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SHORT COMMUNICATION

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Chemotaxonomic characterization of rice seedling blight complex using fatty acid methyl ester (FAME) profiles

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Abstract Rice seedling blight is an important disease caused by a complex of fungi that include *Fusarium*, *Rhizopus*, *Pythium*, and *Trichoderma* species. A modified MIDI method was used for extraction of fatty acids from these causal pathogens, and fatty acid methyl ester (FAME) profiles were characterized. Factors that might affect fatty acid production, such as period of culture and saponification in extraction, were also evaluated. A total of 14 fatty acids were detected, and FAME profiles showed quantitative and qualitative variations by discriminant analysis and principal component analysis. Genus-specific FAME profiles consisting of the types of fatty acid produced and remarkable components of individual fatty acids were observed. The possibility of application as chemotaxonomic methods based on the FAME profiles for diagnosis of the rice seedling blight complex is also discussed.

Key words Fatty acid methyl ester · Modified MIDI method · Rice seedling blight

Rice has been grown under transplant systems in flooded fields in most parts of Japan for hundreds of years. When rice plant seedlings are young, severe blights and damping-off frequently occur. Several causal pathogens were identified as fungal genera including *Fusarium*, *Pythium*, *Rhizopus*, and *Trichoderma* species (Ohata et al. 1978). Rapid and accurate diagnosis of this disease is important because of severe yield loss and economic damage in the rice-growing regions of the world. Although many researchers have developed methods for detection of these causal pathogens based on the techniques of molecular and genetic characteristics, most of these require high levels of technical knowledge, expensive reagents and equipment, and an innovated laboratory. In addition, understanding of disease epidemiology and host–pathogen interactions is greatly dependent on the genetic knowledge of their complexity. Consequently, it is necessary to establish technically simple and economically superior methods for characteristics traits of these fungal genera.

Analysis of cellular fatty acids is routinely used to characterize, differentiate, and identify genera, species, and strains of fungi. Taxonomic traits based on fatty acid profiling are distinguishable by the types of fatty acid produced (qualification) and the relative concentrations of individual fatty acid (quantification). In addition, fatty acid composition is strongly influenced by the three primary environmental variables of growth substrate, incubation temperature, and incubation time. However, results that are obtained from fatty acid profiling are shown to be highly reproducible, consistent, and conserved among different fungal genera when these factors are held constant (Sasser 1990). The fact is that fatty acid profiles may also be useful for identification and characterization of fungi (Muller et al. 1994; Stahl and Klug 1996; Pankhurst et al. 2001; Ruess et al. 2002). Moreover, recent work has shown that variation in the relative concentration of the fatty acids is sufficient to enable effective differentiation for subspecific groups of *Penicillium* (Lopes da Silva et al. 1998), *Rhizoctonia* (Matsumoto et al. 1997; Stevens and Jones 2001; Matsumoto 2005), *Pythium* (Pankhurst et al. 2001), *Phytophthora* (Larkin and Groves 2003), and *Fusarium* (Matsumoto 2006).

A common protocol used for those identification techniques is the MIDI (Microbial Identification System; Microbial ID Inc., Newark, DE, USA), which may have good potential for differentiation by fatty acid methyl ester (FAME) profiles (Sasser 1990). In 1997, Matsumoto et al. described a new protocol by modifying the MIDI method, and Lanoiselet et al. (2005) successfully used this for diagnosis of the causal *Rhizoctonia* species of rice sheath blight.

The objective of this study was to investigate the potential of FAME profiles for identification, characterization, and differentiation of causal pathogens of rice seedling

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Table 1. Fungal species, isolates and tester strains used in this study

