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Trial of Direct Diagnosis of *Bradyrhizobium japonicum* and *B. elkanii* from Soils by Fatty Acid Analysis Based on Plant Bait Technique

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Previous studies have already reported that cellular fatty acid analysis is a useful taxonomic tool for classifying and identifying *Bradyrhizobium* strains, including *B. japonicum*, *B. elkanii* and *Bradyrhizobium* sp. obtained from mungbean. In this study, the existence of species–specific fatty acids, 16:1ω5*cis*, 17:0 cyclo and 19:0 cyclo ω8*cis* were remarked as the identification profiles within *B. japonicum* and *B. elkanii*. Direct detection of *B. japonicum* and *B. elkanii* from plant grown soils were performed by whole cellular fatty acid analysis combined with plant baiting technique. Tested plant materials, soybean, mungbean, groundnut and cowpea were used as the plant baiting experiment. Soil materials, contaminated soils to single strain and mixture of *B. japonicum* and *B. elkanii*, and natural infested soils were prepared for whole cellular fatty acid analysis. Bacteria trap experiment with plant baiting technique revealed that whole cellular fatty acid analysis was effectively detected *B. japonicum* and *B. elkanii* from all soil materials by using soybean plant.

INTRODUCTION

Physiological and cultural characterization was differentiated between genus *Agrobacterium* spp. and *Bradyrhizobium* spp. A cultural criterion of *Rhizobium* spp. is shown to 2–4 mm of colony growth for 3–5 days but colony growth of *Bradyrhizobium* spp. is less than 1 mm for 5–7 days. Most significant difference is the site of nitrogen fixation gene (nif) and nodule forming gene (nod), existing on Sym plasmid of *Rhizobium* spp. and on chromosome region of *Bradyrhizobium* spp., respectively.

The genus *Bradyrhizobium* was recognized as the slow–growing members of the genus *Rhizobium* (Jordan, 1982). The taxonomy of the *Bradyrhizobium*, *B. japonicum* (Hollis *et al.*, 1981) and *B. elkanii* (Kuykendall *et al.*, 1992) were established species (Young and Haukka, 1996). Recent research have proposed a new species, *B. liaoningense* was an extra–slow growing member and characterized as unique phenotype and genotype (Xu *et al.*, 1995). Moreover, *Bradyrhizobium* sp. isolated from the root nodules of peanut (*Arachis hypogaea*) was also performed on the study (Zhang *et al.*, 1999).

These species of *Bradyrhizobium* is now aspired to rapid and easy identification at the species revel because of changeable of taxonomy and possibility to additional species. Previous research showed that whole cellular fatty acid analysis was successfully characterized and evaluated the *Bradyrhizobium* strains belonging to five species (Jarvis and Tighe, 1994; Tighe *et al.*, 2000). This analysis is based on the extraction of fatty acid–methyl ester (FAME) from bacterial cells, determination of the cellular fatty acid compositions and comparison of the fatty acid profiles among strains.

The aim of the experiment is to establish the direct detection method of *Bradyrhizobium* spp. from soils by using plant baiting trap method to analyze whole cellular fatty acid originated from bacterial cells. In this experiment, rapid and easy non–isolation method of *B. japonicum* and *B. elkanii* were created by plant bait trapping system.

MATERIALS AND METHODS

Bacterial strains

The strains of *Bradyrhizobium* used in this experiment are listed in Table 1. They were seven strains of *B. japonicum* and six strains of *B. elkanii* isolated from root nodule of mungbean as the bait trap method from newly cropped areas. All strains analyzed in this experiment were grown using methods previously described (Graham *et al.*, 1995; Jarvis and Tighe, 1994).

Table 1. Lists of strains of *B. japonicum* and *B. elkanii* used in this experiment.

