Physiology and Biochemistry

Determination of Whole-Cell Fatty Acids in Isolates of *Rhizoctonia solani* AG-1 IA

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ABSTRACT

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The Microbial Identification System (Microbial ID, Inc., Newark, DE), used to identify and characterize fatty acids of bacteria, was modified to characterize fatty acids of isolates of *Rhizoctonia solani* AG-1 IA. The major fatty acid found was linoleic (18:2 cis 9, 12), which constituted 75.3–78.5% of the whole-cell fatty acid content. This fatty acid, along with palmitic (16:0) and oleic (18:1 cis 9), accounted for 95–96% of the C9–C20 fatty acids present. Smaller amounts of nine other fatty acids were consistently identified.

Additional keywords: sheath blight of rice.

Sheath blight of rice (*Oryzae sativa* L.) is caused by *Rhizoctonia solani* Kühn (teleomorph = *Thanatephorus cucumeris* (A. B. Frank) Donk) anastomosis group (AG)-1 (13). The intraspecific group of *R. solani* responsible for sheath blight is AG-1 IA (17). Isolates of *R. solani* AG-1 IA are also responsible for aerial blight of soybean, and banded leaf and sheath spot of sorghum and corn (12).

Gottlieb and Van Etten (7) studied the effect of cell age on fatty acid composition of an isolate of *R. solani* of unknown

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AG. They identified seven fatty acids including: myristic (14:0), pentadecanoic (15:0), palmitic (16:0), palmitoleic (16:1 cis 9), stearic (18:0), oleic (18:1 cis 9), and linoleic (18:2 cis 9, 12). The principal fatty acid was 18:2 cis 9, 12; it constituted 66.6% of the total fatty acids in young cells (0–16 h) and 54.4% in old cells (56–80 h).

Unique whole-cell fatty acid profiles have been used to differentiate closely related strains of bacteria (3,8,9). Genera and species of bacteria are distinguishable by qualitative or quantitative differences in fatty acid content (5,6,11,15). Miller and Berger (16) developed a rapid, sensitive, and reproducible bacterial sample preparation technique employing fatty acid methyl ester (FAME) derivatives. In addition, a computer software package (Microbial Identification System [MIS], Microbial ID, Inc., Newark, DE) was developed to identify fatty acids and to study relationships between different bacteria using quantitative and qualitative differences in constituent fatty acid methyl esters. While MIS has been used to identify and differentiate bacteria, few eukaryotic organisms have been studied.

We chose to use MIS to examine isolates of *R. solani* AG-1 IA. *R. solani* is an important worldwide pathogen of rice. Our objectives were to explore the application of MIS methods to a population of isolates from *R. solani* AG-1 IA with respect to repeatability within an isolate and the capacity to distinguish isolates.

MATERIALS AND METHODS

Fungal isolates and sample preparation. Ten isolates of *R. solani* AG-1 IA were analyzed. These isolates were collected from diseased rice plants in Texas during 1984–1987 (12). Isolates were maintained on potato-dextrose agar (Difco Laboratories, Detroit, MI) amended with 50 mg/L each of streptomycin sulfate and penicillin G (PDA+).

In the following experiments, one isolate, 1-1-006, was randomly selected from a population of 10 isolates and used in each experiment unless otherwise noted. Plates were centrally inoculated with a 4-mm plug taken from an actively growing PDA+ culture. All cultures were grown at 28 C in darkness for 48 h. At 48 h, a doughnut-shaped plug (inner diameter of 3 cm and an outer diameter of 7 cm) of actively growing mycelium was cut from each plate. After harvest, individual mycelial plugs were immersed in 50 ml of distilled water in 250-ml glass beakers and microwaved at 600 watts for 1 min (until boiling). Mycelial mats were removed, weighed, and placed in a 13 × 100 mm screw-top test tube for fatty acid extraction. Variations to this procedure will be described within each experiment.

