml of LG (N-free) broth in 21 ml test tube and incubated for seven and two days, respectively at 28

± 2°C. Ten percentage (v/v) of gas phase in the headspace was replaced with acetylene and further incubated at 28 ± 2°C for 24 h, and the free-living N_2-fixing activity was examined by acetylene reduction assay (ARA) following Hardy et al. (1968). Ethylene production was measured by gas chromatograph (GC) with a flame ionization detector equipped with PE-Alumina column (50 m x 0.32 mm x 0.25 µm) (Perkin Elmer, USA). Standard curve of ethylene was constructed by varied concentration of pure ethylene following Nuntagij et al. (1997).

Indole-3-acetic acid (IAA) production

IAA production of selected bacterial strains was colorimetrically determined as described by Fukuhara et al. (1994). Pure IAA at different concentrations of 0, 10, 20, 50, 100, 150 and 200 µM were used as a standard.

After completion of ARA and IAA assays, total protein concentrations of the concerned cell suspensions were determined using Lowry's method (Lowry et al., 1951).

Identification of selected bacteria

The chromosomal DNA of the selected rhizobacterium (Isolate 3) was extracted following Prakamhang et al. (2009) and 16S rRNA gene was amplified by using the primer pair fD1 and rP2 (Weisburg et al., 1991). The resulted PCR product was purified by using the QIA quick PCR purification kit (Qiagen, Hilden, Germany) and ligated into the pGEM-T Easy Vector System (Promega, USA) for further transformation into *Escherchia coli* DH5α competent cells by following the manufacturer's instructions. DNA sequencing was performed by MACROGEN Company (Korea) and the most closely related sequences were obtained from the NCBI database.

Single and co-inoculation effects of selected rhizobacteria and *B. japonicum* strains

The experimental soils used in both pot experiment and the field experimental sites were selected from non-soybean growing area of Muang District, Nakhon Ratchasima, Thailand (14° 52' 10" N and 102° 00' 42.24" E) which had no history of any leguminous crops cultivation.

Quantification of indigenous soybean-nodulating bradyrhizobia

Soil samples were collected from 15 randomized sites of the experimental field at depth of 10 to 15 cm. The amount of indigenous soybean-nodulating bradyrhizobia present in experimental soil samples was determined by a modification of the plant infection test using the most probable-number (MPN) technique (Vincent, 1970). Plants were grown on a growth shelf at 27/20°C under 16/8 h light/dark photoperiod. MPN estimations based on nodulation were determined at three weeks after inoculation.

Pot experiment

The soils were amended with organic and inorganic fertilizers including eucalyptus compost, P_2O_5, K_2O, $CaSO_4.2H_2O$ and $CaCO_3$ at the rate of 37.50, 0.75, 0.75, 15.00 and 2.50 g kg^{-1} soil, respectively, and thoroughly mixed to ensure uniform distribution of the fertilizers. The physicochemical analysis of amended soil showed loamy sand in texture, having a pH 5.25, 0.39% organic matter, 4.03 and 34.5 mg kg^{-1} of available P and exchangeable K, respectively. Nine kilograms of amended soils were filled into pots (20 cm diameter x 20 cm height), and ten pre-sterilized and pre-germinated soybean seeds (Chiang Mai 60) were sown in each pot. *B. japonicum* strains (CB 1809 and USDA 110), *Azospirillum* sp. (AB 114190), and *Bacillus solisalsi* Isolate 3 were cultured as described before, and single or mixed bacterial broth culture was inoculated onto seed (10^8 cells ml^{-1} $seed^{-1}$). The treatments included: 1 - 4) single inoculation of each of *Azospirillum* sp., *B. solisalsi* Isolate 3, USDA 110, and CB 1809, 5 - 7) co-inoculation in 1: 1 (v/v) of CB 1809 with each of USDA 110, *Azospirillum* sp. and *B. solisalsi* Isolate 3, 7 - 9) co-inoculation in 1: 1 (v/v) of USDA 110 with each of *Azospirillum* sp. and *B. solisalsi* Isolate 3, 10-12) co-inoculation in 1: 1: 1 (v/v/v) of CB 1809, USDA 110 and either *Azospirillum* sp. or *B. solisalsi* Isolate 3, 13) combined inoculation in 1:1:1:1 (v/v) of all tested bacterial cultures, and 14) bulk soil (no planted and non-inoculated control).

The pots were laid out in a CRD design with three replications. Plants were thinned down to uniformity (six plant pot^{-1}) and watered by tap water whenever necessary. Regular agricultural practices were done except pesticide spraying. Plants were sampled and nodule number, nodule dry weight, and biomass dry weight (dried at 70°C) were recorded at 30 and 45 DAI. Statistical significance was determined by analysis of variance (Steel et al., 1980) and means were compared by the Duncan's Multiple Range Test (DMRT) ($p \leq 0.05$) (Duncan, 1955). Based on this experiment, the most effective rhizobacteria was selected to evaluate its potential under field conditions.

Field experiment

Before sowing, the field soil was fertilized with 50 kg ha^{-1} of each P_2O_5 and K_2O fertilizers. The soil was sandy soil in texture, having pH 6.41, 0.39% organic matter, and available P and exchangeable K was 4.78 and 70.64 mg kg^{-1}, respectively. Each subplot size was 2 and 3 m^2 in size with four rows. The experiment was arranged in a Randomized Complete Block Design (RCBD) with three replications. The treatments consisted of non-inoculated control, single inoculation with USDA 110, CB 1809, and *Azospirillum* sp. alone, and co-inoculated in 1:1 (v/v) ratio of *Azospirillum* sp. and each of CB 1809 and USDA 110. For each plot, bacterial inoculation was done immediately before sowing by mixing 10 ml of bacterial broth cultures with 40 to 50 g (70 kg ha^{-1}) of soybean seeds (Chiang Mai 60) to obtain approximately 10^6 bacterial cells $seed^{-1}$.

During the experiment, regular agricultural practices were done as needed. At 30, 45 and 70 DAI, five soybean plants per each plot were randomly sampled for assessment of nodulation and plant growth parameters. At 70 DAI, the dried plant materials were analyzed for dry matter and total plant nitrogen percent. Soybean yield and yield components were determined from a random sample of 10 plants from two inner rows per plot at maturity (90 DAI). Statistical significance was determined as described in pot experiment.

Denaturing gradient gel electrophoresis (DGGE) and principle component analysis (PCA) from pot and field experiments

Total genomic DNAs were extracted following Prakamhang et al. (2009) from selected bacteria which were used for inoculation in pot and field experiments and extracted DNAs were kept at -20°C before using as the marker. Both eubacterial and fungal community structures were evaluated from pot experiment and only eubacterial community structure was analyzed in field experiment at 0, 7, 14, 30 and 45 DAI. Soil microbial DNAs were directly extracted from 0.5 g rhizosphere soils by using the Ultra Clean soil DNA kit (MoBio

Laboratories, Solana Beach, Califonia, USA) following the manufacturer's instructions. Eubacterial 16S rRNA (V6-V8 variable regions, ~ 400 bp) and fungal 18S rRNA (~1,650 bp) gene fragments were amplified by using universal primers F984 and R1378 (Heuer et al., 1997) and fungus-specific primers NS1 and FR1 (Oros-Sichler et al., 2006), respectively. A GC-clamp (Costa et al., 2006) was added to the 5'end of the forward primers F984 and NS1 to prevent the complete melting of PCR products during separation in the denaturing gradient gel.

PCR products were subjected separately for DGGE analysis by using a Dcode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA). About 45 µl of PCR products were loaded onto 1 mm thick (20 x 20 cm) gel with 6% (w/v) polyacrylamide gel (37.5:1 of Acrylamide: Bisacrylamide, Bio-Rad Laboratories, Inc.) prepared with a linear denaturing gradient ranging from 40 to 70% denaturant (100% denaturant consisted of 40% (v/v) formamide and 7M urea) and 10% (w/v) polyacrylamide gel with 18 to 43% denaturant for 16S and 18S rRNA, respectively. PCR products from inoculated bacteria were loaded at both left and right sides of the sample lanes as markers.