Species	Isolate/tester strain no.	Host	Source
Fusarium roseum	237348	Tulip	MAFF 237348
	306358	Wheat	MAFF 306358
	FUN-521	Rice	M. Matsumoto
	FUK-63	Rice	M. Matsumoto
	GIY-5-6	Rice	M. Matsumoto
	FUK-36	Rice	M. Matsumoto
	TMK23	Rice	M. Matsumoto
	KK147	Rice	S. Matsumoto
	21SEC	Rice	S.S. Aye
	Shurin8	Rice	S.S. Aye
	Shurin41	Rice	S.S. Aye
F. solani	235426	Wheat	MAFF 235426
	235975	Potato	MAFF 235975
Rhizopus chinensis	4737	–[a]	NBRC 4737
	4768	–	NBRC 4768
	KNK-96-36	Rice	M. Matsumoto
	HKT-66	Rice	M. Matsumoto
	R12–36-6	Rice	S.S. Aye
	G1+	Rice	S.S. Aye
	Mik-36	Rice	S.S. Aye
	Mis-14	Rice	S.S. Aye
	TMK14	Rice	S.S. Aye
	HTC78	Rice	S.S. Aye
	61Gi-S74	Rice	S.S. Aye
	Ken77	Rice	S.S. Aye
R. oryzae	238040	Green pea	MAFF 238040
	725501	Green pea	MAFF 725501
R. javanicus	305207	–	MAFF 305207
Trichoderma viride	236542	Rice	MAFF 236542
	236543	Rice	MAFF 236543
	TD-23	Rice	S.S. Aye
	TD-36	Rice	S.S. Aye
	TD-35-3	Rice	S.S. Aye
	TD-18	Rice	S. Matsumoto
	TD-55	Rice	M. Matsumoto
	TD-T41	Rice	M. Matsumoto
	TDD-7	Rice	S.S. Aye
	MAT-63	Rice	S.S. Aye
	AATD1	Rice	S. Matsumoto
	SSP-63	Rice	M. Matsumoto
	7C-147	Rice	M. Matsumoto
Pythium graminicola	238432	Rice	MAFF 238432
	238433	Rice	MAFF 238433
	Py-65-3	Rice	M. Matsumoto
	Py-1-23	Rice	M. Matsumoto
	Tr-69-3	Rice	M. Matsumoto
	GTR-7	Rice	M. Matsumoto
	T 24-41	Rice	M. Matsumoto
	Hha 85	Rice	M. Matsumoto
	GenTi-4	Rice	M. Matsumoto
	GenTi-7	Rice	M. Matsumoto
	WakTPy-66	Rice	M. Matsumoto
	WakTPy-32	Rice	M. Matsumoto
P. spinosum	425452	Strawberry	MAFF 425452
	425464	Soil	MAFF 425464
P. irregulare	237501	Soil	MAFF 237501
	425433	Soil	MAFF 425433
P. sylvaticum	235102	Tulip	MAFF 235102
	712264	Chrysanthemum	MAFF 712264

[a] Unknown host

blight complex. In this experiment, protocols of the extraction of cellular fatty acids were furthermore modified from the MIDI method previously reported, and factors that might affect growth and fatty acid extraction were also evaluated.

Fungal isolates used in this experiment are listed in Table 1. Tester strains of *Fusarium roseum* (MAFF237348 and MAFF306358), *F. solani* (MAFF235426 and MAFF235975), *Rhizopus chinensis* (NBRC4737 and NBRC 4768), *R. oryzae* (MAFF238040 and MAFF725501),

Pythium graminicola (MAFF238432 and MAFF238433), *P. spinosum* (MAFF425452 and MAFF425464), *P. irregulare* (MAFF237501 and MAFF425433) and *P. sylvaticum* (MAFF235102 and MAFF712264), and *Trichoderma viride* (MAFF236542 and MAFF236543) were distributed from NITE Biological Resource Center (NBRC) and National Institute of Agricultural Sciences (MAFF). Isolates obtained from diseased rice seedlings in this study were collected from University Farm, Faculty of Agriculture, Kyushu University at the period of April in 2003–2004 and stored at the laboratory of Institute of Tropical Agriculture, Kyushu University, Fukuoka, Japan. Diseased rice (*Oryza sativa* subsp. *japonica*) cv. Nipponbare seedlings were washed with 100 ml sterile distilled water five times. The seedlings were then cut into 1-cm-long segments and placed on water agar (WA) plates (pH 4.0–4.2) or potato dextrose agar (PDA) plates (pH 4.0–4.2) with streptomycin sulfate (500 ppm). The WA and PDA plates were then incubated at 25°C for 3–5 days, and isolates were established by transferring a colony of a fresh 18-cm slant tube of PDA amended with 50 mg/l penicillin-G and 10 mg/l refampicin and incubated at 25°C in the dark. Finally, all isolates were stored at the conditions of lyophilized mycelial samples.

