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ORIGINAL PAPERS

Impact of rhizobial inoculants on rhizosphere bacterial communities of three medicinal legumes assessed by denaturing gradient gel electrophoresis (DGGE)

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Abstract Denaturing Gradient Gel Electrophoresis (DGGE) was used to study the impact of rhizobial inoculants on the rhizosphere bacterial communities of three medicinal legumes: *Indigofera tinctoria*, *Pueraria mirifica* and *Derris elliptica* Benth. Rhizosphere soils were collected from these legumes grown naturally in 11 provinces of Thailand. The host-specific rhizobial strains were inoculated to their hosts planted in the collected rhizosphere soils of each legume. Four months after planting, total bacterial communities DNA was extracted from the uninoculated rhizosphere soils and the inoculated rhizosphere soils. DGGE fingerprints of PCR-amplified 16S rDNA were obtained from the bacterial communities. PCR-DGGE analysis showed that the bacterial community structures in native rhizospheres of the three legumes were different from each other based on the generated dendrogram and Sorensen's index. These results suggest that different plant species and soil characteristics synergically affected the rhizosphere bacterial communities. The bacterial diversity of *I. tinctoria* and *P. mirifica* native rhizospheres were significantly different from that of *D. elliptica* Benth. native rhizosphere. Our results also showed that the inoculants contributed to the slight changes in rhizosphere community structures. In comparison with each other, the plants appeared to have a much stronger influence on the bacterial communities rather than the inoculants. Hierarchical cluster analysis revealed that the community structure of the inoculated rhizosphere of *D. elliptica* Benth. was more divergent from those of inoculated rhizospheres of *I. tinctoria* and *P. mirifica*. The ribotype richness which indicates species diversity, was highest in *I. tinctoria* rhizosphere, followed by *P. mirifica* rhizosphere and *D. elliptica* Benth. rhizosphere, respectively.

Keywords DGGE · Medicinal legume · Rhizobial inoculants · Rhizosphere bacterial community

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Introduction

The rhizosphere is the zone of the soil that is directly influenced by plant roots and is characterized by complex interactions between beneficial and deleterious microorganisms and their host plants (Estrada et al. 1998). The rhizosphere represents unique and diverse microorganisms that are controlled by organic materials derived from the root of plants and root exudates (Merckx et al. 1987; Rovira 1956). Previous studies reported that microbial diversity is affected not only by host plants but also by soil compositions (Kotani-tanoi et al. 2007). The rhizosphere contains PGPR (plant-growth promoting rhizobacteria), which are described as a heterogeneous group of bacteria that live abundantly in the rhizosphere and root surface, and also improve the quality of plants directly or indirectly (Glick 1995; Kloepper et al. 1989). The relationship between microbial diversity and function in soil is still unclear. It is also not understood what factors are responsible for the high microbial diversity

in soil (Kotani-tanoi et al. 2007). Conventional culturing techniques such as culture-based methods may not be suitable for studying soil microbial diversity because those techniques lack the ability to isolate unculturable microbes (Amann et al. 1995). Some isolation methods for rhizosphere soil produce an imbalance on the abundance of bacteria because fast-growing groups and dominant species can grow rapidly and compete successfully on isolation plates, ultimately leading to the obtaining of only small amounts of the slow-growing groups. It has been estimated that only 0.1–1.0% of the microorganisms found on typical agricultural soils would be culturable by using current culture media formulations while culture-independent methods based on 16S rRNA gene amplification permit the detection of over 90% of microorganisms that can be observed microscopically in situ (Hill et al. 2000).

