World J Microbiol Biotechnol (2012) 28:615–626
DOI 10.1007/s11274-011-0855-7

ORIGINAL PAPER

Genotypic diversity among rhizospheric bacteria of three legumes assessed by cultivation-dependent and cultivation-independent techniques

Neelawan Pongsilp · Pongrawee Nimnoi · Saisamorn Lumyong

Received: 19 October 2010 / Accepted: 1 August 2011 / Published online: 30 August 2011

Abstract The genotypic diversity of rhizospheric bacteria of 3 legumes including *Vigna radiata*, *Arachis hypogaea* and *Acacia mangium* was compared by using cultivation-dependent and cultivation-independent methods. For cultivation-dependent method, Random amplified polymorphic DNA (RAPD) profiles revealed that the bacterial genetic diversity of *V. radiata* and *A. mangium* rhizospheres was higher than that of *A. hypogaea* rhizosphere. For cultivation-independent method, Denaturing gradient gel electrophoresis (DGGE) profiles of PCR-amplified 16S rRNA genes revealed the difference in bacterial community and diversity of rhizospheres collected from 3 legumes. The ribotype richness which indicates species diversity, was highest in *V. radiata* rhizosphere, followed by *A. hypogaea* and *A. mangium* rhizospheres, respectively. Three kinds of media were used to cultivate different target groups of bacteria. The result indicates that the communities of cultivable bacteria in 3 rhizospheres recovered from nutrient agar (NA) medium were mostly different from each other, while *Bradyrhizobium* selective medium (BJSM) and nitrogen-free medium shaped the communities of cultivable bacteria. Nine isolates grown on BJSM were identified by 16S rRNA gene sequence analysis. These isolates were very closely related (with 96% to 99% identities) to either one of the three groups including *Cupriavidus-Ralstonia* group, *Bacillus* group and *Bradyrhizobium-Bosea-Afipia* group. The rhizospheres were also examined for their enzymatic patterns. Of 19 enzymes tested, 3 rhizospheres were distinguishable by the presence or the absence of leucine acrylamidase and acid phosphatase. The selected cultivable bacteria recovered from NA varied in their abilities to produce indole-acetic acid and ammnonia. The resistance to 10 antibiotics was indistinguishable among bacteria isolated from different rhizospheres.

Keywords Denaturing gradient gel electrophoresis (DGGE) · Legume rhizosphere · Random amplified polymorphic DNA (RAPD) · Soil enzymatic pattern · 16S rRNA gene

N. Pongsilp (✉)
Department of Microbiology Faculty of Science, Silpakorn University, Nakhon Pathom 73000, Thailand
e-mail: neelawan@su.ac.th

P. Nimnoi
Department of Microbiology, Faculty of Liberal Arts and Sciences, Kasetsart University, Nakhon Pathom 73140, Thailand
e-mail: umco_perra@hotmail.com

S. Lumyong
Microbiology Division, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand
e-mail: scboi009@chiangmai.ac.th

Introduction

The rhizosphere refers in general to the portion of soil adjacent to the roots of living plants. It supports a diversely and densely populated microbial community, and is subjected to chemical transformations caused by the effect of root exudates and metabolites of microbial degradation (Albareda et al. 2006). Plant roots release a wide variety of compounds into the rhizospheres that create unique microenvironments for soil microorganisms. It is commonly recognized that root exudates differ according to plant species, cultivar and plant growth stage (Xu et al. 2009). Thus, it has been hypothesized that different root exudates produced by distinct plant species influence the microbial community in the rhizosphere. Rhizosphere

microorganisms exert strong effects on plant growth and health by nutrient solubilization, nitrogen fixation or the production of plant hormones (Höflich et al. 1994; Patten and Glick 1996; Smalla et al. 2001). Functional and genotypic diversities of rhizospheric bacteria have been reported in previous studies. By using Biolog ECO plates, effects of soil type and genotype of *Arabidopsis thaliana* on functional diversity of rhizospheric bacteria were found (Velasco et al. 2009). To estimate genotypic diversity, molecular techniques, especially fingerprinting techniques, allow easy and quick comparison of microbial community profiling. These techniques include cultivation-dependent and cultivation-independent techniques. A variety of cultivation-dependent techniques include Random amplified polymorphic DNA (RAPD), repetitive sequence-based Polymerase chain reaction (rep-PCR), Restriction fragment length polymorphism (RFLP) and PCR–RFLP. Instances of cultivation-independent techniques are Denaturing gradient gel electrophoresis (DGGE), Temperature gradient gel electrophoresis (TGGE) and Terminal restriction fragment length polymorphism (T-RFLP). DGGE, in particular, has commonly been used to analyze microbial populations in a variety of samples (Morgan et al. 2005). DGGE revealed that bacterial community structures in native rhizospheres of 3 medicinal legumes, *Indigofera tinctoria*, *Pueraria mirifica* and *Derris elliptica*, were different from each other (Nimnoi et al. 2011). DGGE was used to analyze the impact of plant species and sampling site on the rhizosphere microbial communities of strawberry (*Fragaria ananassa* Duch.) and oilseed rape (*Brassica napus* L.) (Costa et al. 2006).

Even though the major problem of cultivation-based analysis is that only a small proportion of the bacterial populations can be recovered from the rhizosphere and soil by traditional cultivation techniques (Amann et al. 1995), cultivation-dependent methods could provide remarkable results in some cases. Xu et al. (2009) examined the impact of soybean genotype on bacterial communities in the soybean rhizosphere. While a significant impact of soybean genotype on the bacterial communities was not observed by DGGE, a plate culture experiment targeting the culturable bacterial communities detected a remarkable difference, suggesting that a small portion of the total bacteria was influenced by plant genotype. In order to cultivate bacteria from soils, several media have been developed for targeting specific groups of bacteria. Nutrient agar (NA) medium is used in general purpose, allowing the growth of many bacteria. *Bradyrhizobium* selective medium (BJSM) (Tong and Sadowsky 1994) allows the direct isolation of bradyrhizobia from soils. Nitrogen-free modified Burk medium (Bishop et al. 1986) can be used to cultivate free-living nitrogen-fixing bacteria from soils. Comparisons of diversity of rhizospheric bacteria obtained from these media are of interest for estimating the selectivity of these media. Among plant species, legumes are known to have a positive effect on soil microorganisms (Wardle et al. 2003) as they have root exudates rich in nitrogen (Warembourg et al. 2003). In this study, we compared the genotypic diversity of rhizospheric bacteria of 3 legumes including *Vigna radiata* (mungbean), *Arachis hypogaea* (peanut) and *Acacia mangium* by cultivation-dependent and cultivation-independent methods. For cultivation-dependent method, 10 bacterial isolates derived from each rhizosphere were selected and the genotypic diversity was assessed by RAPD. Bacterial strains recovered from NA were examined for plasmid profiles, antibiotic resistance as well as production of urease, indole-acetic acid (IAA) and ammonia. Nine bacteria grown on BJSM were identified by 16S rRNA gene sequence analysis. Comparisons of diversity of rhizospheric bacteria obtained from 3 kinds of media were examined by DGGE. For cultivation-independent method, total DNA was directly extracted from rhizospheres, 16S rRNA gene fragments were amplified in PCR and further analyzed by DGGE. In addition, rhizospheres of these 3 legumes were compared for their enzymatic patterns.