Samples for fatty acid profile analysis were prepared from mycelia of each isolate grown on PDA for 3 days at 25°C. Three plugs (9 mm each in diameter) with the grown mycelia were placed in 20 ml potato dextrose broth (PDB; Difco, Franklin Lakes, NJ, USA) amended with 50 mg/l streptomycin sulfate and incubated without shaking for 4 days at 25°C in the dark. The mycelia were harvested by vacuum filtration through Whatman no. 1 filter paper in a Buchner funnel, rinsed with sterile distilled water, and placed on a paper towel to remove excess moisture. The fungal mycelia were lyophilized and stored at –20°C until use.

Thirty milligrams of lyophilized mycelia was methylated with 0.5 ml 5% (v/v) HCl-methanol at 100°C for 1 h in a capped, heat-resistant glass tube. The resultant FAMEs were cooled to room temperature, diluted with 0.5 ml distilled water, and extracted with 1 ml *n*-hexane by shaking. The tubes were then centrifuged at 15000 rpm for 1 min. The solvent (organic) phase was transferred to a new glass tube, washed with an equal volume of distilled water to remove HCl, and dehydrated by mixing with 0.5 mg anhydrous sodium sulfate. The mixture was concentrated by volatilization with nitrogen gas. Samples were stored at –20°C.

The extracted fatty acids were analyzed by gas chromatography using a Shimadzu gas chromatograph GS18A fitted with a Shimadzu Hicap CBP20 fused silica capillary column (0.22 mm × 25 m) and equipped with a flame ionization detector, used to separate FAMEs from each isolate. Nitrogen was used as the carrier gas; its pressure was maintained at 95 kPa. The temperature program was initiated at 160°C and increased 1°C/min to a final temperature of 250°C. The FAME peaks were identified by the MIDI programs Ver. 4.5 based on their Equivalent Chain Length. The concentrations of individual fatty acids presented by FAME profiles were expressed as a percentage of the fatty acid content. Fatty acid data were averaged by repeating the fatty acid extraction three times.

Variations and compositions of fatty acid consisted of carbon chain lengths ranging from 14 to 20. The most commonly and abundantly contained fatty acids were 16:0, 18:0, 18:1ω9c, and 18:2ω6c, which often made up greater than 50% of the total fatty acid content of the tested strains and isolates (Table 2). These four fatty acids were present in all the fungi examined in this experiment. Other fatty acids (14:0, 15:0, 16:1ω7c, 18:3ω6c, 20:0, 20:1ω9c, 20:2ω6c, 20:3ω6c, 20:4ω6c, and 20:5ω3c) were also detected, and all were found in *Pythium* spp. (Table 2). Nine of the detected fatty acids were recovered from the *Fusarium* spp., 12 fatty acids were recovered from the *Rhizopus* spp., and 12 fatty acids were recovered from the *Trichoderma viride*, respectively. Seven fatty acids were common to all isolates.

There were significant differences ($P < 0.05$) in the qualitative and quantitative variations of fatty acids in genera *Fusarium*, *Rhizopus*, *Pythium*, and *Trichoderma* (see Table 2). Isolates of *Fusarium* spp. contained large quantities of fatty acids of 16:0, 18:1ω9c, and 18:2ω6c, more than 19%. Fatty acids of 20:0, 20:3, 20:4, and 20:5 were not present in the isolates of *Fusarium* spp. (Table 2). Fatty acid composition of *Rhizopus* spp. was also abundantly contained in 16:0, 18:1ω9c, and 18:3ω6c, more than 18%, respectively. Other fatty acids, 18:2ω6c, were also contained in *Rhizopus* spp., more than 9% and 6%, respectively. Fatty acids of 20:1 and 20:3 were not present in the isolates of *Rhizopus* spp. (Table 2). Isolates of *Pythium* spp. showed large variations and components of fatty acid compared to the other fungal genera. The fatty acids of 16:0, 18:1ω9c, and 18:2ω6c were significantly detected in the isolates of *Pythium* spp., more than 10% amounts, and especially 14:0 and 16:1ω7c were specific in this genus (Table 2). On the other hand, isolates of *Trichoderma viride* abundantly contained the fatty acids 16:0, 18:1ω9c, and 18:2ω6c, more than 20%, respectively (see Table 2). Fatty acids of 20:2ω6c and 20:5ω3c were not detected in this species.