More recently, a molecular technique, denaturing gradient gel electrophoresis (DGGE), has been developed to study the microbial community and to monitor microbial populations in many ecological systems. This technique can be used to analyze specific genes of interest such as small subunit ribosomal RNA genes (Lui et al. 2010), nitrogenase reductase gene (Diallo et al. 2004) and antibiotic synthetic genes (Bergsma-Vlami et al. 2005). DGGE has been employed to investigate the microbial communities within the different plant rhizospheres and to monitor the effects of microbial inoculants on the rhizosphere communities. van Dillewijn et al. (2002) determined the effect of *Sinorhizobium meliloti* on the rhizosphere microbial community of its host plant, alfafa (*Medicago sativa*), and found that the plant appeared to have a much stronger influence on the microbial community compared with a *S. meliloti* inoculation. Schwieger and Tebbe (2000) studied the impact of rhizobial inoculant, *S. meliloti* L33, on the microbial communities of *M. sativa* rhizosphere and found that both plant species and inoculant had an effect on the rhizosphere structure. Lioussanne et al. (2010) reported that the arbuscular mycorrhizal (AM) fungi, *Glomus intraradices* and *G. mosseae*, caused a significant change in the bacterial community structure of tomato rhizosphere by physical or chemical factors associated with the mycelium volatiles and/or root surface bound substrates rather than by compounds present in root exudates of the mycorrhizal plants. Costa et al. (2006) analyzed the impact of plant species and sampling sites on the rhizosphere microbial communities of strawberry (*Fragaria ananassa* Duch.) and oilseed rape (*Brassica napus* L.). The plant species was found to exert a greater influence on the rhizosphere microbial communities than the sampling sites.

Leguminous plants can be nodulated by symbiotic bacteria, collectively known as rhizobia. In Thailand, three leguminous plants: *Indigofera tinctoria*, *Pueraria mirifica* and *Derris elliptica* Benth. are widely consumed in many applications of medicine. Phytochemical compounds from *I. tinctoria* have revealed their several biological activities such as antioxidant activity (Bakasso et al. 2008; Sreepriya et al. 2001) and anticancer activity (Han 1994). *Pueraria mirifica* has been known as a source of phytoestrogens (Okamura et al. 2008). Phytochemical compounds from *D. elliptica* Benth. have been reported for their antioxidant activity (Palasuwan et al. 2005). These legumes are naturally grown across Thailand without introducing microbial inoculants so their rhizosphere communities are considered as indigenous communities. In addition, the influence of these plants on the rhizosphere microbial communities has not been investigated. Therefore, we were interested to evaluate whether the plant hosts affect the rhizosphere bacterial community, and whether the inoculation of their microsymbionts can induce significant changes in the community structures. The objectives of this study were to determine the bacterial community and the bacterial diversity of the native rhizosphere soils from these leguminous plants and to evaluate the impact of 10 rhizobial inoculants on the rhizosphere bacterial communities by using the DGGE technique.

Materials and methods

Bacterial strains and medium The 10 rhizobial strains used in this study are indigenous strains isolated from root nodules of *I. tinctoria*, *P. mirifica* or *D. elliptica* Benth. grown naturally in Thailand without introducing microbial inoculants or chemical fertilizers. All strains were verified as capable of nodule formation on their original hosts. *Indigofera tinctoria*-nodulating strains include the unidentified strain DASA 57050, *Rhizobium* sp. DASA 57053 and the unidentified strain DASA 57075. *Pueraria mirifica*-nodulating strains include *Bradyrhizobium* sp. DASA 64008, *Bradyrhizobium* sp. DASA 64011, *Rhizobium* sp. DASA 64026 and *Rhizobium* sp. DASA 64027. *Derris elliptica*-nodulating strains include *Rhizobium* sp. DASA 68006, *Rhizobium* sp. DASA 68020 and *Rhizobium* sp. DASA 68025. Yeast Mannitol (YM) medium (Keele et al. 1969) was used for growth and maintenance.

Rhizosphere soils collections Rhizosphere soils were collected from *I. tinctoria*, *P. mirifica* and *D. elliptica* Benth. grown naturally in 11 provinces of Thailand. *Indigofera tinctoria* rhizospheres were pooled from samples collected from 3 provinces: Chiang Mai, Tak and Phrae. *Pueraria mirifica* rhizospheres were pooled from samples collected from 7 provinces: Pichit, Kanchanaburi, Lampang, Lopburi, Burirum, Phayao and Nakhon Ratchasima. *Derris elliptica* Benth. rhizosphere was collected from Petchaburi province.