Characterization of isolates of *R. solani* is typically done on PDA (19,20) or on Czapex-Dox agar (1,4), whereas trypticase soy broth agar (TSBA) was used by Miller and Berger (16). An experiment was designed to determine the effect of different media on fatty acid composition of an isolate of *R. solani.* Five media, each amended with 50 mg/L each of streptomycin sulfate and penicillin G, were tested: PDA+, Czapex-Dox agar (CDA+), CDA+ amended with 3.4 mg/L of thiamine hydrochloride (CDA+T), TSBA (TSBA+), and water agar (WA+). Three plates of each medium were inoculated with isolate 1-1-006. Mycelia were harvested and prepared for fatty acid extraction as described. The experiment was repeated once.

A time course experiment was conducted over a 96-h period. A 22-cm square plate was filled with 200 ml of PDA+. The plate was inoculated along one edge with 20 1-cm plugs of an actively growing PDA+ culture of isolate 1-1-006 (Fig. 1). The plate was incubated for 96 h at 28 C in darkness. At 96 h, 0.5 × 22 cm strips were sequentially cut from the leading edge of the mycelium back to the point of inoculation. A total of 30 strips were harvested. Each strip was prepared for fatty acid extraction as previously described. The experiment was repeated two times.

An additional time course experiment was conducted over a 28-day period. Plates of PDA+ were inoculated with isolate 1-1-006. Due to the length of the experiment, the plates were sealed with Parafilm and placed in plastic bags in an incubator. Plates were removed for fatty acid extraction at 2, 7, 14, and 28 days. Mycelia were harvested and prepared for fatty acid extraction as described. Three replicate plates were used in the experiment, which was repeated once.

To test the variation between plates of the same isolate, 10 replicate PDA+ plates were inoculated with isolate 1-1-006. These were harvested, and the mycelia were prepared for fatty acid extraction.

To test the variation among isolates, 10 isolates of AG-1 IA were used in the following experiment. Three replicate plates of PDA+ were inoculated for each isolate. Mycelia were harvested and prepared for fatty acid extraction. The experiment was repeated once.

Cellular fatty acid analyses. Fatty acid composition was determined by gas chromatography procedures described in Hewlett-Packard application note 228-41 (Hewlett-Packard, Pittsburgh, PA) and similar to that used by Gudmestad et al (9). Saponification was accomplished by adding 1.0 ml of a 7.5 M NaOH-50% methanol (1:1, v/v) reagent, vortexing for 5–10 s, and heating for 5 min at 100 C in a water bath. Tubes were vortexed for 5–10 s and returned to the water bath for an additional 25 min. After cooling to room temperature by holding tubes under cold running tap water, the saponificate was acidified and methylated by adding 2.0 ml of a 6 N HCl-100% methanol (13:11, v/v) solution, vortexing for 5–10 s, and heating at 80 C in a water bath for 10 min. Tubes were again cooled under cold running tap water. The FAMEs were extracted by adding 1.25 ml of hexane-*tert*-butyl methyl ether (1:1) and by rotating the tubes using a hematology mixer (model 346, Fisher Scientific, Avondale, PA) for 10 min. Tubes were then uncapped and the lower aqueous phases and remaining mycelia were removed with a Pasteur pipet and discarded. The remaining organic phases were each washed by adding 3.0 ml of 0.3 M NaOH and rotating the tubes again on the hematology mixer for 5 min. The upper (organic) phases were each transferred with a Pasteur pipet to a glass gas chromatography vial, which was capped with a septum. Samples were analyzed within 48 h.

FAMEs were analyzed by gas chromatography with a Hewlett-Packard 5890 gas chromatograph (GC) equipped with a 25 m × 0.2 mm phenyl methyl silicone fused silica capillary column (Hewlett-Packard, Ultra 2), with an automatic sampler and flame