DGGE was performed in 1x TAE buffer at 60°C with constant voltage of 75 V for 10 min and thereafter 110 V for 18 h for eubacteria PCR and at 180 V for 16 h for fungal PCR. The gels were stained with SYBR Green (3 µl in 15µl 1x TAE buffer) for 30 min and rinsed for 3 min in running water before photographing. DNAs from excised bands of interest in DGGE gels were eluted by incubation in 30 µl ddH_2O at 4°C overnight. Supernatant (~0.5 µl) was used as a template for PCR amplification as described above by using with the same primer pair without a GC-clamp. The PCR products were purified by using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) followed by sequencing and analyzing of DNA as described above.

Cluster analysis and principle components analysis (PCA) were performed according to the presence and absence of bands occurring in DGGE gels based on Unweighted Pair Group Method with Arithmetic Means (UPGMA) algorithms by the NTSYSpc (2.2, Exeter Software, USA) (Rohlf, 2000). Based on the DGGE results, 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

Antagonistic test and screening of rhizobacterial isolates for co-inoculation with *B. japonicum* strains

Totally 152 out of 195 tested rhizobacterial isolates were detected as '*Bradyrhizobium* non-inhibitors' and they were screened for co-inoculation with *B. japonicum* on soybean under controlled conditions. Among them, Isolates 1, 3, 13, and 15 showed an increase in nodule numbers when each was co-inoculated with either CB 1809 or USDA 110; however, these numbers were not significantly higher than that of individual bradyrhizobial inoculation (data not shown). Therefore, five additional rhizobacterial strains did not inhibit tested bradyrhizobia in *in vitro* cultures; namely, *Bacillus* sp. SUT 1, *Pseudomonas* sp. SUT 16 and SUT 19, which are prominent in most of the experimental research at Laboratory of School of Biotechnology, SUT (Piromyou et al., 2011), *Azotobacter* sp., and *Azospirillum* sp. which are being commercialized as PGPR inocula for various crops cultivation by Suranaree University of Technology (Teaumroong et al., 2009), were selected to be added in screening test. Out of the nine rhizobacterial isolates, co-inoculation of Isolate 3 or *Azospirillum* sp. with either CB 1809 or USDA 110 gave significantly higher nodule numbers than bradyrhizobial single inoculation, and thus those two isolates were selected for further experiments (Table 1).

Characterization of selected bacteria

Based on 16S rRNA sequence analysis, Isolate 3 was related to *Bacillus solisalsi* with 98% homology (JX 290169). This *B. solisalsi* Isolate 3 gave the significantly highest IAA production, and *Azospirillum* sp. produced higher but not significantly different amount of IAA as compared to *B. japonicum* CB 1809 and USDA 110 (Table 2). ARA results revealed that CB 1809 gave the maximum N_2-fixation in free-living bacterial stage followed by USDA 110. N_2-fixation given by CB 1809 was significantly different from those by *Azospirillum* sp. and *B. solisalsi* Isolate 3. The *B. solisalsi* Isolate 3 has the lowest N_2-fixation ability when compared with others.

Pot experiment

No nodule formation was observed in MPN plant infection counting from soil samples. In pot experiment, nodule formation was not observed in non-inoculated control and rhizobacterial inoculation alone as expected. The lowest shoot dry weight was noted in non-inoculated control.

Either single bradyrhizobial inoculation or co-inoculation with tested rhizobacteria gave the significantly highest biomass dry weight as compared to PGPR inoculation alone or non-inoculated control (Figure 1C). The nodule formation was significantly increased when *B. solisalsi* Isolate 3 was co-inoculated with CB 1809; however, a similar trend was not observed in co-inoculation with USDA 110 (Figure 1A). Maximum nodulation, nodule dry weight and biomass dry weight of soybean were accomplished by altogether combined inoculation of tested bradyrhizobia and rhizobacterial isolates (Figure 1A, B and C). Positive responses on nodule number and shoot dry weight of soybean were observed by co-inoculation of either *B. japonicum* CB 1809 or USDA 110 with *Azospirillum* sp. at 45 DAI.

DGGE and PCA analysis from pot experiment

DGGE profiles of eubacterial community structures were

Table 1. Single or co-inoculation effects of *B. japonicum* strain (CB 1809 or USDA 110) and promising rhizobacterial isolates on nodulation of soybean (Chiang Mai 60) under controlled environmental conditions at 21 DAI.

Treatment (Bacterial isolate no.)	Nodule number plant^{-1} CB 1809	Nodule number plant^{-1} USDA 110
Isolate 1	14.3^{cd} ± 2.9	14.8^{ab} ± 2.3
Isolate 3	24.0^{ab} ± 2.7	20.1^{a} ± 2.7
Isolate 13	19.6^{bc} ± 2.7	11.8^{bc} ± 1.1
Isolate 15	14.2^{cd} ± 2.6	14.8^{ab} ± 4.7
SUT 1 (*Bacillus* sp.)	26.8^{a} ± 3.3	6.2^{c} ± 2.7
SUT 16 (*Pseudomonas* sp.)	16.9^{cd} ± 4.3	9.3^{bc} ± 0.9
SUT 19 (*Pseudomonas* sp.)	14.4^{cd} ± 1.7	18.9^{a} ± 2.8
Azotobacter sp.	17.4^{cd} ± 2.7	18.8^{a} ± 3.7
Azospirillum sp.	19.2^{bc} ± 2.7	19.2^{a} ± 3.3
None (*B. japonicum* inoculation alone)	12.4^{d} ± 1.7	11.1^{bc} ± 5.3
F- test	**	**

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 2. Characterization of selected bacteria for nitrogenase activity and IAA production.

Treatment	Nitrogenase activity of free-living bacteria (nmole of ethylene mg protein^{-1} hr^{-1})	IAA (µM mg protein^{-1})
Azospirillum sp.	3.08^{c} ± 0.5	0.25^{b} ± 0.2
B. solisalsi Isolate 3	1.19^{d} ± 0.1	0.78^{a} ± 0.1
B. japonicum CB 1809	8.21^{a} ± 0.0	0.13^{b} ± 0.0
B. japonicum USDA 110	4.12^{b} ± 0.0	0.10^{b} ± 0.0
F- test	**	**

Different letters in the same column indicate significant differences among treatments ($P \leq 0.05$).

divided into two main clusters. The first cluster mainly included the samples from 0, 7 and 14 DAI samples with 78% similarity and the latter included those mainly from 30 and 45 DAI samples with 81% similarity (Supplementary Figure 1). Eubacterial community structure in bulk soil samples did not form a separate branch from the clustering tree of bacterial inoculation treatments. A clear separation of the DGGE profiles was observed at different sampling times as well as different plant growth stages in 0, 7, 14 and 45 DAI except in 30 DAI. PCA result did not provide any clear separation among treatments (Supplementary Figure 1 and Figure 2).

Banding patterns of the bulk soil and rhizosphere soil samples from 7, 14, 30 and 45 DAI also revealed that there were considerable differences among the sampling times varying from 7-23, 10-22, 13-25 and 16-27 bands, respectively. However, the Shannon diversity indices (H' values) calculated from DGGE profiles of each treatment were not different significantly from each other in each sampling time (data not shown). High recovery of the DGGE bands of the inoculated bacteria was observed at the same position of the reference markers in all plant growth stages (line 1, 2 and 3 in Supplementary Figure 1). There was only one common band that appeared in all samples that is, 100% similar to *Burkholderia* sp. (JX 290164) (PB1). Other two bands which were homologous to *Clostridium* sp. (JX 290165) (98% homology) (PB 2) and *Parasegitibacter luojiensis* (JX 290166) (95% homology) (PB 3) were observed in most of the samples.

