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A Study on Fatty Acid Analysis as a New Taxonomic Tool for Differentiating *Rhizoctonia* Spp.

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Fatty acid composition of three species of *Rhizoctonia* and seven anastomosis groups including twelve intraspecific groups of *R. solani* was studied by gas-liquid chromatography. The major fatty acids identified were palmitic, palmitoleic, oleic and linoleic acids, which constituted 92.1-96.0% of the whole-cell fatty acids identified. A dendrogram of the three *Rhizoctonia* spp. showed that the isolates of *R. solani* and *R. candida* are closely related in fatty acid composition, whereas the isolates of *R. oryzae* were more distantly related with them. In addition, each of the anastomosis groups and intraspecific groups of *R. solani* was divided into individual clusters by dendrogram analysis. All of the intraspecific groups belonging to the AG-1 and AG-2 were differentiated by cluster analysis based on the percent compositions of fatty acids identified. No correlation could be obtained on the dendrogram of *R.* solani isolates on the fatty acid composition and pathogenicity on their respective hosts. However, this technique clearly differentiated *Rhizoctonia* spp. and especially of anastomosis groups and intraspecific groups of *R. solani.* These works clearly suggested that the fatty acid analysis will be used as one of the taxonomic tools in differentiating *Rhizoctonia* spp.

INTRODUCTION

Fungi is generally classified by their morphological characteristics. However, insufficient morphological features of the genus *Rhixoctonia* have led to the complexity and confusion in their nomenclature as well as taxonomic classification. *Rhixoctonia solani* Kuhn (teleomorph= *Thanatephorus cucumeris* (Frank) Donk) has wide and overlapping range of isolates with varied morphology, physiology and pathogenicity (Adams, 1988). Attempts to distinguish *R. solani* isolates into logical grouping are based on either hyphal anastomosis and differences in pathogenicity (Ogoshi, 1976; 1987) or the morphological and cultural characteristics (Jones and Belmar, 1989; Sherwood, 1969; Watanabe and Matsuda, 1966).

Hyphal anastomosis in *R. solani* was first reported by Matsumoto (1921). Currently, 10 anastomosis groups (AGs) of *R. solani* are recognized (Carling et *al.*, 1987; Ogoshi, 1987; Ogoshi *et al.*, 1990), and are further divided into subgroups (intraspecific groups: ISGs) based on anastomosis frequency (Ogoshi, 1976) and thiamine requirement (Ogoshi and Ui, 1979; Sherwood, 1969). Ogoshi (1975; 1987) suggested that *R. solani* could also be differentiated based on their genetic characteristics including isozyme patterns (Matsuyama *et al.*, 1978), DNA base composition (Kuninaga and Yokosawa, 1980), DNA base sequence homology (Vilgalys, 1988), and ribosomal DNA fragment length polymorphisms (RFLPs) techniques (Vilgalys and Gonzalez, 1990).

Unique whole-cell fatty acid profiles have been used to classify closely related strains of bacterial plant pathogens (De Boer and Sasser, 1986; Graham *et al., 1990).* However, it has not been used to classify closely related isolates of fungi (Gottlieb and Van Etten, 1966). In plant pathogenic bacteria, the genus and species can be distinguished by qualitative and quantitative differences in fatty acid contents (Gitaitis and Beaver, 1990; Maas *et al.,* 1985). Recently, Stevens Johnk and Jones (1992; 1993; 1994) reported the comparison of whole-cell fatty acid composition in ISGs of *R.solani.* They also concluded that fatty acid analysis can be used as one of the tools to differentiate pathological types within AGs of *R.* solani.

In the present study the usefulness of fatty acid analysis for classification of *Rhixoctonia* spp. and the AGs of *R. solani* was discussed.

MATERIALS AND METHODS

1. Fungal isolates

Thirty-six isolates of *R. solani,* eight isolates of *R. oryzae,* and two isolates of *R. candida* were used in this study (Table 1). Each isolate was maintained on a slant culture of potato-dextrose agar (Funakoshi Chem. Co.) amended with 50 mg/L streptomycin sulfate at 25°C in the dark. To prepare samples for fatty acid analysis, each isolate was cultured without shaking in 100ml of a liquid nutrient-broth medium amended with 50 mg/L of streptomycin sulfate at 25℃ for 7 days. After harvest, the mycelia were washed with distilled water, lyophilized and stored at -20°C until use.