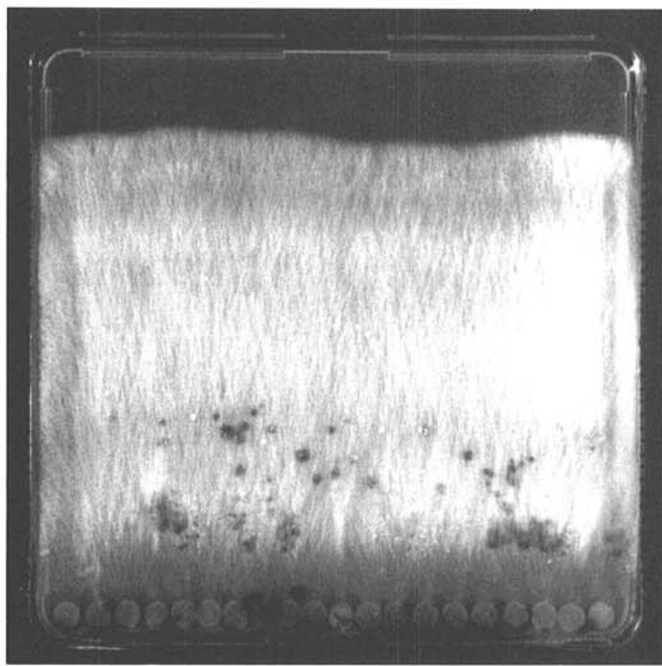

Fig. 1. Cultural appearance, after 96 h of incubation, of isolate 1-1-006 of *Rhizoctonia solani* AG-1 IA in the 96-h time course experiment. The plate is a 22-cm square and contains 200 ml of potato-dextrose agar plus 50 mg/L each of streptomycin sulfate and penicillin G.

ionization detector. Nitrogen was used as the carrier gas. The temperature program was initiated at 170 C and increased at 5 C/min to a final temperature of 270 C.

Before analysis of samples, the GC was calibrated with a commercial external FAMEs mixture (Aerobe Calibration Standard, Microbial ID, Inc.) containing straight chain saturated fatty acids from C9–C20, and five hydroxy fatty acids including: 2-hydroxydecanoic (10:0 2OH), 3-hydroxydecanoic (10:0 3OH), 2-hydroxymyristic (14:0 2OH), 3-hydroxymyristic (14:0 3OH) and 2-hydroxypalmitic acid (16:0 2OH).

Retention times and peak areas were analyzed to meet acceptance criteria of MIS. The retention times and peak areas were compared to known retention times and peak areas of fatty acids in the peak naming table of the Aerobe Method contained in MIS. The peak naming table contains theoretically perfect values for retention times and area-to-height ratios, as well as sample control parameters for accepting a peak including maximum area-to-height ratios of 0.1, and minimum and maximum area counts of 80,000 and 400,000, respectively. Maximum peak area counts were modified to accept samples with up to 550,000 area counts in a single peak. Thus, given peaks of a chromatogram are presumed to be a specified fatty acid if the peak meets all acceptance criteria. Fatty acid profiles (fatty acid name and percentage of composition) were stored separately for each sample of *R. solani* AG-1 IA analyzed.

Library generation. An average of six profiles from six separate extractions of each isolate enabled development of a unique library entry for each isolate. For a fatty acid to be used in creating the library entry, the mean percentage of composition multiplied by the percentage of total cultures that contain that fatty acid was arbitrarily required to exceed 0.25. If, for example, a fatty acid was found in four of the six profiles and the mean percentage of composition was 0.30, that fatty acid would not be used to create a library entry because (0.30 * 4/6) = 0.20. Those failing that quality threshold were excluded from the library entry. This prevented peaks represented only once from being used to create a library entry. Library generation was used to look at populations, and fatty acids used to create library entries must be reproducibly detected in a population of isolates.

The Library Generation Software (Microbial ID, Inc.) compares the profile of a given sample to each library entry to determine its relatedness to the library entry. A similarity index, between 0.0 and 1.0, expresses how nearly the profile of a given sample matches that of the library entry (18). This index reflects the sample's distance in *n*-dimensional space (Gaussian distance) from the mean profile of the closest library entry.