The cluster analysis on DGGE banding profiles of 18S rRNA genes showed different but not clear effects of bacterial inoculation and sampling times on rhizosphere soil fungal community structures (Figure 3) except that they shared some 2-3 common bands (Supplementary Figure 2). Some bands were widely distributed and found in more than half of the samples. The number of bands corresponded to the number of predominant members in the microbial communities. However, most of the excised bands failed to be amplified and could not be sequenced. Two dominant bands which could be sequenced successfully were uncultured ascomycetes (JX 290170)

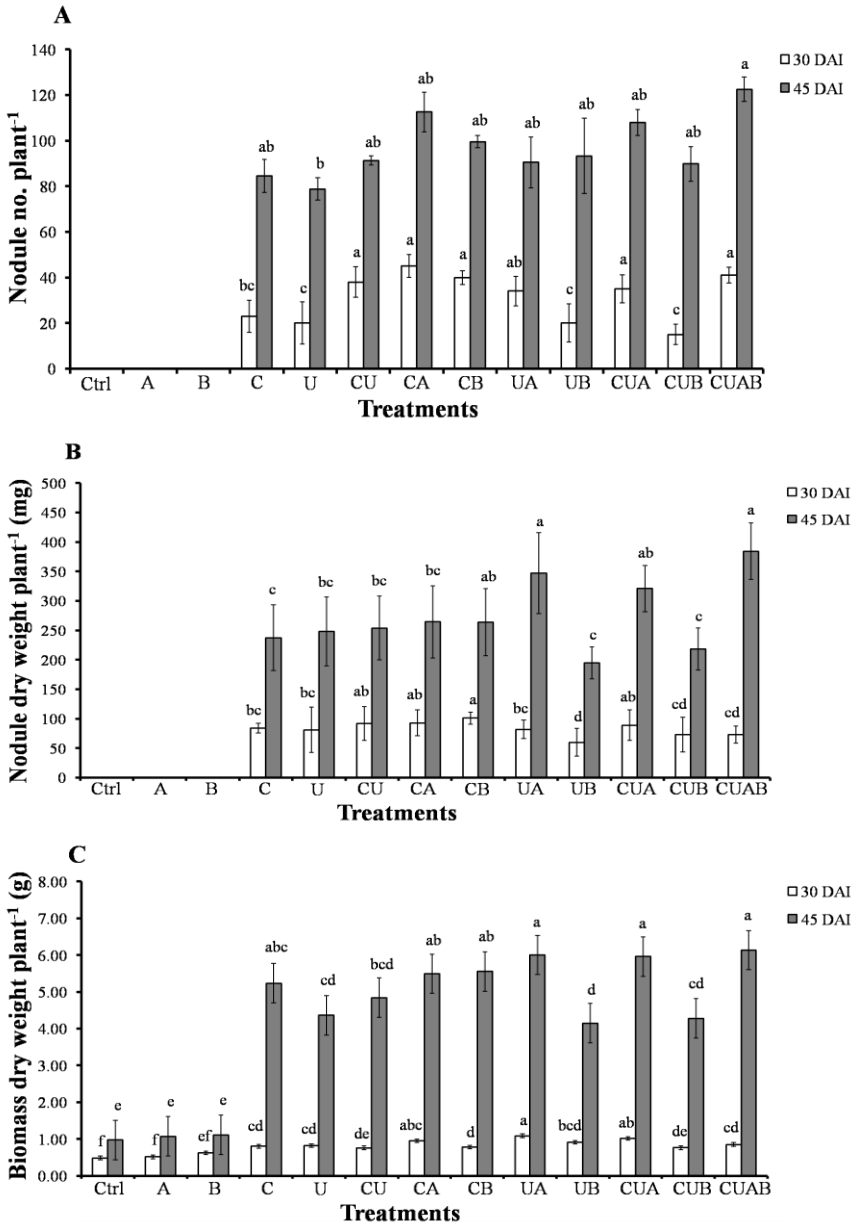


Figure 1. Co-inoculation effects of *B. japonicum* (CB 1809 and USDA 110) and selected rhizobacteria (*Azospirillum* sp. and *Bacillus solisalsi* Isolate 3) on soybean nodulation and plant growth under soybean-nodulating bradyrhizobia-free pot condition at 30 and 45 DAI. (Ctrl) Control; (A) *Azospirillum* sp.; (B) *Bacillus solisalsi* Isolate 3, (U) *B. japonicum* USDA 110; (C) *B. japonicum* CB 1809; and coupled-letters refer to co-inoculated with indicated labels. **A**. Nodule number plant^{-1}, **B**. Nodule dry weight plant^{-1} (mg), and **C**. Biomass dry weight plant^{-1} (g).

(95% homology) (PF1) and *Fusarium oxysporum* (JX 290168) (99% homology) (PF2) (Supplementary Figure 2).

Field experiment

Based on pot experiment results, *Azospirillum* sp. was selected for further study under field condition as it has nodulation and plant growth promoting ability on soybean when co-inoculated with bradyrhizobia. The soybean plants which were obtained without bradyrhizobial inoculation in field experiment and those from MPN plant-infection count were free of nodules. Single inoculation of *Azospirillum* sp. has no prominent effects on soybean

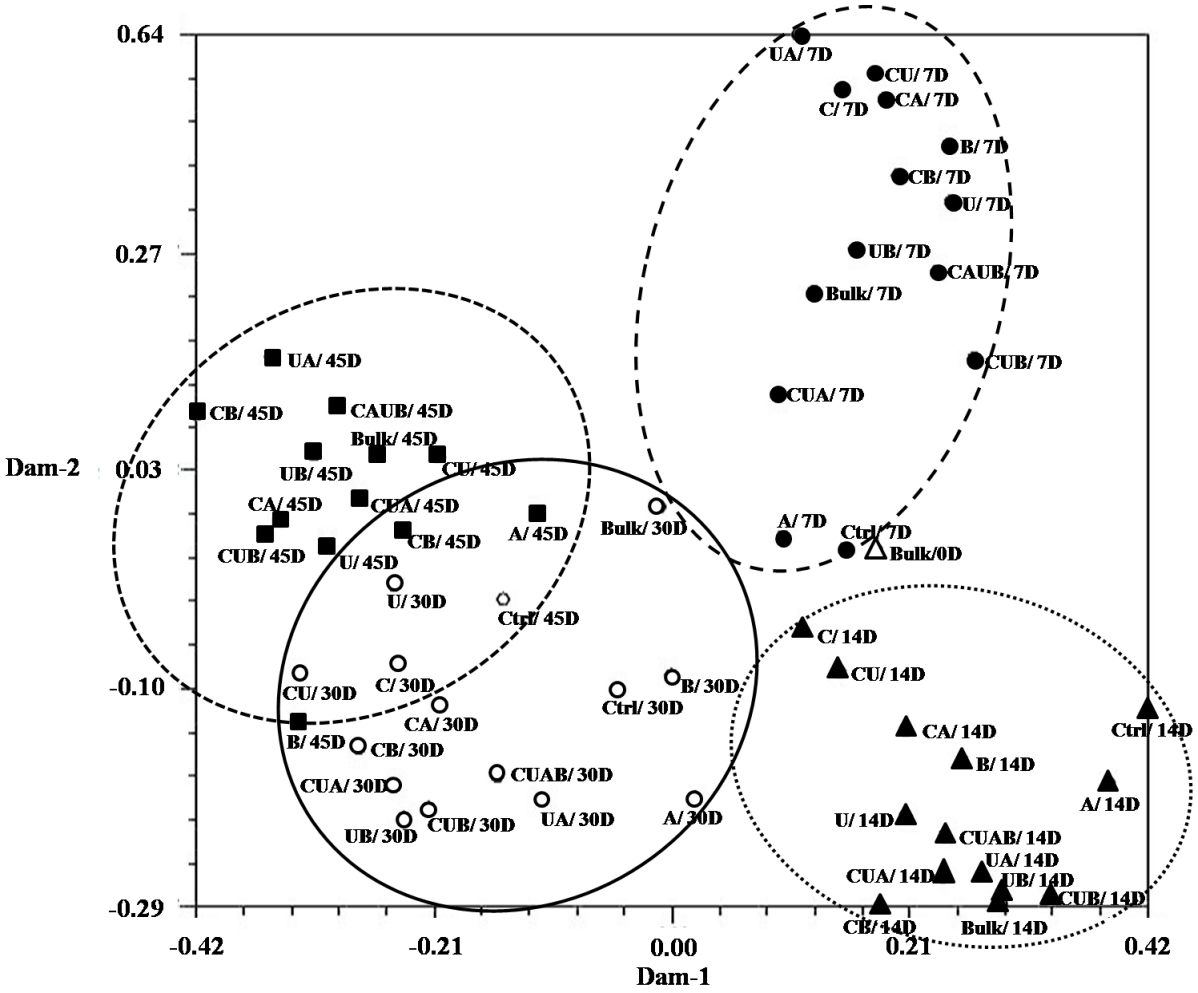


Figure 2. Community analysis derived from PCA of partial 16S rRNA banding profiles of soybean rhizosphere soil under soybean-nodulating bradyrhizobia-free pot conditions. Letters adjacent to marks indicate the treatments: (Bulk) Bulk soil; (Ctrl) Control; (A) *Azospirillum* sp.; (B) *B. solisalsi* Isolate 3 (C) *B. japonicum* CB 1809; (U) *B. japonicum* USDA 110; and coupled-letters refer to co-inoculation due to indicated labels) at different sampling times: (△) 0DAI; (●) 7DAI; (▲) 14 DAI; (o) 30 DAI and (■) 45 DAI. Different samples formed a cluster which is circled by (- - - - , ……,– – – – and ——), which shows a trend of 7, 14, 30 and 45 DAI, respectively.