Materials and methods

Soil sampling

Native rhizospheres of *Vigna radiata* (mungbean) and *Arachis hypogaea* (peanut) were collected from the plantations in Saraburi (Lat 14° 31′N Long 100° 55′E) and Lopburi (Lat 14° 48′N Long 100° 37′E) provinces of Thailand, respectively. Native rhizosphere of *Acacia mangium* was collected from a herbal garden in Nakhon Pathom province (Lat 17° 23′N Long 104° 43′E) of Thailand. These rhizospheres were collected during October–November, 2009.

Soil enzymatic patterns

pH, urease activity, alkaline phosphatase activity and enzymatic patterns of rhizospheres were analyzed. pH of rhizosphere soil sample was measured as described by Renshaw et al. (2003). Urease activity was measured as described by Askin and Kizilkaya (2006), except that the soil suspension was not diluted with distilled water and 5 ml of the soil filtrate was used to make a reaction mixture. The absorbance at 578 nm of the reaction mixture was measured. Controls without the addition of urea were also included. Ammonia concentrations in soils without and with the addition of urea were used to determine the released ammonia resulting from urease activity. The

concentration of ammonia was determined by comparison with a standard curve. A standard curve was constructed by preparing reaction mixtures in which ammonium citrate tribasic at varied concentration (equivalent to 0–50 mg/ml ammonia) was used instead of the soil filtrate. One unit of urease activity is equal to the amount of urease that hydrolyzes 0.5 µmol of urea and liberates 1.0 µmol of ammonia in 1 min at 37°C (Starnes et al. 1984). Alkaline phosphatase activity was determined as described by Eivazi and Tabatabai (1977). Hydrolysis of p-nitrophenyl phosphate was estimated by measuring the concentration of p-nitrophenol with a spectrophotometer at wavelength 405 nm. The concentration of p-nitrophenol was determined by comparison with a standard curve. One unit of phosphatase activity is defined as the amount of enzyme required to liberate 1.0 µmol of p-nitrophenol in 1 min (Tso and Chen 1997). pH, urease activity and alkaline phosphatase activity were measured in triplicate. The presence of 19 different enzymes in rhizospheres was detected with Api®Zym enzymatic quantification test (BioMérieux, Marcy-L'Etoile, France). Two grams of soil was suspended in 2 ml of deionized (DI) water. The soil suspension was centrifuged at 1,500 rpm for 5 min. The upper aqueous phase was pipetted into each cupule of the Api-Zym® strips. The strip was incubated at 37°C for 4½ h. The detection was done according to the manufacture's instruction. The intensity of color developed within 7–10 min after the addition of reagents was scored from 0 to 5. A value of 0 corresponded to negative reaction, 1–2 to weak positive reaction and 3–5 to strong positive reaction. The Api®Zym test was conducted in duplicate.

Cultivation and enumeration of rhizospheric bacteria on 3 kinds of media

Ten grams of rhizosphere soil was suspended in 100 ml of 0.2% $Na_4P_2O_7$, pH 8.5. Soil sample was shaken on a rotary shaker for 30 min at 200 rpm and centrifuged for 5 min at 600 rpm to pellet fungi. The upper aqueous phase was transferred to a sterile test tube and serial dilutions were used for enumeration of bacterial numbers by the standard plate count method on 3 kinds of media including nutrient agar (NA) medium, *Bradyrhizobium* selective medium (BJSM) (Tong and Sadowsky 1994) and nitrogen-free modified Burk medium (Bishop et al. 1986). Plates were incubated at 30°C for 2 weeks. Single colonies on each medium were selected. Pure cultures were maintained on NA slants at 4°C.

Random amplified polymorphic DNA (RAPD) analysis

Ten bacterial isolates recovered from each rhizosphere by using NA were selected to investigate genetic diversity based on RAPD fingerprinting. Genomic DNA was extracted from exponentially grown culture by using a GF-1 Bacterial DNA extraction kit (Vivantis, Selangor Darul Ehsan, Malaysia) according to the manufacture's instruction. The PCR reactions were carried out using an arbitary primer RAPD1 (5′ GGTGCGGGAA 3′) (Nuntagij et al. 1997). PCR-RAPD amplifications were carried out as described by Pongsilp and Nuntagij (2009). Negative controls (no DNA added) were included in all sets of reactions. Approximately 2,000 ng of PCR products were loaded into individual lanes in the gel. The presence and size of the amplified fragments were determined by 1% agarose gel electrophoresis and unweighted pair groups using mathematical averages (UPGMA) dendrograms were constructed using the Image Master 1D Elite Software version 5.20 (GE Healthcare, Munich, Germany). The strains generated individual RAPD patterns were also examined for plasmid profiles, antibiotic resistance as well as production of urease, IAA and ammonia.

Plasmid profiles

The strains were examined for the presence of small plasmids. Small plasmids were extracted from exponentially grown culture by using a GF-1 plasmid extraction kit (Vivantis, Selangor Darul Ehsan, Malaysia) according to the manufacture's instruction. The presence of small plasmids was determined by 1% agarose gel electrophoresis. Sizes of the isolated plasmids were assessed by comparison of their migration distances with those of a supercoiled DNA ladder (Invitrogen, Carlsbad, CA).

Antibiotic resistance

To test antibiotic resistance, bacterial cultures were spread on NA plates and antibiotic discs (Oxoid, Basingstoke, UK) were placed on the agar surfaces. Antibiotic susceptibility was observed as clear zones around antibiotic discs. The test was conducted in duplicate.

Urease production

Bacterial strains were streaked on urea Christensen agar plates (Christensen 1946). Plates were incubated at 30°C until colonies developed. A color change from yellow to pink was indicative of a urease-positive result. Urease test was performed in triplicate.

Colorimetric assay for indole acetic acid (IAA) production

Bacterial strains were propagated in Tris-TMRT broth (Nuntagij et al. 1997) at 30°C in the dark until cell cultures

reached mid-log phase. The IAA concentration in the cultures was determined by the colorimetric assay (Gordon and Weber 1951). Two ml of 0.01 M $FeCl_3$ in 35% $HClO_4$ was added into 1 ml of supernatant of the culture. The reaction was incubated in the dark at 30°C for 25 min. The absorbance at 530 nm was measured. The concentration of IAA was determined by comparison with a standard curve.

Ammonia production

Bacterial strains were propagated in peptone water and incubated at 30°C with shaking at 150 rpm until cell cultures reached mid-log phase. A 0.25 ml of Nessler's reagent was added into 5 ml of the culture (Cappucino and Sherman 1992). After 30 min of incubation, the absorbance at 500 nm was measured (Barnes and Sugden 1990). NH_4Cl that is equivalent to ammonia in the range of 0–10 μg/ml was used in the reactions to prepare a standard curve. The concentration of ammonia was determined by comparison with a standard curve.