A statistical analysis was employed to compare FAME profiles of individual fungi and to determine whether fungi can be differentiated on the basis of fatty acid compositions. The FAME profiles of the most similar species were then compared by an index of similarity and a multivariate statistical test. To quantify the similarity in fatty acid composition of two fungi, an index-of-relationship value was calculated by the following formula (Holman 1978):

$$R_{xy} = (C_x/C_y)_1(C_x + C_y/200)_1 + \ldots + (C_x/C_y)n\ (C_x + C_y/200)_n$$

where x and y represent the fungi being compared, C is the mean fatty acid composition expressed as a percentage of the total content for 1 through n fatty acids, and (C_x/C_y) represents the minor ratio of the fatty acids being used in the experiment. To determine if the fatty acid compositions of two fungi were statistically different, discriminant analysis was used to test the hypothesis that species x (f_{a1}, f_{a2}, f_{a3}, ... f_{an}) = species y (f_{a1}, f_{a2}, f_{a3}, ... f_{an}), where fa is fatty acid. In this experiment, tester strains and isolated strains were compared by these methods to determine how well congeners could be differentiated.

Table 2. Fatty acid methyl ester (FAME) profiles of isolates of *Fusarium*, *Rhizopus*, *Trichoderma*, and *Pythium* species, associated with rice seedling blight

Fungal species	Total fatty acid composition (%)[a]												
	14:0	15:0	16:0	16:1ω7c	18:0	18:1ω9c	18:2ω6c	18:3ω6c	20:0	20:1ω9c	20:2ω6c	20:3ω6c	20:4ω6c	20:5ω3c
Fusarium roseum	0.89	0.00	19.27	1.13	7.00	32.60	38.67	0.19	0.00	0.12	0.13	0.00	0.00	0.00
F. solani	0.71	1.15	20.58	0.05	6.14	25.00	45.95	0.11	0.00	0.15	0.18	0.00	0.00	0.00
Rhizopus chinensis	1.49	0.67	20.56	0.60	10.59	35.88	9.44	18.69	0.59	0.00	1.11	0.00	0.14	0.54
R. oryzae	1.99	0.49	20.00	1.39	9.81	36.97	6.82	20.18	0.12	0.20	0.95	0.00	0.12	0.39
R. javanicus	1.80	0.49	20.07	1.63	8.00	40.08	7.22	19.14	0.01	0.20	0.89	0.00	0.12	0.33
Trichoderma viride	0.44	0.15	22.63	2.26	2.62	20.57	49.47	0.99	0.28	0.33	0.00	0.12	0.15	0.00
Pythium graminicola	12.85	0.60	15.43	10.63	2.52	24.36	16.99	0.63	0.37	0.97	1.17	1.65	5.28	6.87
P. spinosum	15.82	0.00	12.40	10.48	5.33	28.15	16.08	1.12	0.25	0.58	1.15	0.60	3.41	4.62
P. irregulare	10.12	1.12	16.57	13.41	6.26	23.27	12.98	2.17	0.23	1.16	0.83	0.00	8.25	3.62
P. sylvaticum	13.72	0.67	9.86	13.86	8.71	25.19	17.32	0.69	0.41	0.44	1.43	0.00	4.14	3.55
LSD $P < 0.05$	0.67	0.11	1.89	0.52	0.79	4.12	3.58	4.19	0.28	0.05	0.14	0.18	0.53	1.15

[a]FAME profile of each data was derived from all the isolates and tester strains listed in Table 1 and each data set were the average of three times repeated.

Comparison of FAME profiles between tester strains and isolates from infected rice seedlings demonstrated that the strains and isolates of the same species were statistically homologous in the FAME profiles (Table 3). Discriminant analysis showed that the FAME profiles of these isolates are similar at high levels of significance ($P < 0.05$) (Table 3). Index of relationship values for these combinations of fungi ranged from 0.88 to 0.95.