The inoculation of rhizobial strains and plant growth Seeds of *I. tinctoria* and seeds of *P. mirifica* were scarified and

surface sterilized with 3% hydrogen peroxide. The seeds were laid on moistened cotton plates and incubated at 25°C in the dark for 1–2 days. Branches with adventitious roots of *D. elliptica* Benth. were surface-sterilized with 95% ethanol. The germinated seeds and branches were planted in Leonard's jars filled with 500 g of pooled rhizosphere soils from each of the different origins of the host plant. The inoculum was prepared by culturing each rhizobial strain in YM medium at 30°C with rotary shaking (120*g*) for 5 days. Each germinated seed or root was inoculated with 1 ml of bacterial suspension (10^9 CFU/ml) of each host-specific strain and plants were fertilized with N-free nutrient solution (Broughton and Dilworth 1971). The uninoculated controls were included in the experiment. Three replicate jars were prepared for each treatment.

Soil DNA extraction and rDNA amplification Four months after planting, the rhizosphere samples were taken from each treatment. Three replicates of rhizosphere inoculated with each rhizobial strain were pooled together. The total DNA was extracted from the uninoculated rhizospheres and the inoculated rhizospheres by using a GF-1 soil DNA extraction kit (Vivantis, Malaysia) according to the manufacturer's instruction. Total DNA of soil samples was amplified by PCR with a MyCycler™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). The variable V6/V9 regions of 16S rDNA (corresponding to positions 968–1,401 of *Escherichia coli* 16S rRNA gene; Kandeler et al. 2000) were amplified by using a set of primer F984 (5′-AAC GCG AAG AAC CTT AC-3′) with the GC-clamp (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G-3′) and primer R1378 (5′-CGG TGT GTA CAA GGC CCG GGA ACG-3′) (Heuer et al. 1997). PCR reactions were performed in 25-μl reaction volumes, containing 2 μl of DNA template (50 ng), 0.75 μl of *Taq* DNA polymerase (5 U μl^{-1}) (BioLabs, MD), 3 μl of 10× PCR reaction buffer supplied with the enzyme, 1.25 μl of 50 mM $MgCl_2$, 1 μl of each of the primers (10 μM), 13 μl of 2.5 mM dNTPs (Vivantis, Malaysia) and 3 μl of Nuclease free water (Promega, WI, USA). Positive and negative controls were included in every set of PCR. The PCR amplification conditions were as follows: initial denaturation was performed at 95°C for 5 min, followed by denaturation at 94°C for 1 min, primer annealing at 53°C for 1 min, and chain elongation at 72°C for 1 min. These 3 steps were repeated for 35 cycles. Final elongation was performed at 72°C for 10 min and the PCR reactions were cooled to 4°C. The amplified products were separated on 1% agarose gels containing ethidium bromide (EtBr) and visualized under UV light.

Denaturing gradient gel electrophoresis The PCR fragments were separated by using DGGE performed with the BioRad DCode™ Universal Mutation Detection System (Bio-Rad). 25 μl (approx. 2.5 μg) of mixed PCR products from each soil sample and 10 μl (approx. 1 μg) of a PCR product from pure culture of each strain were applied to 6% polyacrylamide gel with a liner gradient of 35–50% denaturant [100% denaturant corresponds to 40% (vol vol^{-1}) of formamide plus 7 M of urea]. Electrophoresis was performed at 150 V for 5 h at a constant temperature of 60°C. Gels were then strained with EtBr for 20 min and visualized under UV light.

Statistical analysis Relatedness of bacterial communities was determined by using similarity coefficients of bands common to two samples. Common bands are defined as bands that migrate the same distance on polyacrylamide gel. The total numbers of different bands was determined for the samples being compared. Then the bands in each sample were scored in a binary matrix based on the presence (1) or absence (0) of bands. Sorensen's index of similarity (C_s) was used to make pairwise calculations of band sharing between samples, C_s was calculated using the formula $C_s=2j/(a+b)$. The definition, j, is the number of bands common to A and B; a is the number of bands in sample A; b is the number of bands in sample B (Sorensen 1948). The bacterial complexity of each sample was expressed by various indices of biodiversity, calculated from the DGGE profile: (1) species diversity (S), which corresponds to the number of bands in a DGGE profile; and (2) simple index (I_i), which was 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 (Silvestri et al. 2007). To examine the structural diversity, number of bands in each rhizosphere was subjected to nonparametric test by using Kruskal-Wallis. A one-way analysis of variance (ANOVA) and Tukey HSD test for post hoc analysis (significant at $P<0.05$) were used to establish significant differences (Zaady et al. 2010). Finally, cluster analysis of the DGGE profiles was performed using the scored data from a binary matrix. The scored data was subjected to classify hierarchical clustering according to the Ward's method with the software package SPSS Version 16.0 (SPSS, Chicago, IL, USA).