Sequence analysis of partial 16S rRNA gene of isolates grown on BJSM

Three isolates recovered from each rhizosphere by using BJSM were identified by sequence analysis of 16S rRNA gene. Partial 16S rRNA gene (approximately 500 bp) of each isolate was amplified using universal primers UN16S 926f (5′ AAACTYAAAKGAATTGACGG 3′) and UN16S 1392r (5′ ACGGGCGGTGTGTRC 3′) (Lane 1991). PCR reactions were carried out and the products were purified as described by Pongsilp et al. (2002). The purified PCR products were sequenced by Bio Basic (Markham, ON, Canada). A sequence similarity search was performed by using BLASTN program (http://www.ncbi.nim.nih.gov/).

Statistical analysis

Experimental data were compared by using the SPSS program version 16.0 (SPSS Inc., Chicago, IL).

Soil DNA, culture DNA extraction and rDNA amplification

The total DNA was extracted from rhizosphere soil samples by using a GF-1 soil DNA extraction kit (Vivantis, Selangor Darul Ehsan, Malaysia) according to the manufacture's instruction. Colonies grown on each medium were pooled together and inoculated into broth of the same medium. Total DNA was extracted from exponentially grown cultures of pooled colonies and a pure culture of *Bradyrhizobium* sp. DASA 64008 by using a GF-1 Bacterial DNA extraction kit (Vivantis, Selangor Darul Ehsan, Malaysia) according to the manufacture's instruction. Total DNA of soil samples and cultures were amplified by PCR with a MyCycler™ Thermal Cycler (Bio-Rad, Hercules, CA). The variable region 3 (V3) within the 16S rRNA gene (corresponding to positions 341–534 of the 16S rRNA gene in *Escherichia coli*; Keyser et al. 2006) was amplified by using a set of primer pairs, F341 (5′- CCT ACG GGA GGC AGC AG -3′) with the GC-clamp (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G-3′) and R534 (5′- ATT ACC GCG GCT GCT GG -3′). PCR reactions and conditions were performed as described in Muyzer et al. (1993). The amplified products were separated on 1% agarose gels containing ethidium bromide (EtBr) and visualized under UV light.

Denaturing gradient gel electrophoresis (DGGE)

The PCR fragments were separated by using DGGE performed with the BioRad DCode™ Universal Mutation Detection System (Bio-Rad, Hercules, CA). Twenty-five μl (approximately 2,500 ng) of mixed PCR products from each soil sample, pooled colonies obtained from each medium and a pure culture of *Bradyrhizobium* sp. DASA 64008 were applied to 8% polyacrylamide gel with a liner gradient of 25–50% denaturant (100% denaturant corresponds to 40% [vol vol^{-1}] of formamide plus 7 M of urea). Electrophoresis was performed at 200 V for 4 h at a constant temperature of 60°C. Gels were then stained with GelStar® Nucleic Acid Gel Stain (Cambrex Bio Science, Rockland, ME) for 30 min and visualized.

Analysis of DGGE profiles

SPSS Version 16.0 (SPSS Inc., Chicago, IL) was used to analyze the community fingerprints of a denaturing gradient gel. The bands in each sample were scored in a binary matrix based on the presence (1) or the absence (0) of each band. The Pearson correlation index (r) for each pair of lanes within a gel was calculated as a measure of similarity between the community fingerprints. Cluster analysis was performed by applying the Ward's method to the matrix of similarities obtained (Costa et al. 2006). The bacterial complexity of each sample was expressed by various indices of biodiversity, calculated from the DGGE profiles. (i) species diversity (S), which corresponds to the number of bands in a DGGE profile; (ii) simple index (I_i), which is calculated using the formula $I_i = n/n_M$, where I is an index number for each band present in a DGGE profile; n is the number of DGGE bands in a given DGGE profile; n_M is the number of bands in the DGGE profile with the highest number of bands (Nimnoi et al. 2010; Silvestri et al. 2007).

Results and discussion

Soil enzymatic patterns

Soil pH and enzymatic patterns were determined for rhizospheres of 3 legumes including *V. radiata, A. hypogaea* and *A. mangium*. The pH values of *V. radiata*, *A. hypogaea* and *A. mangium* rhizospheres were 7.63 ± 0.01, 7.37 ± 0.10 and 6.80 ± 0.13, respectively. Urease activity, alkaline phosphatase activity and soil enzymatic patterns are presented in Table 1. Urease activity in *V. radiata* and *A. hypogaea* rhizospheres did not differ significantly from each other, while urease activity in *A. mangium* rhizosphere was significantly lower than those of the other two rhizospheres. Alkaline phosphatase activity in 3 rhizospheres differed significantly from each other. It was highest in *A. mangium* rhizosphere, followed by *V. radiata* and *A. hypogaea* rhizospheres, respectively. The presence and the absence of 19 enzymes in 3 rhizopheres were similar but not identical. Alkaline phosphatase, esterase (C4) and napthol-AS-BI-phosphohydrolase were detected in all rhizospheres, while leucine acrylamidase and acid phosphatase were detected in only *A. hypogaea* and *A. mangium* rhizospheres, respectively. As enzymes present in rhizospheres might be obtained from root exudates and/or produced by soil microorganisms, the enzymatic activity of soils can be used to compare the influence of specific plant species and used as an indicator of biological activity of the soils.

Cultivation and enumeration of rhizospheric bacteria on 3 kinds of media

Bacterial counts in 3 rhizosphere soil samples obtained from 3 kinds of media are shown in Table 2. Bacterial numbers in *A. hypogaea* and *A. mangium* rhizospheres cultivated on NA did not significantly differ from each other but both were significantly higher from that of *V. radiata* rhizosphere. Bacterial count on BJSM was highest in *V. radiata* rhizosphere, followed by *A. mangium* and *A. hypogaea* rhizospheres, respectively. Cell numbers of free-living nitrogen-fixing bacteria in 3 rhizospheres were not significantly different from each other. Three kinds of media showed the different levels of bacterial recovery from rhizospheres, resulting in different numbers of cultivable bacteria. NA medium is used in general purpose, allowing many types of bacteria to grow. Bacterial cell numbers obtained from all rhizospheres were highest in NA. BJSM allows the direct isolation of bradyrhizobia from soils (Tong and Sadowsky 1994). Nodulating and non-nodulating *Bradyrhizobium* could be isolated from soils by using this medium (Pongsilp et al. 2002). As this selective medium was developed to grow bacterial isolates of the genus *Bradyrhizobium* based on their resistance to some heavy metals, other bacteria resistant to heavy metals could also be cultivated. N-free medium can be used to cultivate free-living nitrogen-fixing bacteria from soils. The variation in numbers of rhizospheric bacteria may be due to different components of the root exudates that

Table 1 Enzymatic patterns of rhizosphere soil samples

Soil enzymatic patterns	Rhizosphere soil samples		
	V. radiata rhizosphere	*A. hypogaea* rhizosphere	*A. mangium* rhizosphere
Urease activity (unit/g soil)	86.30 ± 15.64	67.26 ± 13.07	30.07 ± 12.28
Alkaline phosphatase activity (milliunit/g soil)	58.50 ± 2.10	32.00 ± 2.80	69.50 ± 6.40
Production of enzymes			
Alkaline phosphatase, esterase (C4) and napthol-AS-BI-phosphohydrolase	+(S)	+(S)	+(S)
Leucine acrylamidase	–	+(S)	–
Acid phosphatase	–	–	+(S)
Esterase lipase (C8), lipase (C14), valine acrylamidase, cystine acrylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucoronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase	–	–	–