RESULTS

A total of 12 fatty acids were detected and quantified. They were myristic (14:0), pentadecanoic (15:0), palmitic (16:0), 2-hydroxypalmitic (16:0 2OH), palmitoleic (16:1 cis 9), heptadecanoic (17:0), 9-heptadecenoic (17:1 cis 9), stearic (18:0), oleic (18:1 cis 9), a dimethylacetyl (dma) derivative of 11-octadecenoic acid (18:1 cis 11 dma), linoleic (18:2 cis 9, 12), and an unknown fatty acid with equivalent chain length (ECL) of 18.201. The ECL of the unknown fatty acid was derived from its relative retention time compared to that of straight chain saturated fatty acids contained in the calibration standard (16).

Area counts of less than 80,000 resulted from analysis of 48-h cultures of isolate 1-1-006 on WA+, CDA+, and CDA+T. Area counts from 48-h growth on PDA+ and TSBA+ fell within the 80,000–550,000 parameters set in the modified aerobe method. The same fatty acids were detected from mycelial growth on either PDA+ or TSBA+. Therefore, PDA+ was used for the remaining experiments because most isolates of *R. solani* grow readily on PDA (20).

The large plate used in the 96-h time course study allowed analysis of fatty acids from mycelia of different ages (Fig. 1).

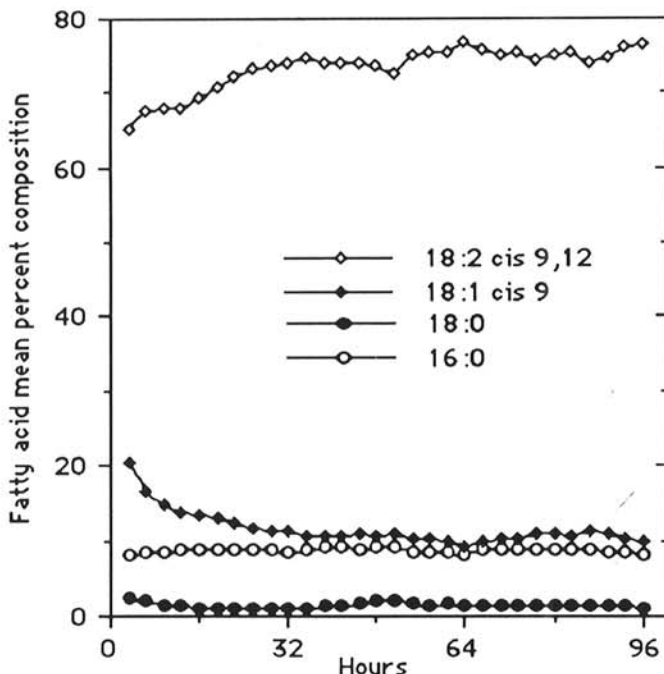

Fig. 2. Mean percentage of composition of four major fatty acids found in isolate 1-1-006 of *Rhizoctonia solani* AG-1 IA over 96 h. Data points are mean values from three experiments.