Results and discussion

The objectives of this study were to compare the bacterial community structures of native rhizosphere soils from three medicinal legumes: *I. tinctoria, P. mirifica* and *D. elliptica* Benth., and to evaluate the impact of 10 rhizobial

inoculants on the rhizosphere bacterial communities by using PCR-DGGE analysis. DGGE band patterns of the native rhizospheres (the uninoculated controls), the inoculated rhizospheres and pure cultures of the inoculants are depicted in Fig. 1. A binary matrix of bands detected in each lane of DGGE profiles is shown in Table 1. Even though the DGGE profiles showed bands common to all native rhizospheres revealing the common bacterial groups in a variety of soils in Thailand, these profiles were different from each other suggesting that plant species and soil characteristics had an impact on the rhizosphere bacterial diversity. As the experiment was performed with pooled rhizosphere soils from each of the different origins of the host plant, therefore it could not separate between the impact of 2 factors, including plant species and soil characteristics, on the rhizosphere bacterial diversity. The impact of plant species and soil type on rhizosphere microbial community has been compared in the previous study of Marschner et al. (2001), who examined the eubacterial community structures in the plant rhizosphere with respect to plant species, soil type, and root zone location. They found that the rhizosphere bacterial community was influenced by plant species and soil type. The bacterial community of some plants such as chickpea was more affected by soil type than root zone and plant species. In contrast, the bacterial community of some plants such as rape and Sudan grass was influenced primarily by root zone, whereas soil type was less important. In this study, the native rhizospheres of *P. mirifica* and *D. elliptica* Benth. presented one and two strong bands, respectively, inferring that there were common dominant species inhabited in the sampling locations. Plant species effects are probably due to differences in the composition of root cell components and root exudates (Marschner et al. 2001). Plants produce root exudates that 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 also create a selective pressure on the microbial community (Kaksonen et al. 2006). The strong effects of soil on the bacterial community in rhizosphere may have resulted from the difference of physical and chemical characteristics of soil such as soil structure, organic matter content and nutrient (Carelli et al. 2000).

In the inoculation experiments, the DGGE fingerprints of *I. tinctoria* rhizosphere inoculated with rhizobial strains DASA 57050, DASA 57053 and DASA 57075 (lanes 2, 3 and 4, respectively) were distinguishable from each other and the uninoculated control (lane 1). Similarity coefficients (Sorensen's index) were calculated from the DGGE profiles. A value of 1.00 indicates all bands are shared and 0.00 indicates no bands are shared. As shown in Table 2, Sorensen's index of the native *I. tinctoria* rhizosphere and its inoculated treatments ranged between 0.66 and 0.90, indicating that the bacterial community structures of the native rhizosphere and the inoculated rhizospheres were different. Based on Sorensen's index, the bacterial community structures of the rhizosphere inoculated