The values shown are the means of 3 replicates ± standard deviation

For production of enzymes: +(s), strong positive; +(w), weak positive; −, negative

Table 2 Bacterial counts in rhizosphere soil samples obtained from 3 kinds of media

Media	Bacterial counts in rhizosphere soil samples (log CFU/g soil)		
	V. radiata rhizosphere	*A. hypogaea* rhizosphere	*A. mangium* rhizosphere
Nutrient agar medium (NA)	6.92 ± 0.04	7.93 ± 0.31	7.80 ± 0.21
Bradyrhizobium selective medium (BJSM)	4.80 ± 0.14	4.08 ± 0.13	4.42 ± 0.03
Nitrogen-free modified Burk medium	6.77 ± 0.16	7.11 ± 0.22	6.98 ± 0.05

The values shown are the means of 3 replicates ± standard deviation

selectively affect bacterial community structure. Pure cultures of 10 isolates recovered from each rhizosphere by using NA were maintained for further studies on genotypic and functional diversity. These isolates were designated by abbreviations: VRRS, AHRS and AMRS are used to refer to the isolates derived from *V. radiata*, *A. hypogaea* and *A. mangium* rhizospheres, respectively.

Random amplified polymorphic DNA (RAPD) analysis

Total 30 isolates generated particular RAPD profiles with amplified fragments ranging between <500 bp to 3,000 bp in size. These isolates could be identified as the individual strains because they generated specific individual patterns. The dendrograms constructed from RAPD profiles of strains derived from *V. radiata*, *A. hypogaea* and *A. mangium* rhizospheres are shown in Figs. 1, 2 and 3, respectively. The highest genetic diversity was observed from bacterial strains in *V. radiata* and *A. mangium* rhizospheres (approximately 10% similarity), followed by *A. hypogaea* rhizosphere (approximately 20% similarity). RAPD is one of the powerful methods for generating DNA fingerprints and evaluating genetic diversity. It has been successfully used to study genetic diversity of several genera in bacterial community and for typing many different organisms (Nimnoi and Pongsilp 2009; Paffetti et al. 1996).

Plasmid profiles

Among 30 strains of rhizospheric bacteria, the only one strain from *V. radiata* rhizosphere harbored one small plasmid with a size of 2 kb and the only one strain from *A. hypogaea* rhizosphere harbored one small plasmid with a size of 9 kb (Figure not shown).

Antibiotic resistance, production of urease, IAA and ammonia

The 30 strains of rhizospheric bacteria were examined for resistance to 10 antibiotics as well as production of urease, IAA and ammonia. The results are shown in Table 3. Among 30 strains recovered from 3 rhizospheres, 9

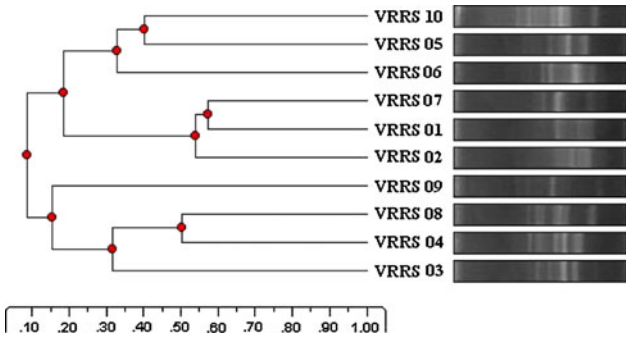

Fig. 1 Dendrogram generated from RAPD profiles of the selected bacteria from *V. radiata* rhizosphere

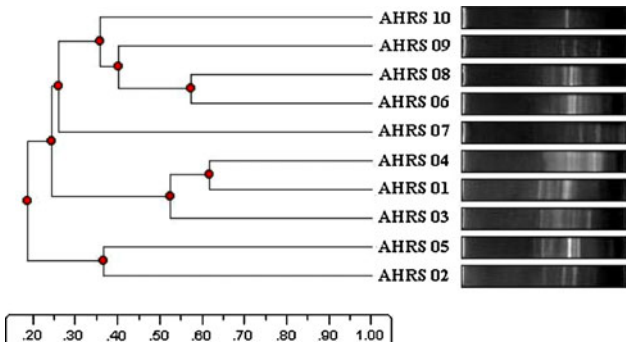

Fig. 2 Dendrogram generated from RAPD profiles of the selected bacteria from *A. hypogaea* rhizosphere

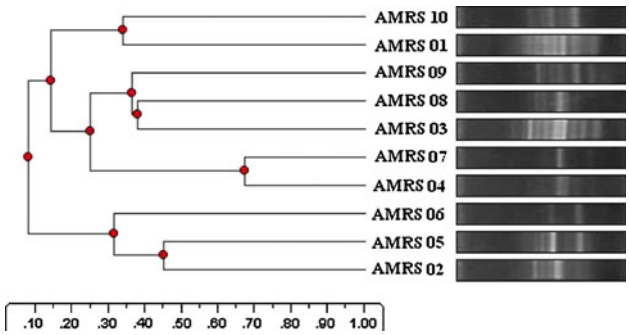

Fig. 3 Dendrogram generated from RAPD profiles of the selected bacteria from *A. mangium* rhizosphere

Table 3 Antibiotic resistance, production of urease, IAA and ammonia of the selected strains derived from 3 rhizospheres

Characteristics	Strains derived from		
	V. radiata rhizosphere ($n = 10$)	*A. hypogaea* rhizosphere ($n = 10$)	*A. mangium* rhizosphere ($n = 10$)
Resistance to antibiotics			
Ampicillin (10 μg)	± (2)	± (1)	± (4)
Ceftazdime (30 μg)	± (4)	± (1)	± (1)
Cefotaxine (30 μg) and streptomycin (10 μg)	± (1)	–	–
Novobiocin (30 μg)	–	± (2)	–
Spectinomycin (10 μg)	–	± (1)	–
Tetracycline (30 μg), kanamycin (30 μg), chloramphenicol (30 μg) and gentamycin (120 μg)	–	–	–
Urease production	± (6)	± (6)	± (4)
Amount of IAA produced (μg/ml)	0.49 ± 0.11 to 19.97 ± 0.72	0.88 ± 0.51 to 10.68 ± 1.42	1.39 ± 0.45 to 13.78 ± 2.23
Average amount of IAA produced per strain (μg/ml)	5.16 ± 0.26	3.12 ± 0.21	9.17 ± 0.63
Amount of ammonia produced (μg/ml)	15.55 ± 1.54 to 38.38 ± 5.21	14.17 ± 1.42 to 43.67 ± 2.00	2.89 ± 2.41 to 40.80 ± 2.46
Average amount of ammonia produced per strain (μg/ml)	26.78 ± 2.51	32.95 ± 1.20	24.20 ± 1.47

n = number of strains tested

For resistance to antibiotics and urease production: −, all strains are negative; ± some strains are positive, numbers in parentheses indicate numbers of positive strains