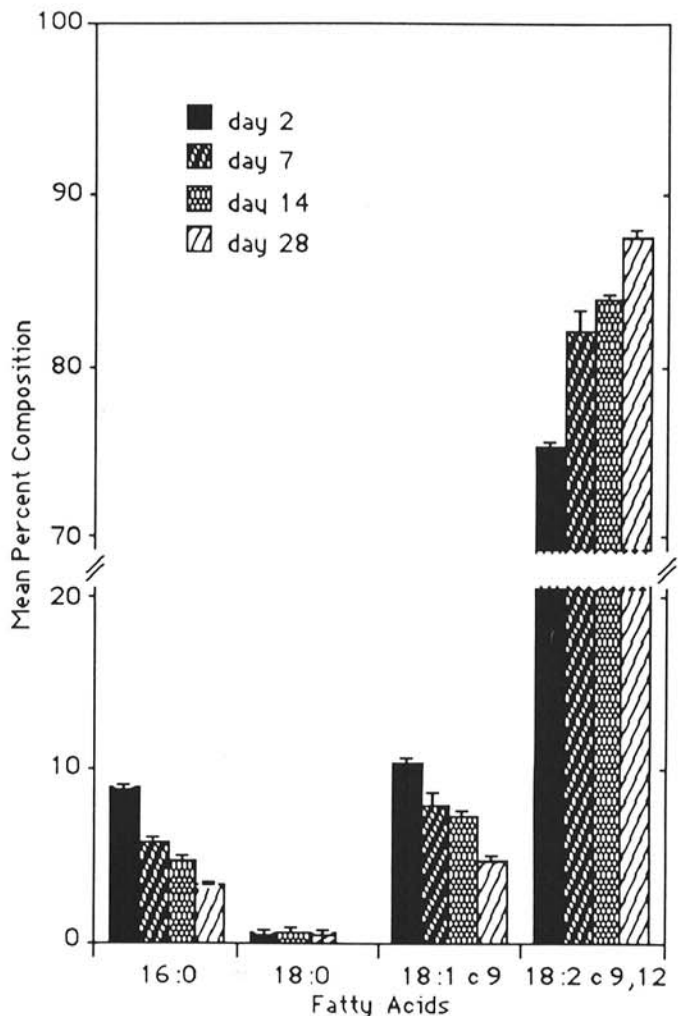

Fig. 3. Mean percentage of composition of four major fatty acids found in isolate 1-1-006 of *Rhizoctonia solani* AG-1 IA at 2, 7, 14 and 28 days. Error bars are standard deviations. The c in 18:1 c 9 and 18:1 c 9, 12 represents cis.

Each 0.5-cm strip represented mycelial growth of approximately 3.2 h. A total of 12 fatty acids were recovered from these preparations. The major fatty acids, accounting for 95% of all fatty acids identified, include 16:0, 18:0, 18:1 cis 9, and 18:2 cis 9, 12 (Fig. 2). Palmitic (16:0) and stearic (18:0) were constant throughout the 96 h. Oleic (18:1 cis 9) decreased from 20.3 to 11.0% in the first 32 h and was constant for the remaining 64 h. Linoleic (18:2 cis 9, 12) increased from 65.2 to 74.5% in the first 32 h and then remained constant through the remaining 64 h. Eight other fatty acids were detected and the mean fatty acid percentage of composition ± the standard deviation of these throughout the 96 h were: 14:0 (0.10 ± 0.07), 15:0 (0.58 ± 0.17), 16:0 2OH (0.09 ± 0.08), 16:1 cis 9 (0.76 ± 0.18), 17:0 (0.69 ± 0.37), 17:1 cis 9 (1.72 ± 0.46), 18:1 cis 11 dma (0.38 ± 0.14) and ECL 18.201 (0.60 ± 0.23). Substantial changes in the percentages of composition of these eight minor fatty acids were not observed over the 96-h time course.

In the 28-day time course, all 12 fatty acids were detected in isolate 1-1-006 at day 2. With time, certain fatty acids were not detected. At day 7, fatty acids not detected include 16:0 2OH, 18:1 cis 11 dma, and ECL 18.201. At day 14, those not detected include those not detected at day 7 plus 14:0. At day 28, those not detected include those not detected at day 14 plus 18:0. The fatty acids 16:0, 18:0, and 18:1 cis 9 all decreased over the 28-day period, whereas 18:2 cis 9, 12 increased (Fig. 3). Because all 12 fatty acids were present at 48 h and because the number of fatty acids recovered decreased by day 7, it was determined that the remaining experiments should be harvested at 48 h.

Eleven fatty acids were detected in the 10 replicate plates of isolate 1-1-006 (Table 1). The fatty acid, 16:0 2OH, was not detected in any of the 10 plates, whereas it was detected in the large plate experiment. There was little variation among the 10 replicate plates. Some variation can be accounted for by variation in the amount of mycelium extracted for each sample.