plant growth as compared to non-inoculated control (Figure 4C). However, when it was co-inoculated with either CB 1809 or USDA 110, nodulation and plant growth were significantly increased when compared with non-inoculated control or *Azospirillum* sp. inoculation alone at 30, 45 and 70 DAI (Figure 4A, B, and C). Better performance in root development was observed in co-inoculation with *Azospirillum* sp. as compared to single inoculation of *B. japonicum*. Based on all sampling times, co-inoculation of either CB 1809 or USDA 110 with *Azospirillum* sp. increased 32.23 and 16.85% of nodulation and 26.51 and 18.83% of nodule dry weights over single inoculation of CB 1809 and USDA 110, respectively. Co-inoculation of USDA 110 with *Azospirillum* sp. increased ~36.99% of soybean nodulation over USDA 110 single inoculation at 45 DAI, leading to significantly higher and evident response to biomass dry weight.

Percentages of total plant nitrogen of soybean given by CB 1809 or USDA 110 inoculation and their co-inoculation with *Azospirillum* sp. were higher than those given by non-inoculated control and inoculation of *Azospirillum* sp. alone at 70 DAI (Table 3). Although, the nodules obtained by inoculation with CB 1809 or co-inoculation with *Azospirillum* sp. gave the significantly highest number and effective nodules with appearance of pink-red color inside the nodules, the plant growth were less than those in USDA 110 and its co-inoculation.

Co-inoculation of USDA 110 with *Azospirillum* sp. gave the significantly highest number of seeds plant^{-1} and higher number of pods, 100 seeds weight and seed weight plant^{-1}; however, these were not significantly

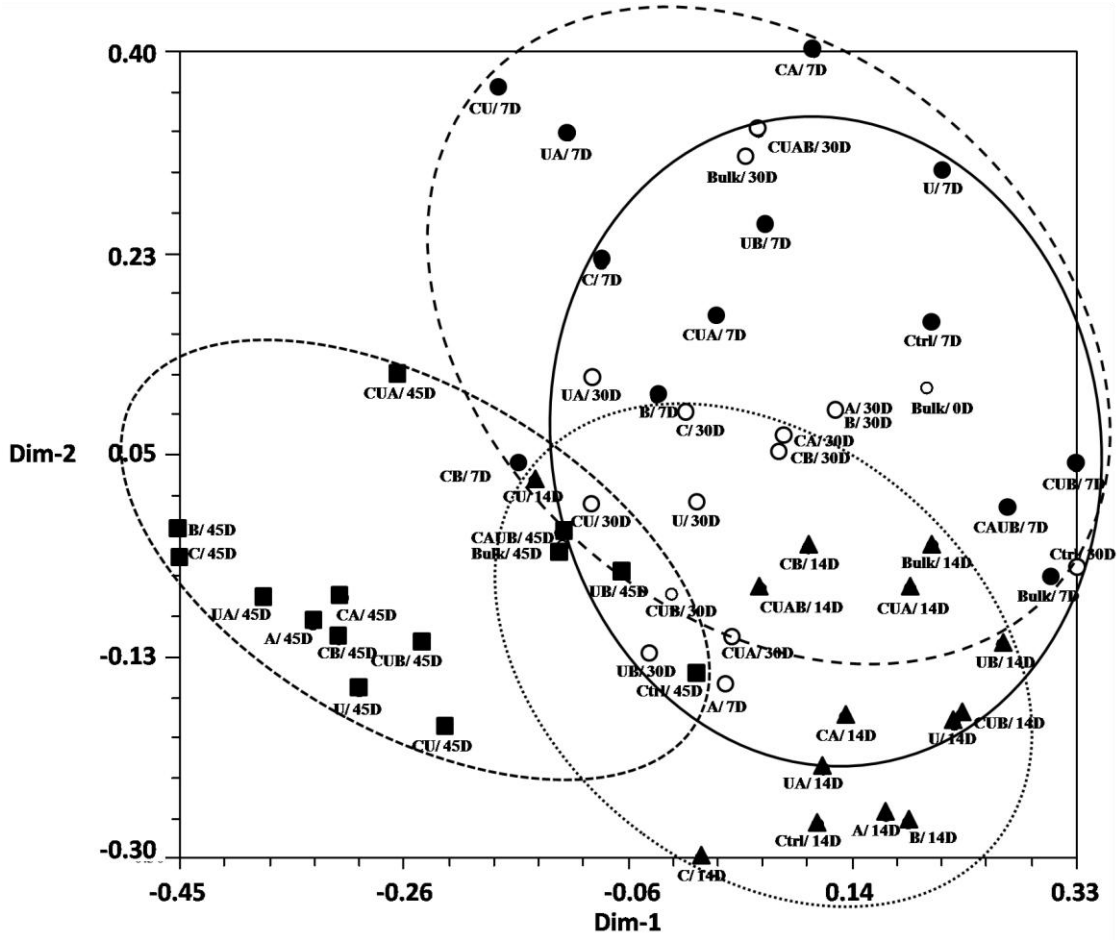


Figure 3. Community analysis derived from PCA of partial 18S rRNA banding profiles of soybean rhizosphere soil samples under soybean-nodulating bradyrhizobia-free pot conditions. Letters adjacent to marks indicate the treatments: (BS) Bulk soil; (Ctrl) Control; (A) *Azospirillum* sp.; (B) *Bacillus* sp. Isolate 3; (C) *B. japonicum* CB 1809; (U) *B. japonicum* USDA 110; and coupled-letters refer to co-inoculation due to indicated labels at different sampling times: (△) 0DAI; (●) 7DAI; (▲) 14 DAI; (o) 30 DAI and (■) 45 DAI. Different samples formed a cluster which is circled by (- - - - , ……,– – – – – and ——), which shows a trend of 7, 14, 30 and 45 DAI, respectively.

different from those of USDA 110 inoculation alone. The lowest yield (304 kg ha^{-1}) was obtained in non-inoculated control. Co-inoculation of *Azospirillum* sp. and either of CB 1809 or USDA 110 gave 23.65 and 34.92% higher seed yields over CB 1809 or USDA 110 single inoculation, respectively. Healthier and bigger seed size obtained by co-inoculation of USDA 110 and *Azospirillum* sp. gave the significantly highest yield with 1727.00 kg ha^{-1} and it was almost 5 to 6 times more yields with respect to the control plants.

DGGE and PCA analysis from field experiment

DGGE profiles of soil eubacteria community structures in the field experiment revealed two main clusters with 76% similarity; one included the samples from four sampling times (0, 7, 30 and 45 DAI), and later from the sampling times at 14 DAI (Supplementary Figure 3).

Except at 14 DAI, the DGGE patterns generated in the rhizosphere soil samples of *Azospirillum* sp. inoculated and its co-inoculation with bradyrhizobia were clearly separated into small cluster with 88 to 91% similarity at different sampling times. The detected band numbers were increased from 7 to 14 DAI and, generally, most of the treatments gave higher number of band detection at 14 DAI and decreased at later stages. PCA analysis provided the grouping of the DGGE band profiles into four main groups and the changes were influenced by plant age (Figure 5).