For IAA and ammonia production: the values shown are the means of 3 replicates ± standard deviation

antibiotic resistance patterns were observed. About half of them (4, 7 and 6 strains derived from *V. radiata*, *A. hypogaea* and *A. mangium* rhizospheres, respectively) were susceptible to all 10 antibiotics. Eight strains (4, 1 and 3 strains derived from *V. radiata*, *A. hypogaea* and *A. mangium* rhizospheres, respectively) were resistant to a single antibiotic (either ampicillin or ceftazidime). Five strains (2, 2 and 1 strains derived from *V. radiata*, *A. hypogaea* and *A. mangium* rhizospheres, respectively) exhibited multiple drug resistance up to 3 antibiotics. Among 13 resistant strains, most of them (8 strains) were resistant to ampicillin. None strains were resistant to tetracycline, kanamycin, chloramphenicol and gentamycin. None of the antibiotics tested were found as selective substances in the rhizopheres since there was no antibiotic to which all strains derived from the same rhizosphere were resistant. Among 30 strains, 16 strains (6, 6 and 4 strains derived from *V. radiata*, *A. hypogaea* and *A. mangium* rhizospheres, respectively) produced urease. The strains varied in the abilities to produce IAA and ammonia. The highest amounts of IAA and ammonia were produced by bacteria derived from *A. mangium* and *A. hypogaea* rhizospheres, respectively. In general, rhizosphere microorganisms are known to exert strong effects on plant growth and health by nutrient solubilization, nitrogen fixation or production of plant hormones (Hoflich et al. 1994; Patten and Glick 1996; Smalla et al. 2001). In this study, bacterial strains in the rhizospheres also exhibited the activities associated with the increase of nutrients and plant hormones. Urease-producing bacteria catalyze the hydrolysis of urea to ammonia and carbon dioxide (Spayd et al. 1978). Ammonia-producing bacteria produce ammonia by deamination of peptone (Vince et al. 1973). Therefore, both groups of rhizospheric bacteria provide ammonia that can be utilized by plants. IAA is the most abundant naturally occurring phytohormone with auxin activity (vande Broek et al. 1999) that promotes root proliferation (Lambrecht et al. 2000; Patten and Glick 1996), cell division and shoot growth (Davies 1995). The ability of IAA-producing bacteria on root and shoot development of *Raphanus sativus* and *Brassica oleracea* has been reported (Nimnoi and Pongsilp 2009). These results demonstrate that rhizospheric bacteria could provide nutrient and plant hormone available to plants.

Sequence analysis of partial 16S rRNA gene of isolates grown on BJSM

Partial sequences of 16S rRNA gene were obtained from 3 isolates recovered from each rhizosphere by using BJSM.

The isolates BJSM1, BJSM2 and BJSM 3, derived from *V. radiata* rhizosphere, were very closely related to the genera *Ralstonia* and *Cupriavidus* with 98% to 99% identities. These genera share an almost identical 16S rRNA gene sequence. The related species included *C. metallidurans* (formerly *R. metallidurans*), a heavy metal resistant bacterium (Goris et al. 2001), *C. taiwanensis* (formerly *R. taiwanensis*), a root-nodule symbiont isolated from *Mimosa* (Chen et al. 2001), *C. pauculus*, *C. gilardii* and *C. oxalaticus*. For the isolates derived from *A. hypogaea* rhizosphere, the isolates BJSM4 and BJSM6 were identified as members of *Bacillus* with 99% identities. The isolate BJSM5 was closely related to bacteria in the genera *Bosea*, *Afipia* and *Bradyrhizobium* with 96% to 99% identities. These genera belong to the family *Bradyrhizobiaceae* and the 16S rRNA gene alone is not variable enough to allow discrimination among these close relatives. For the isolates derived from *A. mangium* rhizosphere, the 16S rRNA gene sequences of the isolates BJSM7 and BJSM8 showed high identities (99%) with those of *Bacillus* spp. The 16S rRNA gene sequence of isolate BJSM9 exhibited high degrees of identity (97–99%) with those of *Bradyrhizobium*, *Bosea* and *Afipia*. Among 9 isolates identified, 3 of them were close relatives of *Ralstonia* and *Cupriavidus*. Three isolates were members of *Bacillus* spp. The remaining isolates were closely related to *Bradyrhizobium*, *Bosea* and *Afipia*. As BJSM was developed to grow bacterial isolates of the genus *Bradyrhizobium* based on their resistance to some heavy metals, other bacteria resistant to heavy metals could also be cultivated. In this study, the other bacteria could also be obtained from BJSM, indicating that this medium is not completely selective for the growth of *Bradyrhizobium*. Therefore, it should be noted that further investigations are required for direct isolation of *Bradyrhizobium* from soils by using BJSM. The partial 16S rRNA gene sequences of these isolates are available from the GenBank database under accession numbers JF437703-JF437711.

DGGE community fingerprinting of 3 rhizosphere soils

To obtain DGGE fingerprintings of the total bacterial community without cultivation, total DNA was directly extracted from the samples and PCR-DGGE was performed by amplification of V3 region of 16S rRNA gene. Repeated amplification and electrophoresis of samples were performed. The identical patterns were generated from 3 replicates of each sample (Figure not shown). As displayed in Fig. 4, DGGE profiles revealed the difference in bacterial community and diversity of rhizosphere soils collected from 3 legumes. These profiles exhibited the dominant species and the intensity of each band indicated its relative abundance. For rhizosphere soils of *V. radiata* (lane 1) and *A. hypogaea* (lane 2), the DGGE patterns consisted of 2 and 1 stronger

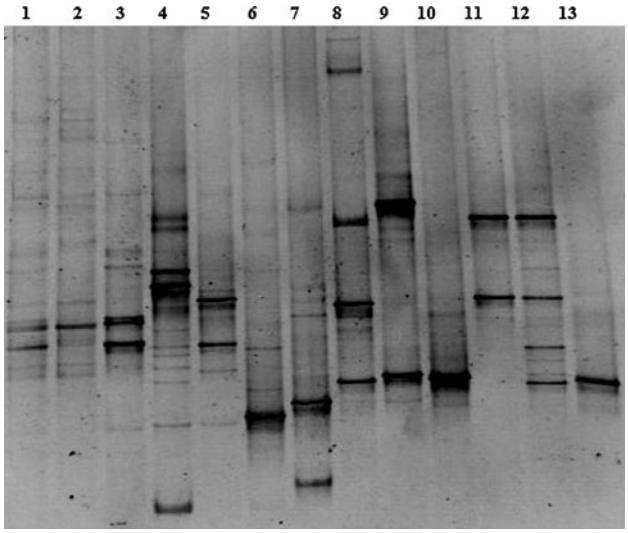

Fig. 4 DGGE patterns of rhizospheres of 3 legumes and pooled colonies recovered from 3 kinds of media. *Lane 1 V. radiata* rhizosphere; *2 A. hypogaea* rhizosphere; *3 A. mangium* rhizosphere; *4* cultivable bacteria in *V. radiata* rhizosphere recovered from NA; *5* cultivable bacteria in *A. hypogaea* rhizosphere recovered from NA; *6* cultivable bacteria in *A. mangium* rhizosphere recovered from NA; *7* cultivable bacteria in *V. radiata* rhizosphere recovered from BJSM; *8* cultivable bacteria in *A. hypogaea* rhizosphere recovered from BJSM; *9* cultivable bacteria in *A. mangium* rhizosphere recovered from BJSM; *10* pure culture of *Bradyrhizobium* sp. DASA 64008; *11* cultivable bacteria in *V. radiata* rhizosphere recovered from N-free medium; *12* cultivable bacteria in *A. hypogaea* rhizosphere recovered from N-free medium; *13* cultivable bacteria in *A. mangium* rhizosphere recovered from N-free medium. NA, nutrient agar medium; BJSM, *Bradyrhizobium* selective medium; N-free medium, nitrogen-free modified Burk medium