Species	Strain	Origin	Host
B. japonicum	MAFF303138	Kumamoto	Soybean
	MAFF303154	Kumamoto	Soybean
	MAFF303174	Kumamoto	Soybean
	MAFF303178	Fukuoka	Soybean
	MAFF303166	Nagasaki	Soybean
	MAFF303195	Kagoshima	Soybean
	MAFF303199	Kagoshima	Soybean
B. elkanii	MAFF303126	Wakayama	Soybean
	MAFF303142	Tottori	Soybean
	MAFF303168	Kagawa	Soybean
	MAFF303193	Niigata	Soybean
	MAFF303196	Kagoshima	Soybean
	MAFF303203	Kagoshima	Soybean

Plant baiting method

For plant baiting trap method, host plants of *Bradyrhizobium* spp., soybean, pigeon pea, groundnut, cowpea, mungbean, black gram and azuki bean, were used in this experiment. After finishing harvest, residue plant materials were dried with natural condition for 20 days and removed leaf and small stem (below than 5 mm in diameter). More than 7 mm in diameter of plant materials were then cut with 5 cm, autoclaved and dried with electric oven at 50 °C for 2 hour.

Soil samples for direct detection of *B. japonicum* and *B. elkanii* strains were prepared as a single species–contaminated soil, two species–contaminated soil, and naturally infested soils obtained from mungbean growing field. 250 ml of vermiculite were autoclaved at 120 °C for 90 min in 500 ml grass beaker, inoculated bacterial suspension at the concentration of 10^8cfu/ml for 10 days. Then plant–baiting materials were inserted into inoculated vermiculite and naturally polluted soils and incubated at 30 °C for 5–7

days. After incubation, bait plant materials were picked out, washed with distilled water, dried on sterile filter paper and used following experiment.

Whole cellular fatty acid analysis

Cultivation for extraction of FAME was performed on the method of Jarvis and Tighe (1994) with slight modifications, that 7-days incubation periods were essential for achieving satisfactory of cell mass. Saponification, methylation and recovery of fatty acids in the tester strain were reported by previously described. Analysis of FAME was performed by a Shimadzu GC-17A gas chromatograph equipped with a HR-SS-10 capillary column. The fatty acid compounds for each strain were identified by first converting the total cellular fatty acids to FAME. Cellular fatty acids were identified by comparing the equivalent chain length (ECL) of each compound to a retention time that represents the manufactured fatty acid compounds. The quality of each compound in a strain was determined as a percentage of the total amount of fatty acid compounds present for that strain.

RESULTS

Fatty acid composition

Table 2. shows that fatty acid compounds detected in identified strains included 16:0, 16:1 ω5*cis*, 16:1 ω7*cis*, 17:0 cyclo, 17:1 ω8*cis*, 18:0, 19:0 cyclo ω8*cis*, 20:2 ω6, 9*cis* and summed feature 7. Summed feature 7 is consisting 18:1 ω7*cis*/ω9*trans*/ω12*trans* and 18:1 ω7*cis*/ω9*cis*/ω12*trans*. Species specific fatty acids contained 17:1 ω8*cis* and 16:1 ω5*cis* in *B. japonicum* and 17:0 cyclo in *B. elkanii*. Fatty acids detected in all tested strains were also presented the existence of subgroup within *B. japonicum* and *B. elkanii*.

Strains of *B. japonicum* were categorized from FAG-I and FAG-II based on com-

Table 2. Mean fatty acid compositions of *B. japonicum* and *B. elkanii*.

Fatty acid	*B. japonicum*		*B. elkanii*	
	FAG-I	FAG-II	Group I	Group II
16:0	12.38	11.74	11.92	9.77
16:1 ω5*cis*	2.78	nd	nd	nd
16:1 ω7*cis*	0.88	0.96	0.50	0.52
17:0	nd	0.14	nd	nd
17:0 cyclo	nd	nd	0.97	0.53
17:1 ω6*cis*	0.14	0.15	nd	nd
17:1 ω8*cis*	0.46	nd	nd	nd
18:0	0.72	0.54	0.35	0.14
19:0 cyclo ω8*cis*	nd	1.66	19.12	8.67
20:2 ω6,9*cis*	nd	nd	0.28	nd
Summed feature 7	82.64	84.81	66.86	80.38