Sequencing of partial 16S rRNA genes from the commonly detected bands revealed that *Bacillus* sp. (JX 290163) (99% homology) (FB1) (Supplementary Figure3) was detected in all samples at all sampling times except

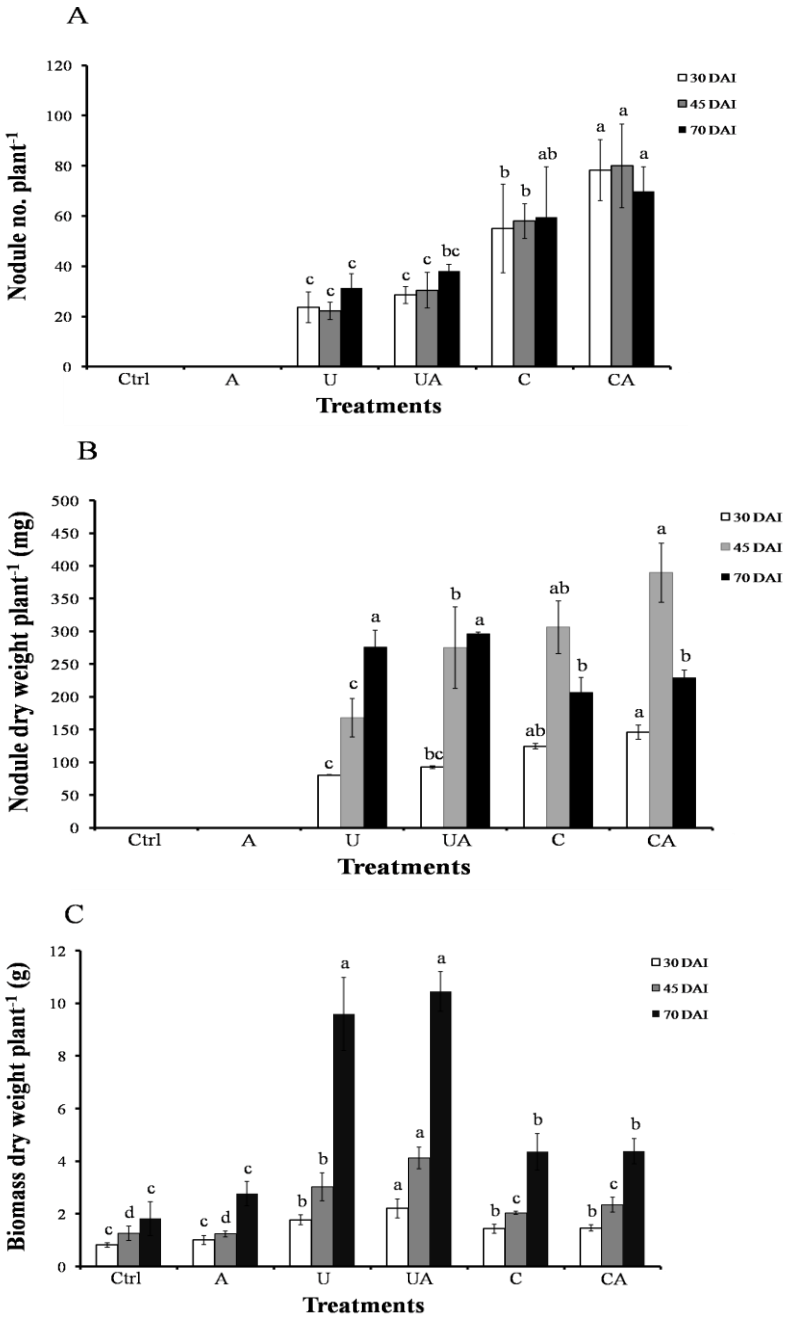


Figure 4. Co-inoculation effects of *B. japonicum* (CB 1809 and USDA 110) and *Azospirillum* sp. on soybean nodulation and plant growth under soybean-nodulating bradyrhizobia-free field conditions at 30, 45, and 70 DAI. (Ctrl) Control; (A) *Azospirillum* sp.; (B) *B. solisalsi* Isolate 3; (U) *B. japonicum* USDA 110; (C) *B. japonicum* CB 1809; and coupled-letters referr to co-inoculation related with indicated labels. **A**. Nodule number plant^{-1}, **B**. Nodule dry weight plant^{-1} (mg), and **C**. Biomass dry weight plant^{-1} (g).

the band intensities that appeared different. However, *Propionibacterium freudenreichii* (JX 290167) (95% homology) (FB2) was detected in late sampling (30 and 45 DAI) and that band seems to be propagated in the later season of soybean growing.

DISCUSSION

Compatibility of the microorganisms needs to be evaluated before they are used as co-inoculants because of the possibility of antagonistic interactions among them

Table 3. Single and co-inoculation effects of *B. japonicum* CB 1809, USDA 110 and *Azospirillum* sp. on N_2-fixation, plant growth, yield and yield components of soybean under soybean-nodulating bradyrhizobia-free field conditions.

Treatment	Number of pods plant^{-1}	Number of seeds plant^{-1}	100 seeds weight (g)	Seed weight (g plant^{-1})	Yield (kg ha^{-1})	Total N (%)
Non-inoculated control	4.7^{e} ± 0.4	7.8^{d} ± 0.3	12.7^{d} ± 1.0	0.62^{c} ± 0.5	304^{e} ± 12.6	0.45^{c} ± 0.1
Azospirillum sp. alone	5.7^{de} ± 0.5	9.2^{d} ± 1.4	13.7^{cd} ± 0.7	0.71^{c} ± 0.6	353^{e} ± 47.0	0.83^{b} ± 0.3
USDA 110 alone	10.3^{ab} ± 0.5	19.9^{b} ± 2.1	15.4^{ab} ± 0.7	2.56^{ab} ± 0.5	1280^{b} ± 62.5	1.03^{ab} ± 0.2
USDA 110 + *Azospirillum* sp.	12.3^{a} ± 2.8	27.2^{a} ± 2.9	16.5^{a} ± 0.7	3.45^{a} ± 1.3	1727^{a} ± 186.5	1.29^{a} ± 0.2
CB 1809 alone	7.2^{cd} ± 0.7	13.1^{c} ± 1.2	14.1^{bcd} ± 0.9	1.56^{bc} ± 0.1	778^{d} ± 54.6	1.25^{a} ± 0.1
CB 1809 + *Azospirillum* sp.	8.8^{bc} ± 0.9	15.0^{c} ± 0.9	15.4^{ab} ± 0.7	1.93^{bc} ± 0.2	962^{c} ± 17.4	1.07^{ab} ± 0.2
F- test	**	**	**	**	**	**