TABLE 1. Variation in mean percentage of composition of the fatty acids found in isolate 1-1-006 of *Rhizoctonia solani* AG-1 IA

Fatty acid	ECL[a]	Percentage of composition[b] (± SD)
Myristic (14:0)	14.000	0.05 (0.10)
Pentadecanoic (15:0)	15.000	0.39 (0.05)
Palmitoleic (16:1 cis 9)	15.817	0.93 (0.12)
Palmitic (16:0)	16.000	8.95 (0.27)
9-Heptadecenoic (17:1 cis 9)	16.792	1.74 (0.14)
Heptadecanoic (17:0)	17.000	0.39 (0.14)
2-Hydroxypalmitic (16:0 20H)	17.235	0.00[c]
Linoleic (18:2 cis 9, 12)	17.720	75.33 (0.52)
Oleic (18:1 cis 9)	17.769	10.29 (0.21)
Stearic (18:0)	18.000	0.83 (0.14)
Unknown ECL 18.201	18.201	0.59 (0.08)
11-Octadecenoic diacetyl (18:1 cis 11 dma)	18.280	0.51 (0.05)

[a] Equivalent chain length (ECL) was calculated from retention times of saturated straight chain fatty acids contained in the calibration mix.
[b] Values represent means of 10 replicate samples.
[c] The fatty acid 16:0 20H was detected in the 96-h time-course experiment but was not detected in the present experiment.

Twelve fatty acids were detected in the 10 isolates of *R. solani* AG-1 IA (Table 2). Three fatty acids were not detected in all of the isolates. They included 14:0 (present in nine of 10 isolates), 16:0 2OH (present in seven of 10 isolates) and 17:0 (present in nine of 10 isolates).

A library entry was created from all 60 samples (10 isolates) of AG-1 IA. Fatty acids present in amounts too low to be reproducibly detected or not present in all samples of an isolate were not used to create the library entry. Fatty acids used to generate the library entry included 15:0, 16:0, 16:1 cis 9, 17:1 cis 9, 18:0, 18:1 cis 9, 18:1 cis 11 dma, 18:2 cis 9, 12, and ECL 18.201.

A comparison of the fatty acid profile from an individual sample with the library entry yielded similarity indices from 0.821 to 0.997. The indices were less than 1.0 because the library entry is the mean of 60 individual profiles from 60 separate extractions. The profiles from each extraction differed slightly because of variation in the amount of mycelium extracted.

An additional library was created in which each of the 10 isolates analyzed in the last experiment were included as entries (six samples each). Fatty acid profiles of the 10 plates of 1-1-006 were compared with the library entry of 1-1-006 created from the last experiment. Given 10 isolate choices, all 10 samples were identified correctly as isolates 1-1-006 with similarity indices from 0.866 to 0.992.

DISCUSSION

MIS has been successfully used to characterize and differentiate plant pathogenic bacteria (3,5,6,8,9,11,15). These systems are currently employed in more than 80 diagnostic laboratories and clinics throughout the United States. (M. J. Sasser, *personal communication*). With slight modifications, the aerobe method of MIS can successfully be used to profile cellular fatty acids from fungi, including *R. solani*.

The MIS method for aerobic bacteria uses the medium TSBA and bacterial strains are grown for 24 h at 28 C. Cells are streaked in four quadrants on each plate of TSBA, and 40–50 mg are harvested from the third quadrant at 24 h. Such cells are in the log phase of replication and are approximately the same age. Saponification, methylation, and extraction of fatty acids allows analysis by gas chromatography. Most bacterial strains contain 10–12 fatty acids (16), although as few as four to as many as 26 are found (Aerobe Library, Microbial ID, Inc.).

For analysis of *R. solani* there were no differences in recovered fatty acids between PDA+ and TSBA+ so we used PDA+ because most isolates of *R. solani* are known to grow well on PDA (20). Incubation times were extended to 48 h to ensure sufficient growth to meet minimum area count requirements and to reproducibly

TABLE 2. Variation in mean percentage of composition of fatty acids found in 10 isolates of *Rhizoctonia solani* AG-1 IA