Based on the data of variations and components of fatty acids, comparison of FAME profiles among isolates of *Fusarium* spp., *Rhizopus* spp., *Pythium* spp., and *Trichoderma viride* were carried out using principal component analysis (PCA). Comparison of the individual composition of fatty acids detected from the tissue of isolates was subjected to an analysis of variance (ANOVA). All calculations were performed with StatPartner Ver.2.0 (NEC Software, Tokyo, Japan).

A PCA plot of the FAME profiles generated from the isolates of *Fusarium* spp., *Rhizopus* spp., *Pythium* spp., and *Trichoderma viride* is shown in Fig. 1. Good discrimination was obtained by the results from PCA analysis at the most clearly separated into the four fungal genera. The first 2 of 14 principal components of the PCA analysis accounted for 90.5% of the coefficient of determination in the data. Principal components 1 and 2 accounted for 55.3% and 35.2% of the coefficient of determination, respectively (Fig. 1).

FAME profiles derived from the isolates of *Fusarium* spp., *Rhizopus* spp., *Pythium* spp., and *Trichoderma viride*, respectively, showed close relationships among isolates within the same genera, but were distinct among these four genera. Amounts of the four fatty acids of 16:0, 18:0, 18:1ω9c,

Fig. 1. Relationship of fatty acid methyl ester (FAME) profiles among isolates of *Fusarium* spp., *Rhizopus* spp., *Pythium* spp., and *Trichoderma viride*, and as represented by plots of the first two principal component analyses using the modified MIDI method

Table 3. Comparison of FAME profiles between tester strains and isolates from diseased rice

Data comparisons: tester strains vs. average of isolates	Index of relationship value	Discriminant analysis (*F* statistic)
Fusarium roseum MAFF237348 vs. *F. roseum* isolates	0.91	23.91[a]
F. roseum MAFF306358 vs. *F. roseum* isolates	0.92	24.18[a]
Rhizopus chinensis NBRC4737 vs. *R. chinensis* isolates	0.93	22.97[a]
R. chinensis NBRC4768 vs. *R. chinensis* isolates	0.93	32.68[a]
Trichoderma viride MAFF236542 vs. *T. viride* isolates	0.95	16.74[a]
T. viride MAFF236543 vs. *T. viride* isolates	0.94	16.81[a]
Pythium graminicola MAFF238432 vs. *P. graminicola* isolates	0.88	34.52
P. graminicola MAFF238433 vs. *P. graminicola* isolates	0.85	29.51[a]

[a] $P < 0.05$

and 18:2ω6c accounted for more than 93% of *Fusarium* spp. and *Trichoderma viride*, for 73%–84% in *Rhizopus* spp., and 50%–63% of the amounts in *Pythium* spp., respectively (see Table 2). Discriminant analysis is also revealed that variations of individual fatty acids showed significant similarities between tester strains and isolated strains from infected rice seedlings (see Table 3). Variations in FAME profiles at species level have been reported for other fungi and yeast, and are also sufficient for statistic differentiation among different fungal genera as well as different species within the same genus (Augustyn et al. 1990; Stahl and Klug 1996; Pankhurst et al. 2001). In this experiment, variation based on FAME profiles revealed good discrimination and effective separation among the four fungal genera by comparing the types of fatty acids produced and the relative compositions of individual fatty acids.

In this experiment, the representative species of causal pathogens of rice seedling blight complex were mortified to supply the fatty acid profile analysis. In another experiment, three species of *Pythium*, *Fusarium*, and *Rhizopus* were applied to fatty acid profile analysis and compared among species level. Comparison of FAME profiles among different species within same fungal genera revealed that types of fatty acids produced were common and consistent within the same fungal genera but that relative concentrations of fatty acid were divergent among different species (see Table 2). Therefore, fatty acid profile analysis was used to identify, characterize, and differentiate the isolates of *Fusarium* spp., *Rhizopus* spp., *Pythium* spp., and *Trichoderma viride* in this article.

For the FAME profiles differentiating and characterizing the causal agents of rice seedling blight complex, it is important to reduce the variation in the amounts of fatty acid compositions. Previous reports have shown that culture conditions (growing temperature and age) can influence fatty acid compositions in fungi (Stahl and Klug 1996). In the present study, culture age was an important consideration as the FAME profiles for cultures of 2–3 days growth were compared with cultures of 4–7 days growth (data not shown). Moreover, our data also suggested that qualitative differences of FAME profiles were sufficient to differentiate the four fungal genera based on the principal component analysis (see Fig. 1). Therefore, comparisons of FAME profiles were made on cultures grown for 4 days under standard conditions in this experiment.