2. Preparation of fatty acid

Extraction of fatty acid was done according to Gudmastad et *al. (1988)* with slight modifications. The fatty acids extracted from 10 mg of the lyophilized mycelia were methylated with 0.5 ml of 5% HCl-methanol at 100°C for 3 h in a sealed glass tube to obtain fatty acid methyl ester (FAME) derivatives. The extract was cooled at room temperature and 1 ml of distilled water was added. The FAMEs were extracted with 2 ml of *n*-hexane by shaking. The solvent phase was washed with an equal volume of distilled water to remove HCl and dehydrated by mixing with 0.5 mg of anhydrous sodium sulfate. The organic phase was concentrated by nitrogen gas blowing. Samples were stored at -20°C.

3. Fatty acid analysis

FAMEs were analyzed by a GLC chromatograph (Shimadzu GC 7AG) equipped with a 3 m × 4 mm DEGS column. The column and injection temperature were maintained at 180°C and 250℃, respectively, and the flow rate of nitrogen gas was 50 ml/min. Three replicated analyses were made for each isolate. Average values of the fatty acid composition were used to differentiate the isolates of *Rhixoctonia* spp. For each isolate, a data record was constructed in percentages of the respective fatty acids. Relative similarities among isolates were determined by cluster analysis. A resemblance matrix based on Euclidean distance coefficients was computed from pairwise comparison of each isolate with every other isolate based on the analysis of percent fatty acid composition. Dendrogram was constructed by an unweighted paired group method with arithmetic

Table 1. Isolates of *Rhizoctonia* spp. analyzed.

Isolate	AG/ISG	Source	Origin
R. solani			
Cs-Ka	1 IA	AHU[a]	Rice
C-325	1 IA	AKU[b]	Rice
C-326	1 IA	AKU	Rice
B-19	1 IB	AHU	Sugar beet
HK-616-23	1 IB	AKU	Soil
RI-86	1 IB	AKU	Sugar beet
BV-7	1 IC	NIAS[c]	Sugar beet
F-2	1 IC	AHU	Flax
P-10	1 IC	AHU	Potato
PS-4	2-1	AHU	Pea
F-15	2-1	AKU	Flax
TG-1	2-1	AKU	Tulip
C-96	2-2 IIIB	M. Oniki	Mat rush
c-321	2-2 IIIB	AKLJ	Mat rush
C-116	2-2 IIIB	AKLJ	Mat rush
RI-64	2-2 IV	IAES[d]	Sugar beet
BV-28	2-2 IV	AKLJ	Sugar beet
BV-34	2-2 IV	AKU	Sugar beet
ST-11-6	3	AHU	Potato
ST-2	3	AKU	Potato
ST-7	3	AKU	Potato
GM-8	4	AKU	Soybean
HI-822	4	AKU	soil
SN-1	4	AKU	Soil
GM-10	5	AHU	Soybean
SH-25		AKU	Soil
SH-26	5	AKU	soil
OHT-1-l	6	S. Kuninaga	Soil
NAT-3-1	6	S. Kuninaga	Soil
HAM-l-1	6	S. Kuninaga	Soil
HO-1556		Y. Homma	Soil
MAFF 305552		Y. Homma	Soil
MAFF 305553		Y. Homma	Soil
AI-l-4	BI	S. Kuninaga	Soil
CH-l-2	BI	S. Kuninaga	Soil
TE-2-4	BI	S. Kuninaga	Soil
R. oryzae			
c-505	WAG-O	NIAS	Rice
T-1101	WAG-O	KU	Soil
KAES-14	WAG-O	KAES[f]	Rice
M-2122	WAG-O	KAES	soil
RO-1	WAG-O	SU[g]	Rice
T-5112	WAG-O	KU	Soil
T-5122	WAG-O	KU	Soil
T-5133	WAG-O	KU	Soil
R. *candida*			
IFO 7032	AG-A (CAG-2)	IFO[h]	Strawberry
IFO 7033	AG-A (CAG-2)	IFO	Beet