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

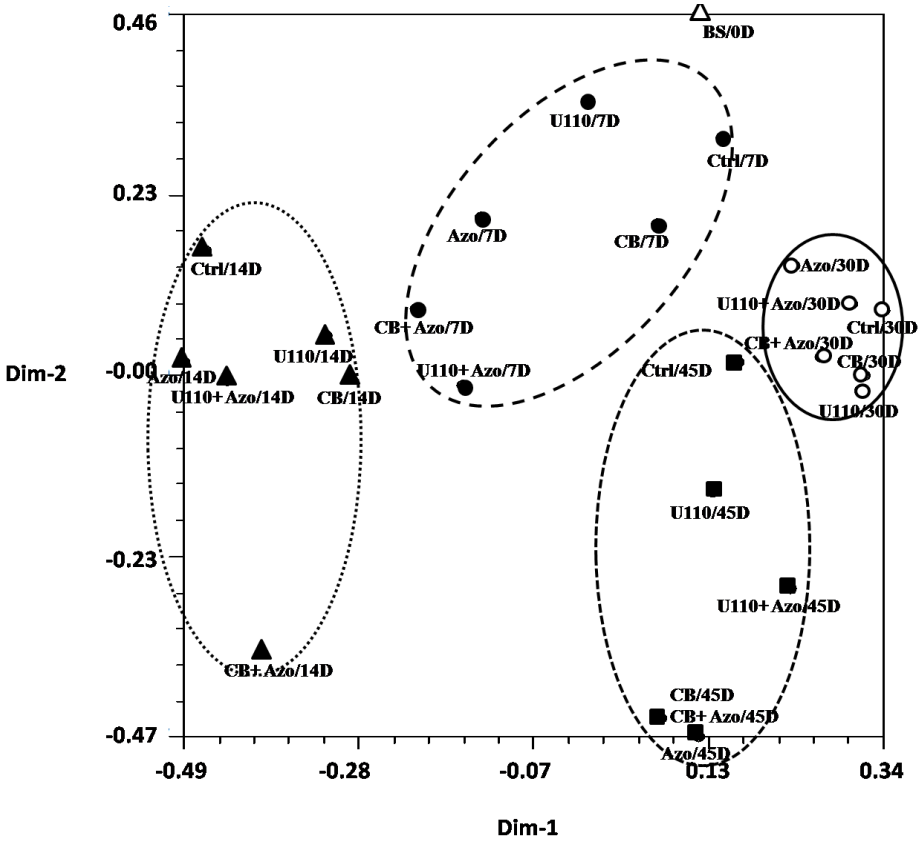
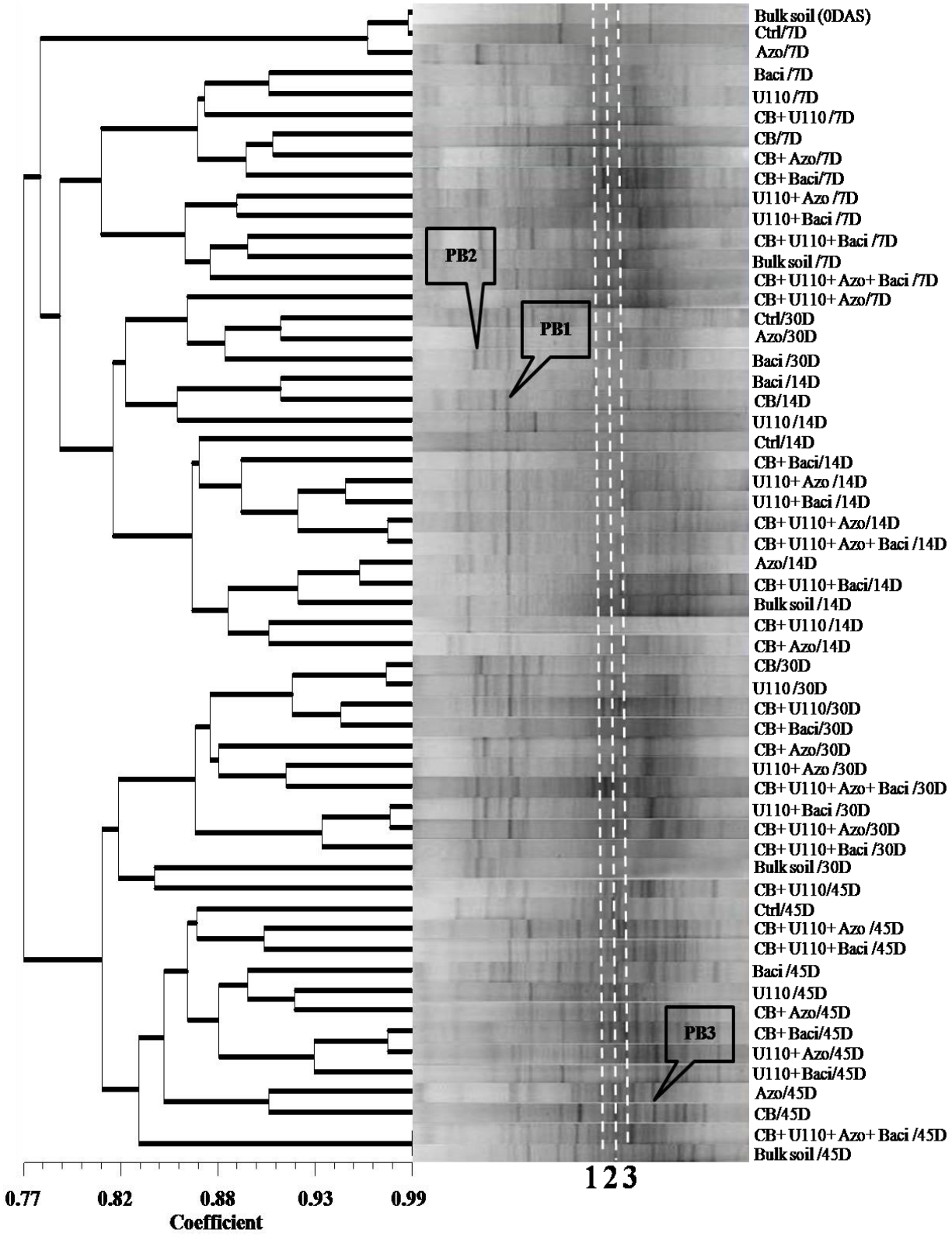
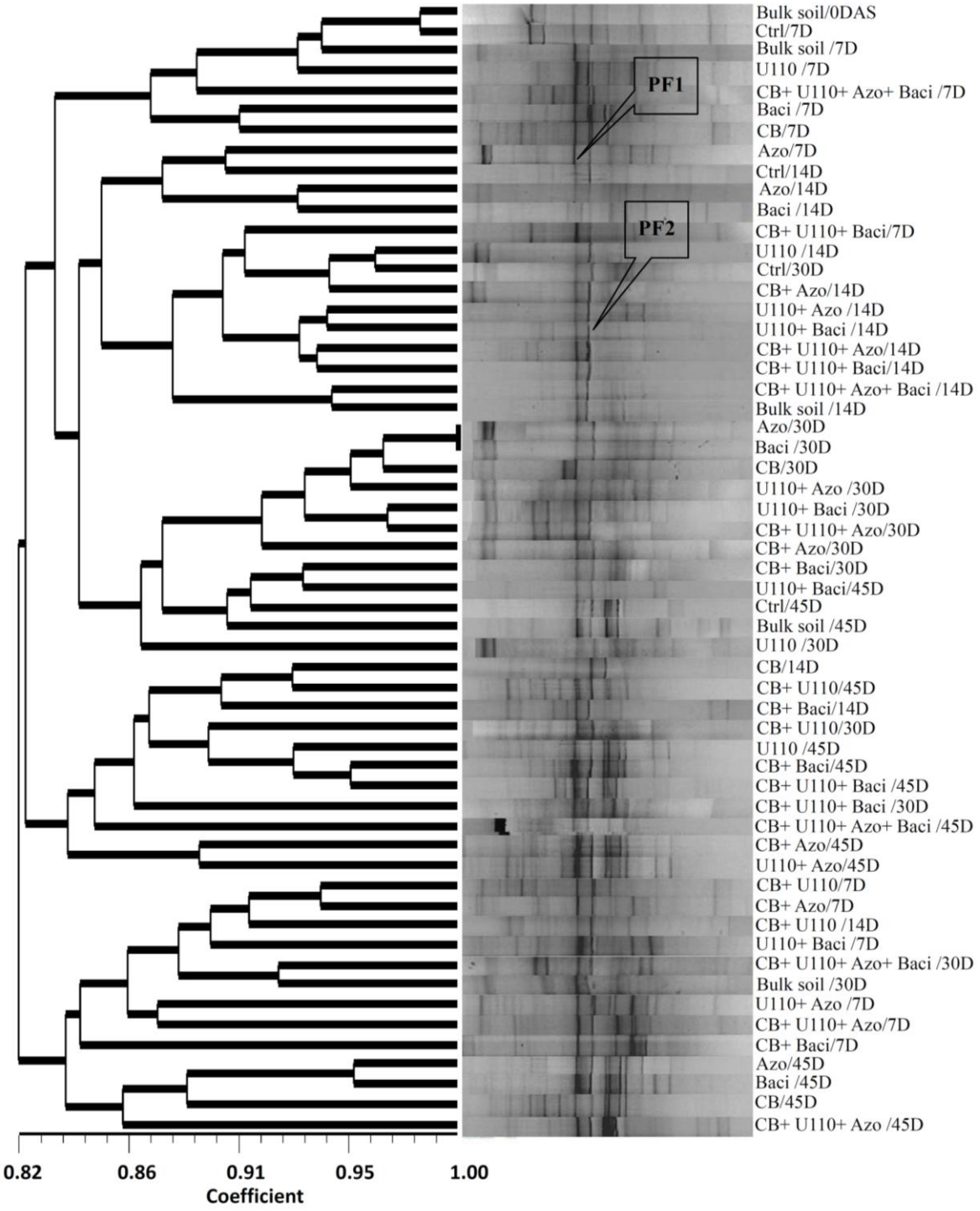