Matsumoto et al. (1997) and Lanoiselet et al. (2005) reported the usefulness of the extraction of fatty acids using a modified MIDI method that differed at the saponification step, which is 2.5 h longer compared to the usual MIDI method. Although saponification time of 30 min is a standard step for fatty acid extraction from bacterial cells and has been used successfully to differentiate and characterize species and genera of fungi, saponification time of 3 h is initiated to break the cells of *Rhizoctonia oryzae* and *R. oryzae-sativae* to expose the entire phospholipids bilayer membrane (Lanoiselet et al. 2005). Our results suggest that saponification of 1 h successfully breaks down the cell walls of the isolates and will allow characterizing and differentiating the FAME profiles.

Fatty acid profile analysis of the isolates of *Fusarium* spp., *Pythium* spp., *Rhizopus* spp., and *Trichoderma viride* had been previously reported and indicated the production of the same predominant fatty acids, especially higher compositions of fatty acids 16:0, 18:0, 18:1ω9c, and 18:2ω6c, respectively (Muller et al. 1994; Stahl and Klug 1996; Pankhurst et al. 2001; Larkin and Groves 2003). Moreover, FAME profiles from rice seedling blight pathogens revealed that genus-specific types of fatty acid and/or remarkable compositions of fatty acids were detected from the four species, containing 18:1ω9c and 18:2ω6c in *Fusarium*, 18:3ω6c in *Rhizopus*, 18:2ω6c in *Trichoderma viride*, and 14:0, 16:1ω7c, 20:4ω6c, and 20:5ω3c in *Pythium* species, respectively (see Table 2). These facts imply that FAME profiles will become the chemotaxonomic marker for the possibility of application of direct detection and diagnosis in the future. However, results of FAME profiles revealed insufficient information to characterize and differentiate species levels of the genera *Fusarium*, *Rhizopus*, and *Pythium*, respectively (Table 2). Moreover, our experiment could not compare the FAME profiles between pathogenic and nonpathogenic isolates within the species among three genera (data not shown). Therefore, adoption of a future practice for fatty acid analysis will allow the recognition of plural species within the same genus such as *Fusarium*, *Rhizopus*, and *Pythium* and comparison of FAME profiles between pathogenic and nonpathogenic isolates.

In summary, fatty acid analysis has applicability for use in characterizing and differentiating causal pathogens of rice seedling blight complex as one of the chemotaxonomic tools. Although more research is needed to make this a

useful tool, it is clear that there is potential for expanding the available information that can be derived from causal pathogens of the rice seedling blight complex. However, several potential disadvantages to fatty acid analysis may interfere with their routine use for characterization of these pathogens. Dominant problems are the need for rigorous control and standardization of culture conditions and extraction protocols. In this experiment, application of culture conditions (4 days growth) and extraction protocols (1 h saponification) succeeded to establish the standardization of FAME profile analysis. Another problem is that individual samples are not definitive, and repeating tests is required to produce reliable results. In this experiment, fatty acid analysis also revealed reliable results by comparing the FAME profiles between tester strains and isolated strains. Compared to the molecular approach, where the chemotaxonomic approach is kept at a distance, this may seem cumbersome. On the other hand, once the appropriate equipment is obtained, fatty acid analysis is relatively simple, quick, and economical. Considering that it may provide a way to diagnose and detect causal pathogens and living isolates that are characterized qualitatively and quantitatively, FAME profiles could prove very useful. It should be emphasized, however, that FAME profiling is intended as a supplemental and complementary tool for characterization of causal pathogens of the rice seedling complex and should not be considered an alternative or replacement for more established means of cultural and genetic characterization. The greatest utility of these FAME profiles may be in their potentially greater genus specificity than other tests, possibly resulting in establishing relationships between profile characteristics and genus-specific traits, such as pathogen diagnosis, disease management, or other characteristics that may be useful in determining epidemiological consequences.

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