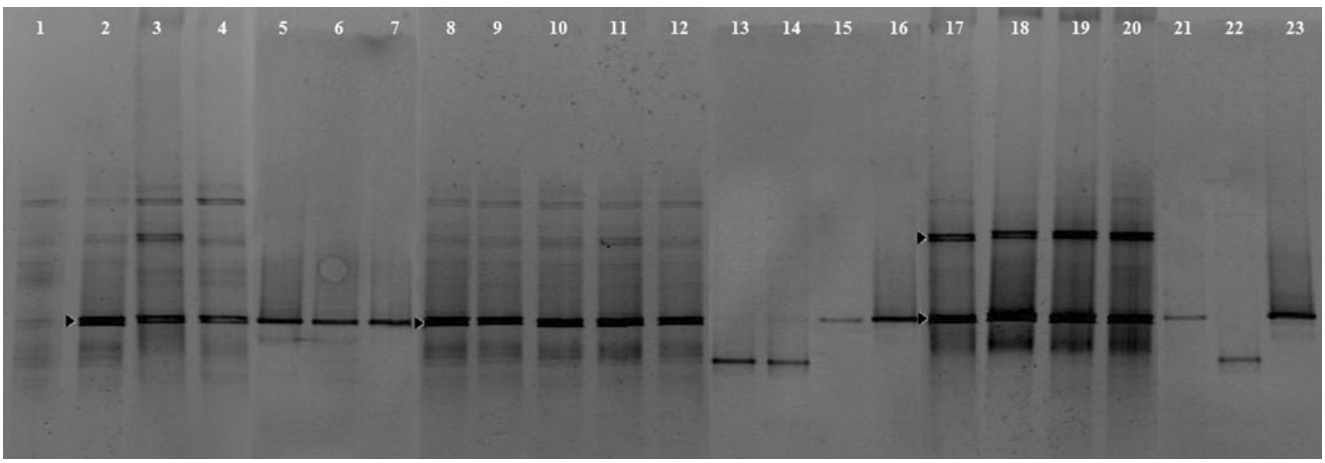

Fig. 1 DGGE band patterns of the native rhizospheres from 3 plants and the rhizospheres inoculated with 10 rhizobial strains compared with pure cultures of rhizobial inoculants. *Lane 1 I. tinctoria* rhizosphere (the uninoculated control), *2 I. tinctoria* rhizosphere inoculated with DASA 57050, *3 I. tinctoria* rhizosphere inoculated with DASA 57053, *4 I. tinctoria* rhizosphere inoculated with DASA 57075, *5* DASA 57050 pure culture, *6* DASA 57053 pure culture, *7* DASA 57075 pure culture, *8 P. mirifica* rhizosphere (the uninoculated control), *9 P. mirifica* rhizosphere inoculated with DASA 64008, *10 P. mirifica* rhizosphere inoculated with DASA 64011, *11 P. mirifica* rhizosphere inoculated with DASA 64026, *12 P. mirifica* rhizosphere inoculated with DASA 64027, *13* DASA 64008 pure culture, *14* DASA 64011 pure culture, *15* DASA 64026 pure culture, *16* DASA 64027 pure culture, *17 D. elliptica* rhizosphere (the uninoculated control), *18 D. elliptica* rhizosphere inoculated with DASA 68006, *19 D. elliptica* rhizosphere inoculated with DASA 68020, *20 D. elliptica* rhizosphere inoculated with DASA 68025, *21* DASA 68006 pure culture, *22* DASA 68020 pure culture, *23* DASA 68025 pure culture

Table 1 Binary matrix of bands detected in each lane of DGGE profiles

Lane[a]	Number of bands															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	0[b]	1[c]	1	0	1	0	1	1	1	1	1	0	1	0	1	1
2	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
3	1	1	1	1	1	0	1	0	1	1	1	1	1	0	1	1
4	0	1	1	0	1	0	1	1	1	1	1	0	0	0	1	0
5	0	1	1	0	1	0	0	1	0	1	1	1	1	0	1	1
6	0	1	1	0	1	0	0	1	0	1	1	1	1	0	1	0
7	0	1	1	0	1	0	0	1	0	1	1	1	1	0	1	0
8	0	1	1	0	1	0	1	1	0	1	1	1	1	0	1	1
9	0	1	1	0	1	0	0	1	0	1	1	1	1	0	1	0
10	0	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0
11	0	1	0	0	0	1	1	0	1	0	1	1	0	0	0	0
12	0	1	0	0	0	1	1	0	1	0	1	1	0	0	0	0
13	0	1	0	0	0	1	1	0	1	0	1	1	0	0	1	0

[a] *Lane 1 I. tinctoria* rhizosphere (the uninoculated control), *2 I. tinctoria* rhizosphere inoculated with DASA 57050, *3 I. tinctoria* rhizosphere inoculated with DASA 57053, *4 I. tinctoria* rhizosphere inoculated with DASA 57075, *5 P. mirifica* rhizosphere (the uninoculated control), *6 P. mirifica* rhizosphere inoculated with DASA 64008, *7 P. mirifica* rhizosphere inoculated with DASA 64011, *8 P. mirifica* rhizosphere inoculated with DASA 64026, *9 P. mirifica* rhizosphere inoculated with DASA 64027, *10 D. elliptica* rhizosphere (the uninoculated control), *11 D. elliptica* rhizosphere inoculated with DASA 68006, *12 D. elliptica* rhizosphere inoculated with DASA 68020, *13 D. elliptica* rhizosphere inoculated with DASA 68025