Figure 5. Community analysis derived from PCA of partial 16S rRNA banding profiles of soybean rhizosphere soil samples under soybean-nodulating bradyrhizobia-free field conditions. Letters adjacent to marks indicate the treatments: (BS) Bulk soil; (Ctrl) Control; (Azo) *Azospirillum* sp.; (CB) *B. japonicum* CB 1809; (U110) *B. japonicum* USDA 110; and (+) co-inoculation refer to co-inoculation of indicated labels at different sampling times: (Δ) 0DAI; (●) 7DAI; (▲) 14 DAI; (0) 30 DAI and (■) 45 DAI. Different samples formed a cluster which is circled (- - - - , **……**, ——— , and – – – – –), which shows a trend of 7, 14, 30 and 45 DAI, respectively.

(Abd-Alla et al., 2001). In this study, 157 out of 200 tested rhizobacterial isolates which did not inhibit the two tested *B. japonicum* growth in *in vitro* cultures were selected as *Bradyrhizobium* non-inhibitors for further screening studies for co-inoculation with bradyrhizobia on soybean under controlled (aseptic) conditions and under potted

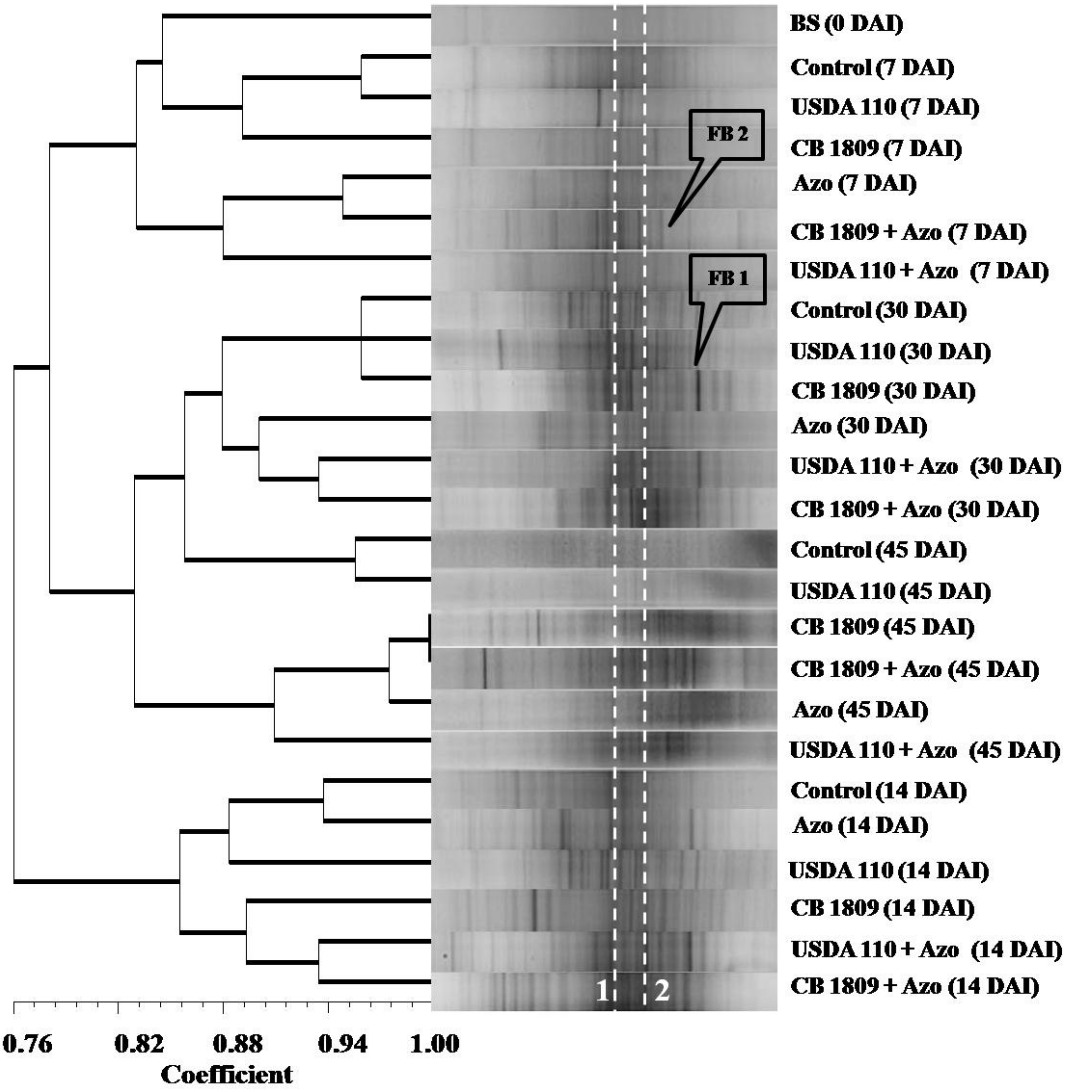


Supplementary Figure 1. Cluster analysis of eubacterial community structures of partial 16S rRNA PCR-DGGE fingerprints of different soybean rhizosphere samples after inoculation with different bacterial inocula. (Bulk) Bulk soil; (Ctrl) Control; (Azo) *Azospirillum* sp.; (Baci) *Bacillus* sp. Isolate 3; (CB) *B. japonicum* CB 1809; (U110) *B. japonicum* USDA 110 and (+) co-inoculation refer to indicated labels at different sampling times (0, 7, 14, 30 and 45 DAI) under soybean-nodulating bradyrhizobia-free pot conditions. Labels on fingerprints were subjected to sequence. Line 1, 2 and 3 refer to inoculated bacteria, *Azospirillum* sp., *B. solisalsi* Isolate 3 and *B. japonicum*, respectively.



Supplementary Figure 2. Cluster analysis of fungal community structures of partial 18S rRNA PCR-DGGE fingerprints of different soybean rhizosphere samples after inoculation with different bacterial inocula. (Ctrl) Control; (Azo) *Azospirillum* sp.; (Baci) *Bacillus* sp. Isolate 3; (CB) *B. japonicum* CB 1809; (U110) *B. japonicum* USDA 110; and (+) co-inoculation refer to indicated labels at different sampling times (0, 7, 14, 30 and 45 DAI) under soybean-nodulating bradyrhizobia-free pot conditions. Labels on fingerprints were subjected to sequence.

field soil condition, in which no specific indigenous soybean-nodulating bradyrhizobia is present in tested soils as indicated by no nodule formation on plant with un-inoculation. Among the selected isolates, *Azotobacter*



Supplementary Figure 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: (BS) Bulk soil; (Ctrl) Control; (Azo), *Azospirillum* sp.; (CB 1809) *B. japonicum* CB 1809; (USDA 110) *B. japonicum* USDA 110; and (+) refer to co-inoculation of indicated labels at different sampling times (0, 7, 14, 30 and 45 DAI) under soybean-nodulating bradyrhizobia-free field conditions. Line 1 and 2 refer to inoculated bacteria *Azospirillum* sp. and *B. japonicum*, respectively.

sp. and *Azospirillum* sp. are being commercialized as PGPR inocula for various crops cultivation by SUT (Piromyou et al., 2011; Teaumroong et al., 2009) and their positive responses on soybean nodulation were observed in this study. Rhizospheric microorganisms may not only influence the inoculated rhizobia adversely through saprophytic competition, but also help them in survival through synergism resulting in an increase in their nodulation ability and N_2-fixing efficiency (Gupta et al., 2003). Different responses on co-inoculation such as interactions among different *B. japonicum* and PGPR strains were observed in this study. In the case of *Bacillus* sp. SUT 1, it gave different responses on nodulation (nodule number) of soybean when it was co-inoculated with *B. japonicum* CB 1809 and USDA 110. It can be possible that SUT 1 did not support nodulation sites on tested soybean roots for USDA 110 as in CB 1809, or it competed for nutrient absorption instead of sharing nutrients with USDA 110, or plant autoregulation system control the amount of nodule in different combinations of two bacteria. In this study, two isolates namely *Azospirillum* sp. and *B. solisalsi* Isolate 3 were selected as soybean nodulation enhancers. It has been reported that co-inoculation of *Azospirillum lipoferum* with rhizobia stimulates the formation of epidermal cells that become infected root hair cells, or create additional infec-

tion sites that are later occupied by rhizobia (Tchebotar et al., 1998). Araújo and Hungria (1999) demonstrated the viability of co-inoculating soybean seeds with crude or formulated metabolites, or with cells of *B. subtilis*, to increase the contribution of the biological nitrogen fixation process.