Note Figures in parentheses indicate the standard deviation. nd, values less than 0.1% of total or in which the standard deviation exceeds the mean.
Summed feature 7 is composed of 18:1 ω7*cis*/ω9*trans*/ω12*trans*, 18:1 ω9*cis*/ω12*trans*/ω7*cis* and 18:1 ω*trans*/ω9*trans*/ω7*cis* fatty acids.

positions qualitative differences compared to quantitative differences of three fatty acids. FAG–I contains 16:1 ω5*cis* and 17:1 ω8*cis* and FAG–II contains 19:0 cyclo ω8*cis*. Strains of *B. elkanii* were consisting two differential groups, group I and II based on the variation of quantitative differences of fatty acid compositions. Group I is high fatty acid composition of 17:0 cyclo and 19:0 cyclo ω8*cis*, and Group II has high concentration of summed feature 7.

Direct detection of several soils using fatty acid analysis based on plant baiting method

Table 3. shows that fatty acid analysis based on plant baiting method revealed that soybean and mungbean were useful plant materials as the bait trap for direct detection of *Bradyrhizobium* species. In single species contaminated soil, *B. japonicum* FAG–I and *B. elkanii* Group I were effectively detected by analyzing whole cellular fatty acid components originated from bacterial cells from soybean and mungbean plant cells. Variation of fatty acid profiles of *B. japonicum* and *B. elkanii* in soybean were presented in comparison with that of mungbean. However, composition of whole cellular fatty acids was contained a large range of varied data obtained from mixture of two species contaminated soils using soybean and mungbean bait trap method.

Direct detection of *Bradyrhizobium* species from naturally infested soils using

Table 3. Mean fatty acid compositions of *B. japonicum* and *B. elkanii* obtained from several soil conditions.

Soil Condition	Single species contaminated soil				Mixture of two species contaminated soil		Naturally infested soil		
Bait plant	Soybean		Mungbean		Soybean	Mungbean	Soybean	Mungbean	Cowpea
Baceteria	*B. japonicum*	*B. elkanii*	*B. japonicum*	*B. elkanii*	Mixture	Mixture	Unknown (*B. japonicum*)	Unknown (*B. elkanii* Group I)	Unknown (*B. elkanii* Group II)
16:0	13.65	14.44	12.44	12.21	13.41	11.86	13.41	11.78	10.58
16:1 ω5*cis*	3.14	0.11	3.02	0.11	2.36	2.36	3.55	0.00	0.14
16:1 ω7*cis*	0.96	0.69	0.96	0.69	0.96	0.81	1.14	0.55	0.55
17:0	1.11	0.00	0.00	0.00	0.11	0.00	0.11	0.00	0.00
17:0 cyclo	2.01	1.14	0.00	1.14	0.00	0.00	0.00	1.14	0.96
17:1 ω6*cis*	0.55	0.55	0.22	0.11	0.36	0.11	0.12	0.00	0.00
17:1 ω8*cis*	0.72	0.86	0.64	0.11	0.65	0.36	0.36	0.00	0.00
18:0	0.88	1.40	0.82	0.41	0.69	0.81	0.69	0.46	0.25
19:0 cyclo ω8*cis*	1.12	22.14	0.00	20.25	20.47	21.10	1.11	19.69	9.03
20:2 ω6,9*cis*	0.36	1.58	0.28	0.31	0.63	0.00	0.00	1.26	0.00
Summed feature 7	75.50	57.09	81.62	64.66	60.36	62.59	79.51	65.12	78.49

Note Figures in parentheses indicate the standard deviation. nd, values less than 0.1% of total or in which the standard deviation exceeds the mean.
Summed feature 7 is composed of 18:1 ω7*cis*/ω9*trans*/ω12*trans*, 18:1 ω9*cis*/ω12*trans*/ω7*cis* and 18:1 ω*trans*/ω9*trans*/ω7*cis* fatty acids.

whole cellular fatty acid analysis based on plant bait trap method effectively revealed that plant specific compatibility were presented in the combination of bait trap plant and *Bradyrhizobium* species. Soybean was compatible to *B. japonicum* FAG–I, mungbean was compatible to *B. elkanii* Group–I and cowpea was compatible to *B. japonicum* Group II. Other bait trap plants could detect whole cellular fatty acid composition originated from bacterial cells but fatty acid profiles did not show clear data (data not shown).