[b] 0 represents the absence of band

[c] 1 represents the presence of band

Table 2 Sorensen's index similarity of the bacterial community in each sample

Lanes compared	1	2	3	4	5	6	7	8	9	10	11	12	13
1	1.00[a]												
2	0.76	1.00											
3	0.83	0.85	1.00										
4	0.90	0.66	0.72	1.00									
5	0.85	0.80	0.78	0.73	1.00								
6	0.80	0.75	0.72	0.73	0.94	1.00							
7	0.80	0.75	0.72	0.73	0.94	1.00	1.00						
8	0.90	0.84	0.83	0.80	0.95	0.90	0.90	1.00					
9	0.80	0.75	0.72	0.73	0.94	1.00	1.00	0.90	1.00				
10	0.26	0.42	0.35	0.28	0.28	0.30	0.30	0.40	0.30	1.00			
11	0.47	0.47	0.52	0.50	0.37	0.40	0.40	0.47	0.40	0.80	1.00		
12	0.47	0.47	0.52	0.50	0.37	0.40	0.40	0.47	0.40	0.80	1.00	1.00	
13	0.55	0.54	0.60	0.58	0.47	0.50	0.50	0.55	0.50	0.72	0.92	0.92	1.00

Lane 1 I. tinctoria rhizosphere (the uninoculated control), *2 I. tinctoria* rhizosphere inoculated with DASA 57050, *3 I. tinctoria* rhizosphere inoculated with DASA 57053, *4 I. tinctoria* rhizosphere inoculated with DASA 57075, *5 P. mirifica* rhizosphere (the uninoculated control), *6 P. mirifica* rhizosphere inoculated with DASA 64008, *7 P. mirifica* rhizosphere inoculated with DASA 64011, *8 P. mirifica* rhizosphere inoculated with DASA 64026, *9 P. mirifica* rhizosphere inoculated with DASA 64027, *10 D. elliptica* rhizosphere (the uninoculated control), *11 D. elliptica* rhizosphere inoculated with DASA 68006, *12 D. elliptica* rhizosphere inoculated with DASA 68020, *13 D. elliptica* rhizosphere inoculated with DASA 68025

[a] A value of 1.00 indicates all bands are shared and 0.00 indicates no bands are shared

with the strain DASA 57050 was mostly divergent from the uninoculated control, followed by the rhizosphere inoculated with the strains DASA 57053 and DASA 57075, respectively. Comparison between the band positions of the pure cultures and the inoculated treatments on the DGGE gel infers that the inoculants resulted in the obvious increase in the band intensity of the inoculated treatments presented in the lower part of DGGE gel. These profiles indicate that the inoculant strains DASA 57050, DASA 57053 and DASA 57075 were capable of surviving and proliferating in soils. The nodule formation on their host plant (data not shown) also confirmed the existence of these strains which subsequently contributed to changes in the community structures. For DGGE fingerprints of *P. mirifica* rhizospheres, the profiles of the uninoculated control (lane 8) and treatments inoculated with each of strains DASA 64008 (lane 9), DASA 64011 (lane 10), DASA 64026 (lane 11) and DASA 64027 (lane 12) were highly similar. This result corresponds with Sorensen's index of similarity that presented a high value, up to 0.95. Moreover, Sorensen's index revealed that the bacterial community structure of *P. mirifica* rhizosphere was slightly affected by these 4 strains. For the rhizospheres of *D. elliptica* Benth., the DGGE patterns presented in Fig. 1 show that the profiles of the native rhizosphere inoculated with each of strains DASA 68006, DASA 68020 and DASA 68025 (lanes 18, 19 and 20, respectively) were slightly different when compared with the native rhizosphere. This finding may implies that these inoculants induced slight changes in the community structures. According to the Sorensen's index, the similarity coefficient of the bacterial communities from the *D. elliptica* Benth. native rhizosphere and those inoculated with each of strains DASA 68006 and DASA 68020 conferred higher values than that inoculated with the strain DASA 68025. This suggests that the inoculation of the strain DASA 68025 affected the bacterial community more than the strains DASA 68006 and DASA 68020. In this study, no band presented in the gel located in the high gradient portion, suggesting that bacteria with high GC content were not considered to be numerically dominant populations. The selective pressure of the inoculant on the rhizosphere bacterial community has been reported in the previous study. *Medicago sativa*-nodulating strain, *S. meliloti* L33, affected the composition of the rhizosphere bacterial community by reducing the numbers of the γ subgroup of the *Proteobacteria* and increasing the number of the α subgroup of the *Proteobacteria*. This shift can be interpreted as a replacement of general bacteria by rhizobia. The increase of rhizobia as a consequence of *S. meliloti* inoculation may have been a result of increased production of root exudates by the nodulated plants (Schwieger and Tebbe 2000).