Auxin regulates the expression of different genes in *Rhizobium*-legume interaction that involved plant signal processing and attachment to plant roots. Moreover, changes in auxin balance in host plant are prerequisite for nodule organogenesis (Mathesius et al., 1998; Spaepen et al., 2009). Ali et al. (2009) reported that bacterial strains from different genera have been shown to produce auxin in the form of IAA in the presence of L-tryptophan. When comparing the characteristics of selected bacteria, both selected *Azospirillum* sp. and *B. solisalsi* Isolate 3 produced high amount of IAA. However, the lowest level of ARA was detected in *B. solisalsi* Isolate 3. Adesemoye and Kloepper (2009) confirmed that PGPR such as *Bacillus amyloliquefaciens* and *Bacillus pumilis* can fix nitrogen and can increase plant N uptake from fertilizer via other mechanisms but not with their own nitrogen fixing capability. In this study, free-living *Azospirillum* sp. gave higher nitrogenase activity than *B. solisalsi* Isolate 3, and similar result was reported by Piromyou et al. (2011) that the *Azospirillum* sp. showed the highest nitrogenase activity in free-living stage as compared to *Azotobacter* sp. and other PGPR isolates including *Bacillus* sp. Moreover, Spaepen et al. (2009) also reported that effects of *Azospirillum* inoculation are mainly attributed to improved root development and enhanced water and mineral uptake. These effects were responsible for plant growth promoting substances, mainly IAA secreted by *Azospirillum*.

The inoculation of plants with *Azospirillum* sp. alters the root morphology, increases numerous plant shoot growth parameters, and eventually increases the yield of many crops (Bashan et al., 2004). However, no prominent enhancement in plant growth of soybean by inoculating the soybean with *Azosprillum* sp. alone was observed in this experiment. This may be due to the fact that the tested soil had very low organic matter content (~0.39%) and cannot accumulate the nitrogen. However, co-inoculation of *Azospirillum* sp. and either CB 1809 or USDA 110 enhanced root growth (data not shown), gave higher nodule numbers and plant growth than single inoculation of *B. japonicum*. It may be due to the fact reported by Poi et al. (1989) that the presence of *Azospirillum* sp. in the rhizosphere makes the root hairs more susceptible to rhizobial infection that is reflected in better plant growth. Co-inoculation of *Rhizobium* spp. and *Azospirillum* spp. can increase the number of root hairs, the amount of flavonoids exuded by the roots and the number of nodules formed as compared to single *Rhizobium* inoculation (Remans et al., 2008).

Cluster analysis did not allow a clear distinction of eubacterial community structures in bulk soil samples from the clustering tree of bacterial inoculation treatments. Costa et al. (2006) reported that no differences encountered between the microenvironments were due to the absence of clear characteristic patterns. Four main groups obtained by PCA analysis confirmed that the differences were mainly due to plant growth stages rather than bacterial inoculation. Similar result was reported by Herschkovitz et al. (2005) that *Azospirillum brasilense* inoculation had no effect on the size or on the structure of the bacterial communities.

More abundant and numerous bands detected in later plant growth stages than early stages suggested that bacterial communities are more complex in later plant growth stages. Xu et al. (2009) also suggested that the growth stage is the second major factor in shaping bacterial communities in the soybean rhizosphere because compositions of the root exudates were shown to vary depending on the plant species and the stage of the plant development (Heulin et al., 1987).

High recovery of the inoculated bacterial bands at the same position of the markers confirmed that the introduced bacteria were able to establish along with the plant growth stages. *Burkholderia* sp. was found to be an indigenous bacteria in tested soil as it was detected in all samples. Moreover, *Clostridium* sp. and *Parasegitibacter luojiensis* were found to be dominant bacteria as those that appeared in most of the samples. The composition of the exudates has been shown to exert selective effects towards certain bacterial groups, such as the *Proteobacteria* (Smit et al., 2001).

The fungi represent a dominant component of the soil microflora (Thorn, 1997). However, there are relatively few studies on the effects of bacterial inoculation on the soil fungal community when compared with the number of studies reporting the effects on specific target plant pathogens (Takehara et al., 2003; Browning et al., 2006) and on the bacterial community (Dungan et al., 2003). The 18S rRNA gene of fungi contains a lower amount of variation than others such as 16S rRNA gene across bacteria (Anderson and Cairney, 2004). In this study, the detected density of fungal community was higher than that of eubacterial community. *Fusarium oxysporum* that was dominantly detected in this pot experiment is a plant pathogen and can cause *Fusarium* wilt; however, no wilt symptom was observed during the plant growth development. In contrast to eubacterial communities, bacterial inoculation and sampling times did not clearly affect soil fungal communities.

In field experiments, no chemical source of N was added and the significant increases in the total plant N was obtained in rhizobacterial or bradyrhizobial inoculated plants. The non-inoculated control plants provide the information on the effects of single *B. japonicum* inoculation and its co-inoculaion on N_2-fixation capacity of soybean. The percentage of total plant N which was obtained by co-inoculating soybean with *B.*

japonicum USDA 110 and *Azospirillum* sp. was 20.16% higher than that obtained by USDA 110 single inoculation. Similar result was reported by Groppa et al. (1998) that nitrogen content of dual (*B. japonicum* and *A. brasilense*) inoculated soybean plants was significantly increased (23% over *B. japonicum* single inoculated plants); however, no significant difference in total dry matter production could be detected in their study. They suggested that co-inoculation leads to an increased number of the most active nodules, therefore, to a greater N_2-fixation and assimilation.

In the field conditions, the structural and functional diversity of rhizosphere populations is supposed to be affected by differences in root exudation and rhizodeposition in different root zones and in relation to soil types, plant species, growth stages, cultural practices such as tillage and crop rotation, and other environmental factors (Horwath et al., 1998; Lupwayi et al., 1998). DGGE patterns of *Azospirillum* sp. inoculated treatments were clearly separated from non-inoculated and bradyrhizobial inoculation alone. Because inoculation with azospirilla also leads to an increase in plant root exudation (Landa et al., 2003), both changes in root structure and exudation are potential factors influencing the type of microorganisms colonizing the radicular environment. Gradual and continuous changes from first to last sampling times in PCA analysis were supposed to be dominated by changes of eubacterial community by plant ages and not by bacterial inoculation. *Bacillus* sp. was supposed to be dominant strain in tested soil because it was detected in all samples in all sampling times. However, *P. freudenreichii* was detected in late sampling, and it seemed to be propagated in later soybean growing season.

Conclusion

Co-inoculation of *B. japonicum* and *Azospirillum* sp. gave positive responses in nodulation and plant growth of soybean, and did not shift the soil microbial community structures noticeably under soybean-nodulating bradyrhizobia-free soils. Therefore, *Azospirillum* sp. was selected as the most effective PGPR that has a potential to be used in co-inoculants with *B. japonicum* strains on soybean. However, on-farm competition trials in soybean-nodulating bradyrhizobia-established soil in soybean growing areas are also necessary to determine their potential for competitiveness against native strains.

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