DISCUSSION

In this study, cellular fatty acid analysis is effectively characterized the distinction between *B. japonicum* and *B. elkanii*. Several recent research of the genus *Bradyrhizobium* also showed clear separation between *B. japonicum* and *B. elkanii* by cellular fatty acid analysis and reported the existence of one or more additional subgroups within these species. Graham *et al.* (1995) reported the existence of two subgroups in *B. japonicum* (Group IA and Group IB) and consisting a single species in *B. elkanii*. Tighe *et al.* (2000) also reported existence of two subgroups in *B. elkanii* (Group–I and II) and consisting a single species in *B. japonicum*. In this experiment, cellular fatty acid analysis revealed the existence of subgroups based on the difference of whole cellular fatty acid compositions between *B. japonicum* and *B. elkanii*. Comparing the data of fatty acid profiles presented in this experiment and previous reports, our results obtained from tested strains of *B. japonicum* and *B. elkanii* suggested the good agreement with these reports that are corresponded to *B. japonicum* Group IA and *B. elkanii* Group I, respectively.

Identification and classification of *B. japonicum* and *B. elkanii* using fatty acid analysis revealed the existence of subgroup based on the specific fatty acids and classified into *B. japonicum* FAG–I and FAG–II, and *B. elkanii* Group–I and Group II, respectively. The differentiation of FAG–I and FAG–II indicated the qualitative difference of 16:1 ω 5*cis*, 17:0, 17:1ω8*cis* and 19:0 cyclo ω8*cis*. The differentiation of Group–I and Group–II indicated the quantitative difference of 16:0, 19:0 cyclo ω8*cis* and summed feature 7. Another taxonomic criteria based on species–specific PCR analysis and cultural characteristics within their subgroups were tested and obtained identical results with cellular fatty acid analysis (data not shown). The results from species–specific PCR analysis and cultural characteristics also showed the similar that from cellular fatty acid analysis (data not shown).

Fatty acid analysis combined with plant bait trap method was a convenient tool for direct detection of *Bradyrhizobium* species from several contaminated soils. Soybean and mungbean bait trap plant was efficiently detected the subgroups within *B. japonicum* and *B. elkanii* from single species contaminated soil, however, quantitative and qualitative variation was observed the bait trap plants pigeon pea, groundnut, cowpea, black gram and azuki bean, obtained from single species contaminated soil. Quantitative and qualitative differences of fatty acid profiles were also observed using all bait trap plants obtained from mixture of two species contaminated soils. On the other hand, bait trap plants, soybean, mungbean and cowpea, could be successfully detected the strains of *B. japonicum*, *B elkanii* Group–I and Group–II, obtained from naturally infested soils. In this experiment, we observed the close relationships between isolated

host plants and bait trap plants because of homologues fatty acid profiles by fatty acid analysis based on plant bait trap method without isolating the target strains of *Bradyrhizobium* species. Fatty acid analysis combined with plant bait trap method will be a convenient and useful method for direct detection and diagnosis of various soil microorganisms and will be needed to improve and select the more proper bait trap plants for fatty acid analysis.

Moreover, the needs for additional work on identification and classification of *Bradyrhizobium* species is evident in a number of different studies and will necessitate the evaluation of strains from many different plant legumes and soils. In this experiment, however, the sole use of fatty acid profiles to compare one species with another is not typically employed for the purpose of diagnosis, it can be used in conjugation with other identification and classification methods such as phylogenetical methods. Our research is one of the attractive tools in identification and classification of *Bradyrhizobium* species because it can be rapidly assay a large number of unknown strains and facilitate as the relative simple and rapid procedures.

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