For clustering analysis, the generated dendrogram (Fig. 2) exhibits three clusters. Each cluster consisted of rhizosphere bacterial communities associated with the same host plant. This indicates that the community structures of rhizospheres from three legumes were clearly different from each other. Different plant species and soil characteristics appeared to have a much stronger influence on the bacterial community conpared with the inoculants. Our result agrees with the previous study of Costa et al. (2006) who proposed that plant type and location (sampling site) influenced microbial community structure in the rhizosphere. Different plant species select different bacterial communities in the vicinity of their roots and these plant-specific enrichments can be increased by repeated cultivation of the plant species in the same field (Smalla et al. 2001). van Dillewijn et al. (2002) demonstrated that the host plant, alfala (*M. sativa*), appeared to have a much stronger influence on the microbial community rather than the inoculation of its microsymbiont, *S. meliloti*. Marschner et al. (2004) investigated the important factors such as soil pH, soil type, plant species and plant age on the structure of the rhizosphere bacterial community. They concluded that those different factors contributed to shaping the species composition in the rhizosphere. Root exudate amount and composition are the key drivers for the different community structure. In this study, the generated dendrogram shows a clear separation between the *D. elliptica* Benth. rhizosphere and the rhizospheres of the other plants. The first cluster of the dendrogram contained rhizosphere bacterial communities associated with *I. tinctoria*. The native *I. tinctoria* rhizosphere and the *I. tinctoria* rhizosphere inoculated with the strain DASA 57075 were claded together at a low Euclidean distance of 1, and they were also linked together with the *I. tinctoria* rhizosphere inoculated with the strains DASA 57050 and DASA 57053 at Euclidean distance of 6. These values indicate that the native *I. tinctoria* rhizosphere and the treatment inoculated with the strain DASA 57075 showed higher similarity of the profiles to each other than to other treatments. The second cluster was the cluster of *P. mirifica* rhizosphere that separated from the *I. tinctoria* cluster at Euclidean distance of 9. In this cluster, the native *P. mirifica* rhizosphere and the *P. mirifica* rhizosphere inoculated with the strain DASA 64026 had a close association with a low Euclidean distance of 1 and they were related to the rhizospheres inoculated with each of strains DASA 64008, DASA 64011 and DASA 64027 at Euclidean distance of 3. The *P. mirifica* rhizospheres inoculated with each of strains DASA 64008, DASA 64011 and DASA 6027 were claded together at low Euclidean distance of 1. The results from the second cluster imply that the strain DASA 64026 contributed to a slightly change of the community structures. The third cluster consisted of the rhizosphere bacterial communities associated with *D. elliptica* Benth. In this cluster, the community structure of the rhizosphere inoculated with each of strains