a) AHU: Faculty of Agriculture, Hokkaido University
b) AKU : Faculty of Agriculture, Kyushu University
c) NIAS: National Institute of Agricultural Sciences
d) IAES: Ibaragi Agricultural Experiment Station
e) KU: Faculty of Agriculture, Kagoshima University
f) KAES: Kagoshima Agricultural Experiment Station
g) SU : Faculty of Agriculture, Saga University
h) IFO: Institute for Fermentation

averages (UPGMA) using the statistics software package SYSTAT.

RESULTS

Ten fatty acids were identified and quantified in the isolates of three *Rhizoctonia* spp. They were myristic (C14$_0$), pentadecanoic (C15$_0$), palmitic (C16$_0$), palmitoleic (C16$_1$), heptadecanoic (C17$_0$), 9-heptadecenoic (C17$_1$), stearic (C18$_0$), oleic (C18$_1$), linoleic (C18$_2$) and one unknown fatty acid. These fatty acids were qualitatively similar, but possess quantitative differences. Fatty acids, C16$_0$, C18$_1$ and C18$_2$ were predominant in the three *Rhizoctonia* spp. and comprised 90.5-95.0% of the whole-cell fatty acids identified.

Profiles of FAMEs of the whole-cell fatty acids prepared from the three *Rhizoctonia* spp. were distinct from each other (Table 2). The isolates of *R. oryzae* contained more palmitic and palmitoleic acids than the isolates of *R.* solani and *R. candida.* These observations suggested a close resemblance between isolates of *R. solani* and *R. candida* than between isolates of *R. solani* and *R. oryzae.*

Table 2. Percentage composition of whole-cell fatty acids in the three species of Rhizoctonia

Rhizoctonia spp.	Composition of fatty acid[a]									
	C14$_0$	C15$_0$	C16$_0$	C16$_1$	C17$_0$	C17$_1$	C18$_0$	C18$_1$	C18$_2$	
R. solani	1.0	0.9	*7.4*	*1.3*	*0.4*	*0.8*	*1.4*	*10.5*	*74.5*	*1.8*
R. oryzae	0.5	*0.3*	*15.4*	*3.5*	*0.6*	*0.8*	1.9	*6.2*	*69.7*	*1.0*
R. candida	*0.5*	*0.4*	*6.5*	*1.0*	*0.3*	*0.2*	1.0	6.1	82.4	1.7

a) Values represent the means of test isolates, each with three replicates.
- : Unidentified

Table 3. Percentage composition of whole-cell fatty acids in *Rhizoctonia solani.*

AGs of *R. solani*	Composition of fatty acid"									
	C14$_0$	C15$_0$	C16$_0$	C16$_1$	C17$_0$	C17$_1$	C18$_0$	C18$_1$	C18$_2$	-
AG-1 I A	1.1	*0.7*	9.1	*2.2*	*0.5*	1.1	1.0	9.3	73.8	1.3
AG-1 I B	*1.3*	*1.0*	*6.8*	*0.7*	*0.6*	*0.4*	1.3	9.5	76.1	2.4
AG-1 I C	*0.9*	*0.7*	*6.7*	*2.0*	*0.9*	*1.3*	1.3	13.4	71.7	1.1
AG-2-1	*0.5*	*1.4*	*6.9*	1.0	*0.6*	*2.5*	1.5	10.3	73.0	2.4
XG-2-2 IIIB	*0.7*	1.1	8.1	1.7	0.3	0.7	1.2	8.9	75.8	1.5
XG-2-2 IV	0.6	0.9	6.9	1.8	0.2	1.3	1.3	7.0	78.3	1.8
AG-3	0.6	1.4	6.4	1.3	0.4	0.7	1.1	11.5	74.1	2.6
AG-4	1.2	0.7	7.8	1.0	0.7	0.7	2.4	10.5	73.5	1.6
AG-5	1.1	0.6	7.3	0.6	0.1	0.2	1.5	9.1	77.5	2.0
XG-6	2.5	1.3	7.1	1.6	0.4	0.6	0.6	9.5	73.6	3.0
AG-7	0.6	1.2	8.5	1.1	0.3	0.4	1.3	14.3	70.7	1.6
AG-BI	0.4	0.4	7.5	0.5	0.2	0.2	2.0	12.5	76.2	0.6

a) Values represent the means of test isolates, each with three replicates.
- : Unidentified