bands, respectively, and a large number of less intensive bands. This result indicates that rhizosphere soils of *V. radiata* and *A. hypogaea* consisted of only 2 and 1 dominant ribotypes, respectively, whereas other ribotypes which varied in relative band intensity seemed to be equally present. For rhizosphere soil of *A. mangium* (lane 3), the DGGE fingerprint consisted of 2 dominant ribotypes and some of faint bands, suggesting the selection of bacterial populations in its rhizosphere. Usually, the rhizosphere contains unique and diverse microorganisms that are controlled by organic materials derived from the root exudates (Merckx et al. 1987; Rovira 1956). Plants are able to stimulate the microbial community in the rhizosphere by providing nutrients and easily degradable energy sources from root exudates and dead root cells to soil microorganisms, then root exudates can create a selective pressure on microbial community. Besides legume species, soil characteristics have been reported as ones of major factors in determining bacterial community structure. It was found that the bacterial community structure was driven firstly by soil characteristics (Kowalchuk et al. 2002). Marschner et al. (2004) found that soil pH, soil type, plant species and plant age contributed to

shaping the species composition in the rhizosphere. As the experiment was performed with native rhizosphere soils from the different origins, therefore it could not separate between the impact of 2 factors, including plant species and soil characteristics, on the rhizosphere bacterial diversity. Thus, legume species and soil characteristics were possible to affect the rhizosphere bacterial communities.

For clustering analysis (Fig. 5), a dendrogram was constructed to determine the different levels of similarity shared among samples. Dendrogram generated from DGGE profiles of these rhizospheres of 3 legumes revealed that the community structures of rhizospheric bacteria from 3 legumes were different from each other. Rhizospheres of *V. radiata* and *A. hypogaea* claded together at a low Euclidean distance of 2, and they were linked together with *A. mangium* rhizosphere at Euclidean distance of 13. The community structure in *A. mangium* rhizosphere showed more divergent than those in *A. hypogaea* and *V. radiata* rhizospheres. Different plant species and soil characteristics appeared to have a strong influence on the bacterial community. Our result agrees with the previous study of Costa et al. (2006) who proposed that plant type and location influence microbial community structure in the rhizosphere. Different plant species selects different bacterial communities in the vicinity of their roots (Smalla et al. 2001). The amount and composition of root exudate are the key drivers for the different community structure (Marschner et al. 2004).

Besides the bacterial community structure, we also analyzed the bacterial diversity. The diversity is defined as a function of the species richness (number of present species) and the relative abundance of individual species (Felske and Osborn 2005). According to Nikolcheva et al. (2003), a band in a denaturing gel represents a discrete ribotype and the number of different ribotype is referred to bacterial diversity. Thus, we examined the genetic diversity based on the presence and the absence of ribotype. Table 4 presents the values obtained from species diversity (*S*) and simple (*I*) index for each sample. Simple index and species diversity index indicated that the highest diversity was observed from *V. radiata* rhizosphere, followed by *A. hypogaea* and *A. mangium* rhizospheres, respectively.

DGGE community fingerprinting of cultivable bacteria

In this study, specific groups of bacteria were enumerated by using 3 different culture media. After colony enumeration, DNA was extracted from colonies grown on media. The V3 region of 16S rRNA gene was amplified followed by DGGE analysis. As depicted in Fig. 4, DGGE profiles of lane 4–13 show particular fingerprints corresponding to media and rhizopheres. The differences in genetic diversity and community of cultivable bacteria were found. Based on cluster analysis (Fig. 5), the positions of DGGE profiles of cultivable bacteria in 3 rhizospheres recovered from BJSM and N-free medium were claded together, while the positions of cultivable bacteria in 3 rhizosphere soils recovered from NA were clearly separated from each other within a dendrogram. The result indicates that the communities of cultivable bacteria in 3 rhizosphere soils recovered from NA were mostly different from each other, while BJSM and N-free medium shaped the communities of cultivable bacteria, targeting different specific groups. The communities of cultivable bacteria in *V. radiata* and *A. hypogaea* rhizospheres recovered from N-free medium were more similar to each other than to *A. mangium* rhizosphere, suggesting the existence of closely related groups of free-living nitrogen-fixing bacteria in both *V. radiata* and *A. hypogaea* rhizospheres. The communities of cultivable bacteria in *A. hypogaea* and *A. mangium* rhizospheres recovered from BJSM were more similar to each other than to *V. radiata* rhizosphere. A consistent result was obtained from the 16S rRNA gene sequences. The rhizospheres of *A. hypogaea* and *A. mangium* consisted of bacteria in the same genera, *Bacillus* group and *Bradyrhizobium-Bosea-Afipia* group. Whereas *V. radiata* rhizopshere was found to contain bacteria in *Ralstonia-Cupriavidus* group. As many works succeeded in using the known strains of bacteria and fungi for verification of the species-specific migration positions on DGGE gel (Das et al. 2007; Silvestri et al.

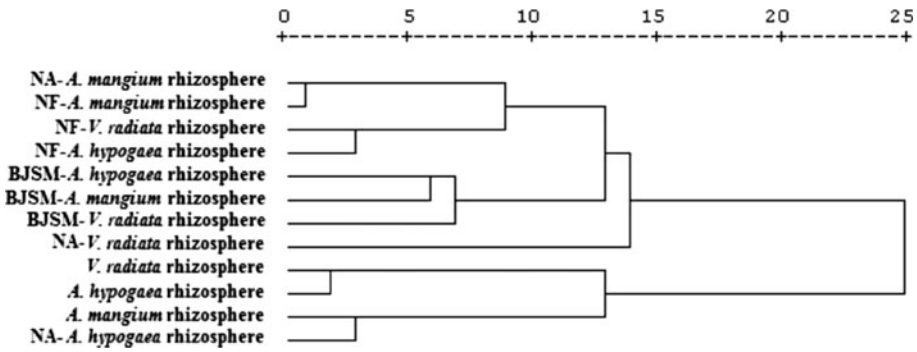

Fig. 5 Cluster analysis of DGGE patterns of rhizospheres of 3 legumes and cultivable bacteria recovered from 3 kinds of media. Dendrogram generated by using Ward's cluster analysis. *Scale* indicates Euclidean distance. NA, nutrient agar medium; BJSM, *Bradyrhizobium* selective medium; NF, nitrogen-free modified Burk medium