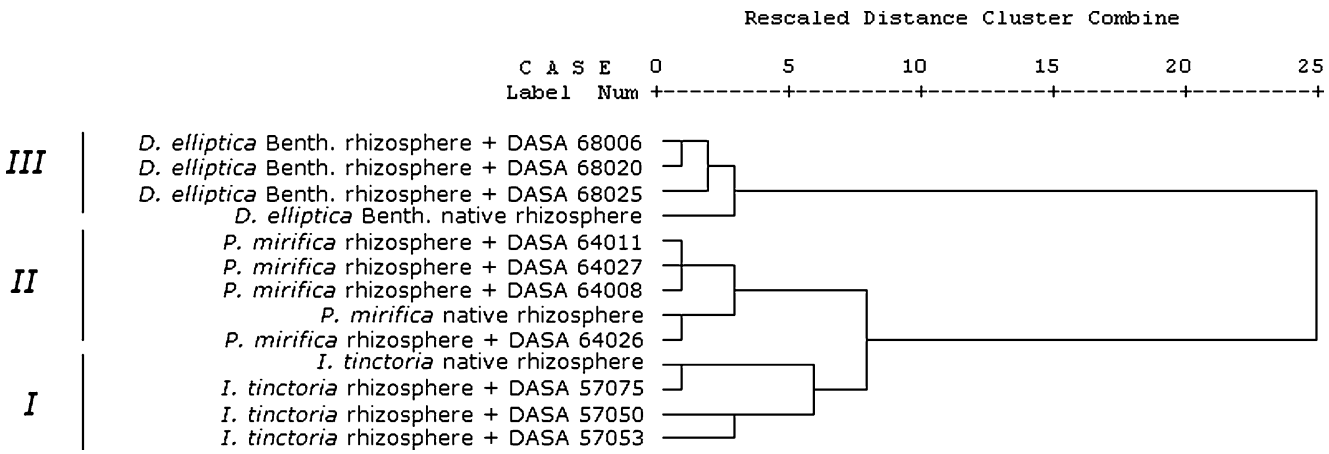

Fig. 2 Hierarchical cluster analysis of banding patterns. Dendrogram generated by using Ward's cluster analysis. Scale indicates Euclidean distance

DASA 68006 and DASA 68020 had the closest association with a low Euclidean distance of 1 and they were linked with the rhizospheres inoculated with the strain DASA 68025 at Euclidean distance of 2. The native *D. elliptica* Benth. rhizosphere showed more divergent lineage than other treatments. This indicates that a shift in the bacterial community might be caused by the inoculation treatments. The results obtained from clustering analysis were consistent with the results obtained from Sorensen's index in all treatments, as discussed above. However, it should be noticed that the similarity coefficients could not be used to estimate the community structures in the case of the presence of weak and smear bands that resulted in a high value of Sorensen's index (Nakatsu et al. 2000).

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. The Kruskal–Wallis test indicated that the genetic diversity in each rhizosphere was different. The Tukey HSD results indicated that the bacterial diversity of *I. tinctoria* rhizosphere and *P. mirifica* rhizospheres was significantly greater ($P<0.05$) than that of *D. elliptica* Benth. rhizospheres. While the bacterial diversity of *I. tinctoria* rhizospheres and *P. mirifica* rhizospheres was not significantly different ($P=0.66$) from each other. Table 3 presents the values obtained from species diversity (S) and simple (I) indexes for each sample. Simple index of rhizospheres from *I. tinctoria* ranged between 0.60 and 1.00, followed by Simple indexes of rhizospheres of *P. mirifica* and *D. elliptica* Benth. that ranged between 0.60 and 0.73 and 0.26 and 0.46, respectively. These indexes indicate

Table 3 The indexes of the biodiversity calculated from each sample

Samples	Rhizospheric bacteria	
	Species diversity	Simple index
I. tinctoria native rhizosphere	11	0.73
I. tinctoria rhizosphere + DASA 57050	15	1.00
I. tinctoria rhizosphere + DASA 57053	13	0.86
I. tinctoria rhizosphere + DASA 57075	9	0.60
P. mirifica native rhizosphere	10	0.66
P. mirifica rhizosphere + DASA 64008	9	0.60
P. mirifica rhizosphere + DASA 64011	9	0.60
P. mirifica rhizosphere + DASA 64026	11	0.73
P. mirifica rhizosphere + DASA 64027	9	0.60
D. elliptica Benth. native rhizosphere	4	0.26
D. elliptica Benth. rhizosphere + DASA 68006	6	0.40
D. elliptica Benth. rhizosphere + DASA 68020	6	0.40
D. elliptica Benth. rhizosphere + DASA 68025	7	0.46

that species diversity was highest in *I. tinctoria* rhizospheres followed by *P. mirifica* and *D. elliptica* Benth. These results were consistent with S indexes.

Using the DGGE technique, we were able to distinguish microbial communities in rhizospheres associated with different plants and found that the differences of the bacterial community structures were greatly dependent on plants and soils rather than the inoculants. This implies that the bacterial communities and the bacterial diversity in rhizosphere highly depend on their abilities to take advantage of a specific environment or to adapt and change conditions, subsequently adjust themselves to utilize or tolerate substances in the vicinity of plant roots.

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