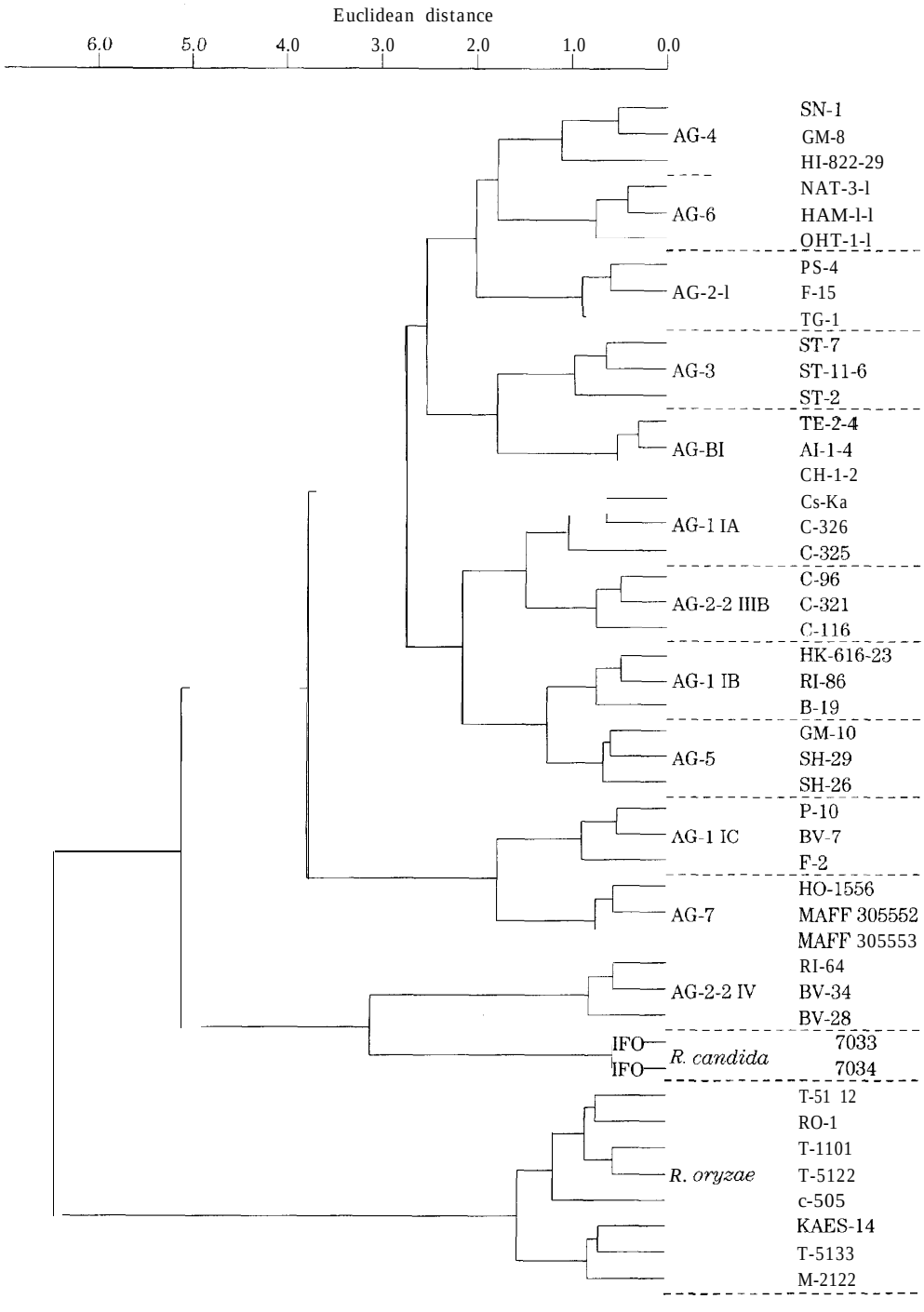

Fig. 1. Dendrogram of cluster analysis on *Rhizoctonia* species based on the percentage of fatty acid composition.