Table 4 The indexes of biodiversity calculated for each sample

Samples	Indexes of biodiversity	
	Species diversity (*S*)	Simple index (*I*)
V. radiata rhizosphere	14	1.00
A. hypogaea rhizosphere	11	0.78
A. mangium rhizosphere	8	0.57
Cultivable bacteria in *V. radiata* rhizosphere recovered from NA	13	0.92
Cultivable bacteria in *A. hypogaea* rhizosphere recovered from NA	6	0.42
Cultivable bacteria in *A. mangium* rhizosphere recovered from NA	5	0.35
Cultivable bacteria in *V. radiata* rhizosphere recovered from BJSM	6	0.42
Cultivable bacteria in *A. hypogaea* rhizosphere recovered from BJSM	9	0.64
Cultivable bacteria in *A. mangium* rhizosphere recovered from BJSM	7	0.50
Cultivable bacteria in *V. radiata* rhizosphere recovered from N-free medium	5	0.35
Cultivable bacteria in *A. hypogaea* rhizosphere recovered from N-free medium	11	0.78
Cultivable bacteria in *A. mangium* rhizosphere recovered from N-free medium	1	0.07

NA, nutrient agar medium, BJSM, *Bradyrhizobium* selective medium, N-free medium, nitrogen-free modified Burk medium

2007), thus we used a pure culture of *Bradyrhizobium* sp. DASA 64008 (lane 10) as a marker strain for verification of the species-specific migration positions. The DGGE patterns of *A. hypogaea* and *A. mangium* rhizospheres showed an intense band that migrated the same position on the gel with the reference strain, suggesting the existence of bradyrhizobia in native rhizospheres of these legumes and the ability of BJSM on the isolation of bradyrhizobia. On the contrary, there was no such a band in the DGGE pattern of *V. radiata* rhizosphere. This was a consistent result with the 16S rRNA gene sequences. The isolates closely related to bradyrhizobia were found in *A. hypogaea* and *A. mangium* rhizopheres, but not in *V. radiata* rhizosphere. It was possible that *V. radiata* rhizosphere might not contain *Bradyrhizobium* or might contain some other *Bradyrhizobium* species that could not tolerate heavy metals used in BJSM.

Moreover, the result obtained from the average values of species diversity (*S*) and simple (*I*) index also indicates that the highest genetic diversity was observed from the profiles of cultivable bacteria recovered from NA, followed by cultivable bacteria recovered from BJSM and cultivable bacteria recovered from N-free medium. This result indicates that NA medium supported the growth of large numbers and diverse groups of bacteria. Furthermore, when compared between the DGGE patterns of DNA directly extracted from the rhizospheres and DNA extracted from pooled colonies cultivated from the rhizospheres, different groups of bacteria were recovered. Several bands presented in the DGGE patterns of rhizospheres could not remain in the DGGE patterns of cultivable bacteria. On the contrary, some bands presented in the DGGE patterns of cultivable bacteria could not be detected in the DGGE profiles of rhizospheres. Some bands in the DGGE patterns of rhizospheres became more intense in the DGGE patterns of cultivable colonies. These results indicate that only a portion of the bacterial populations in rhizospheres could be cultivated by using these media. However, these media were useful for isolation and enrichment of some bacterial groups that might be less competitive in rhizospheres. Therefore, this study demonstrates the advantage of the comparative study by using both culture-dependent and culture-independent techniques. By using both approaches, we obtained overall data that provides a good benefit for evaluating microbial diversity in environmental systems. The culture-dependent methods are required for the study on functional activity of bacterial groups that can be used to evaluate phenotypic diversity and soil biological fertility. The DGGE analysis of rhizosphere community and cultivable community can be used as an alternative to traditional tools for identification of dominant species because it can be used to complete the study of microbial diversity and provides information on concentration ratios among species occurring in a particular environment (Ercolini et al. 2001).

Acknowledgments We thank Dr. Achara Nuntagij, Soil Microbiology Research Group, Department of Agriculture for providing rhizosphere soil samples.

References

Albareda M, Dardanelli MS, Sousa C, Megías M, Temprano F, Rodríguez-Navarro DN (2006) Factors affecting the attachment of rhizospheric bacteria to bean and soybean roots. FEMS Microbiol Lett 259:67–73

Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 59:143–169

Aşkın T, Kızılkaya R (2006) Assessing spatial variability of soil enzyme activities in pasture topsoils using geostatistics. Eur J Soil Biol 42:230–237

Barnes AR, Sugden JK (1990) Comparison of colorimetric methods for ammonia determination. Pharm Acta Helv 65:258–261

Bishop PE, Hawkins ME, Eady RR (1986) Nitrogen fixation in molybdenum-deficient continuous culture by a strain of *Azotobacter vinelandii* carrying a deletion of the structural genes for nitrogenase (*nifHDK*). Biochem J 238:437–442

Cappucino JC, Sherman N (1992) Microbiology: a laboratory manual. Benjamin/Cummings Publishing Company, New York, pp 125–179

Chen WM, Laevens S, Lee TM, Coenye T, de Vos P, Mergeay M, Vandamme P (2001) *Ralstonia taiwanensis* sp. nov., isolated from *Mimosa* species and sputum of a cystic fibrosis patient. Int J Syst Evol Microbiol 51:1729–1735

Christensen WB (1946) Urea decomposition as a means of differentiating *Proteus* and Paracolon cultures from each other and from *Salmonella* and *Shigella* types. J Bacteriol 52:461–466

Costa R, Götz M, Mrotzek N, Lottmann J, Berg G, Smalla K (2006) Effects of site and plant species on rhizosphere community structure as revealed by molecular analysis of microbial guilds. FEMS Microbiol Ecol 56:236–249

Das M, Royer TV, Leff LG (2007) Diversity of fungi, bacteria, and actinomycetes on leaves decomposing in a stream. Appl Environ Microbiol 73:756–767

Davies PJ (1995) The plant hormone concept: concentration, sensitivity, and transport. In: Davies PJ (ed) Plant hormones: physiology, biochemistry and molecular biology. Kluwer Academic Publishers, Dordrecht, pp 13–18

Eivazi F, Tabatabai MA (1977) Phosphatases in soils. Soil Biol Biochem 9:167–172

Ercolini D, Moschetti G, Blaiotta G, Coppola S (2001) The potential of a polyphasic PCR-DGGE approach in evaluating microbial diversity of natural whey cultures for water-buffalo mozzarella cheese production: bias of culture-dependent and culture-independent analyses. Syst Appl Microbiol 24:610–617

Felske A, Osborn AM (2005) DNA fingerprint of microbial communities. In: Osborn AM, Smith CJ (eds) Molecular microbial ecology, 1 st edn. Taylor and francis group, New York, pp 65–90

Gordon SA, Weber RP (1951) Colorimetric estimation of indoleacetic acid. Plant Physiol 26:192–195

Goris J, De Vos P, Coenye T, Hoste B, Janssens D, Brim H, Diels L, Mergeay M, Kersters K, Vandamme P (2001) Classification of metal-resistant bacteria from industrial biotopes as *Ralstonia campinensis* sp. nov., *Ralstonia metallidurans* sp. nov. and *Ralstonia basilensis* Steinle et al. 1998 emend. Int J Syst Evol 51:1773–1782

Höflich G, Wiehe W, Kühn G (1994) Plant growth stimulation by inoculation with symbiotic and associative rhizosphere microorganisms. Experientia 50:897–905

Keyser M, Witthuhn RC, Britz TJ (2006) PCR-based denaturing gradient gel electrophoretic evaluation of changes in the non-methanogenic population of stressed upflow anaerobic sludge blanket granules. World J Microbiol Biotechnol 22:1041–1048

Kowalchuk G, Buma D, de Boer W, Klinkhamer P, van Veen J (2002) Effects of above-ground plant species composition and diversity on the diversity of soil-borne microorganisms. Antonie van Leeuwenhoek 81:509–520

Lambrecht M, Okon Y, Broek AV, Vanderleyden J (2000) Indole-3-acetic acid: a reciprocal signaling molecule in bacteria-plant interactions. Trends Microbiol 8:298–300

Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics, 1st edn. Wiley, New York, pp 115–175

Marschner P, Crowley D, Yang CH (2004) Development of specific rhizosphere bacterial communities in relation to plant species, nutrition and soil type. Plant Soil 261:199–208

Merckx R, Dijkra A, Hartog AD, van Veen JA (1987) Production of root-derived material and associated microbial growth in soil at different nutrient levels. Biol Fertil Soils 5:126–132

Morgan JAW, Bending GD, White PJ (2005) Biological costs and benefits to plant-microbe interactions in the rhizosphere. J Exp Bot 56:1729–1739

Muyzer G, Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700

Nikolcheva LG, Cockshutt AM, Barlocher F (2003) Determining diversity of freshwater fungi on decaying leaves: comparison of traditional and molecular approaches. Appl Environ Microbiol 69:2548–2554

Nimnoi P, Pongsilp N (2009) Genetic diversity and plant growth promoting ability of the indole-3-acetic acid (IAA) synthetic bacteria isolated from agricultural soil as well as rhizosphere, rhizoplane and root tissue of *Ficus religiosa* L., *Leucaena leucocephala* and *Piper sarmentosum* Roxb. Res J Agric Biol Sci 5:29–41

Nimnoi P, Pongsilp N, Lumyong S (2010) Genetic diversity and community of endophytic actinomycetes within the roots of *Aquilaria crassna* Pierre ex Lec assessed by *Actinomycetes*-specific PCR and PCR-DGGE of 16S rRNA gene. Biochem Syst Ecol 38:595–601

Nimnoi P, Lumyong S, Pongsilp N (2011) Impact of rhizobial inoculants on rhizosphere bacterial communities of three medicinal legumes assessed by denaturing gradient gel electrophoresis (DGGE). Ann Microbiol 61:237–245

Nuntagij A, Abe M, Uchiumi T, Seki Y, Boonkerd N, Higashi S (1997) Characterization of *Bradyrhizobium* strains isolated from soybean cultivation in Thailand. J Gen Appl Microbiol 43: 183–187

Paffetti D, Scotti C, Gnocchi S, Fancelli S, Bazzicalupo M (1996) Genetic diversity of an Italian *Rhizobium meliloti* population from different *Medicago sativa* varieties. Appl Environ Microbiol 62:2279–2285

Patten CL, Glick BR (1996) Bacterial biosynthesis of indole-3-acetic acid. Can J Microbiol 42:207–220

Pongsilp N, Nuntagij A (2009) Genetic diversity and metabolites production of root-nodule bacteria isolated from medicinal legumes *Indigofera tinctoria, Pueraria mirifica* and *Derris elliptica* Benth grown in different geographic origins across Thailand. Am Eur J Agric Biol Sci 6:26–34

Pongsilp N, Teaumroong N, Nuntagij A, Boonkerd N, Sadowsky MJ (2002) Genetic structure of indigenous non-nodulating and nodulating populations of *Bradyrhizobium* in soils from Thailand. Symbiosis 33:39–58

Renshaw JC, Halliday V, Robson GD, Trinci APJ, Wiebe MG, Livens FR, Collison D, Taylor RJ (2003) Development and application of an assay for uranyl complexation by fungal metabolites, including siderophores. Appl Environ Microbiol 69:3600–3606

Rovira A (1956) Plant root excretions in relation to rhizosphere effect. I. The nature of root exudates from oats and peas. Plant Soil 7: 178–194

Silvestri G, Santarelli S, Aquilanti L, Beccaceci A, Osimani A, Tonucci F, Clementi F (2007) Investigation of the microbial ecology of Ciauscolo, a traditional Italian salami, by culture-dependent techniques and PCR-DGGE. Meat Sci 77:413–423

Smalla K, Wieland G, Buchner A, Zock A, Parzy J, Kaiser S, Roskot N, Heuer H, Berg G (2001) Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. Appl Environ Microbiol 67:4742–4751

Spayd RW, Bruschi B, Burdick BA, Dappen GM, Elkenberry JN, Esders TW, Figueras J, Goodhue CT, LaRossa DD, Nelson RW, Rand RN, Wu TW (1978) Multilayer film elements for clinical analysis: applications to representative chemical determinations. Clin Chem 24:1343–1350

Starnes SR, Spears JW, Froetschel MA, Croom WJ Jr (1984) Influence of monensin and lasalocid on mineral metabolism and ruminal urease activity in steers. J Nutr 114:518–525

Tong Z, Sadowsky MJ (1994) A Selective medium for the isolation and quantification of *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* strains from soils and inoculants. Appl Environ Microbiol 60:581–586

Tso SC, Chen YR (1997) Isolation and characterization of a group III isozyme of acid phosphatase from rice plants. Bot Bull Acad Sin 38:245–250

vande Broek A, Lambrecht M, Eggermont K, Vanderleyden J (1999) Auxins upregulate expression of the indole-3-pyruvate decarboxylase gene in *Azospirillum brasilense*. J Bacteriol 181: 1338–1342

Velasco AGV, Probanza A, Mañero FJG, Ramos B, García JAL (2009) Functional diversity of rhizosphere microorganisms from different genotypes of *Arabidopsis thaliana*. Community Ecol 10:111–119

Vince A, Dawson AM, Park N, O'Grady F (1973) Ammonia production by intestinal bacteria. Int J Gastroenterol Hepatol 14: 171–177

Wardle DA, Yeates GW, Williamson W, Bonner KI (2003) The response of a three trophic level soil food web to the identity and diversity of plant species and functional groups. Oikos 102: 45–56

Warembourg FR, Roumet C, Lafont F (2003) Differences in rhizosphere carbon-partitioning among plant species of different families. Plant Soil 256:347–357

Xu Y, Wang G, Jin J, Liu J, Zhang Q, Xiaobing L (2009) Bacterial communities in soybean rhizosphere in response to soil type, soybean genotype, and their growth stage. Soil Biol Biochem 41:919–925