The compositions of fatty acids of the various AGs of *R. solani* were shown in Table 3 and Figure 1. The dendrogram analysis gave four major clusters among the AGs of *R. solani.* The AG-4, AG-6, AG-2-1, AG-3 and AG-BI isolates clustered together, with a Euclidean distance of 2.4. The AG-1 IA, AG-2-2 IIIB, AG-1 IB and AG-5 isolates were in another cluster, with a Euclidean distance of 2.3. The AG-1 IC and AG-7 isolates clustered, with a Euclidean distance of 2.1. Interestingly, the AG-2-2 IV isolates were found to be similar with the isolates of *R. candida,* with a Euclidean distance of 3.1. On the other hand, the cluster analysis on the isolates of *R. oryzae* was distinct from those of the isolates of *R. solani* and *R. candida,* with a Euclidean distance of 6.3.

DISCUSSION

Similar studies indicated that the optimum period of incubation of 4 days cultures on PDA was the best for fatty acid analysis of *R. solani* isolates (Stevens Johnk and Jones, 1993; 1994; Steven Johns et al., 1993). In our preliminary studies, we found no quantitative differences in fatty acid analysis between PDA and the liquid nutrient-broth cultures at 7 days incubation. Therefore, incubation period was fixed at 7 days to ensure sufficient growth of hyphae and to recover larger numbers of fatty acids.

Fatty acid analysis revealed major differences among the three *Rhizoctonia* spp. The fatty acid composition of the isolates of *R. solani* was similar to that of *R. candida* but was distinct from that of *R. oryxae* isolates. Fatty acid analysis was also shown to be useful to differentiate *R. solani* AGs. Further the qualitative detection of fatty acids {palmitic (14.5%), oleic (24.9%), and linoleic (54.4%)} detected supported the finding of Gottlieb and Van Etten (1966). However, the same major fatty acids detected in this study had different composition. Similar study by Stevens Johnk and Jones (1993, 1994) suggested the possibility of differentiating AGs of *R. solani* by fatty acid analysis. However, they described that the fatty acid analysis was not useful in differentiating isolates of AG-1 IA and AG-1 IB and morphological and pathological differences would be necessary to distinguish these ISGs (Stevens Johnk and Jones, 1994). Although the fatty acid composition of the isolates of AG-1 IA and AG-1 IB groups was similar in our study, both of the ISGs were separated into different clusters, with a Euclidean distance of 2.3. In addition, fatty acid analysis of three ISGs of AG-2 isolates, AG-2-1, AG-2-2 IIIB and AG-2-2 IV separated them into distinct clusters. Therefore, the results of fatty acid analysis of the ISGs of AG-2 isolates would reflect their grouping based on the morphological and pathological characteristics. These cluster analyses here indicated that the AG-1 IA isolates from rice plant were closely related with the AG-2-2 IIIB isolates from mat rush, with a Euclidean distance of 1.7 (Fig. 1). On the other hand, the fatty acid composition of the AG-1 IB and AG-2-2 IV isolates from sugar beet distinctly differed and the dendrogram analysis gave independent clusters for them (Table 3 and Fig. 1). These results also suggested that fatty acid composition of *R. solani* isolates and their pathogenicity do not always correlate.

In conclusion, fatty acid analysis is useful as a new taxonomic index to classify *Rhizoctonia* spp. and AGs and ISGs of *R. solani.* Further investigation to develop database library of fatty acid composition for AGs and ISGs of *R. solani* isolates is in

progress.

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