Fatty acid	Isolate[a] 1-1-001	1-1-002	1-1-003	1-1-004	1-1-005	1-1-006	1-1-007	1-1-008	1-1-009	1-1-010	Mean of isolates	CV[b]
14:0	0.06	0.23	0.09	0.04	0.12	0.06	0.07	0.09	0.00	0.04	0.08	80.2
15:0	0.36	0.43	0.37	0.30	0.46	0.34	0.31	0.39	0.37	0.17	0.35	22.9
16:1 cis 9	0.99	1.23	0.91	1.00	0.82	0.93	1.03	0.72	0.74	0.75	0.91	17.5
16:0	8.52	8.40	8.67	8.18	8.31	8.60	8.58	8.97	7.93	9.04	8.52	4.0
17:1 cis 9	0.80	0.84	0.97	1.52	0.85	1.58	0.76	0.60	0.97	0.76	0.97	33.8
17:0	0.11	0.18	0.11	0.20	0.21	0.35	0.00	0.30	0.29	0.12	0.19	57.4
16:0 20H	0.00	0.07	0.00	0.04	0.09	0.00	0.12	0.05	0.08	0.05	0.05	86.2
18:2 cis 9,12	75.28	75.84	77.12	75.00	78.30	76.29	78.54	76.45	78.42	77.46	76.87	1.9
18:1 cis 9	11.83	11.95	9.50	11.89	9.12	9.91	8.82	9.88	9.45	9.19	10.15	12.2
18:0	1.00	0.74	0.85	0.68	0.71	0.70	0.61	1.23	0.63	1.16	0.83	26.8
ECL 18.201	0.55	0.60	0.75	0.57	0.54	0.61	0.57	0.66	0.59	0.69	0.61	11.0
18:1 cis 11 dma	0.51	0.48	0.59	0.49	0.43	0.60	0.57	0.55	0.55	0.59	0.54	10.4

[a] Values are the means of two experiments, each with three replicate plates.
[b] Coefficients of variation for the means of 10 isolates.

recover larger numbers of fatty acids. A doughnut-shaped plug was used in an attempt to harvest cells of approximately the same age. The results of the 96-h study indicated that very young cells differ, on average, and exhibited increased variation compared to cells that were greater than 32 h old. The doughnut-shaped mycelial plug allowed for tissue of approximately the same age to be harvested while eliminating the youngest tissue that contained fewer fatty acids. Methods for saponification, methylation, and extraction of the fatty acids were kept the same as for the MIS aerobe method.

Similar fatty acids are found in most fungi although there are differences. In Phycomycetes, there are more C20–C22 fatty acids than in higher fungi and γ-linolenic acid (18:3 cis 6, 9, 12) is present instead of α-linolenic acid (18:3 cis 9, 12, 15) found in higher fungi (22). Palmitic (16:0) is the most common saturated fatty acid, while 18:1 cis 9 and 18:2 cis 9, 12 are the most common unsaturated fatty acids in Ascomycetes and Basidiomycetes, (14,23). The major fatty acid in Basidiomycetes is 18:2 cis 9, 12, however 16:0 and 18:1 cis 9 are also significant (23). In cultivated varieties of *Agaricus campestris*, the major fatty acids were 16:0 (12–15%), 18:0 (4–6%), 18:2 cis 9, 12 (63–74%), and 20:0 (2–9%) (10). In *Sclerotium rolfsii*, a fungus in the same form order as *R. solani*, the major fatty acids included: 16:0 (23.9%), 18:0 (10.2%), 18:1 (17.5%), and 18:2 cis 9, 12 (40.1%) (14).

Fatty acids detected in the present study support the findings of Gottlieb and Van Etten (7). They found the predominant fatty acids of *R. solani* at 54–80 h to include 16:0 (14.5%), 18:1 (24.9%), and 18:2 (54.4%). In addition they detected 15:0 (trace), 16:1 cis 9 (2.5%), and 18:0 (3.9%). While the same major fatty acids were observed in this study, the percentages of composition differed. Differences may be accounted for by improved resolution with the use of capillary columns, better integration of chromatographic peaks, improved reproducibility afforded by automated injections, and by differences in incubation and growth conditions between the two studies. These reasons may also explain why additional fatty acids were identified and quantified in this study.

Changes from more unsaturated to less unsaturated fatty acids have been reported during fungal growth (21). In the isolate of *R. solani* studied by Gottlieb and Van Etten (7), there was a decrease in the percentage of 18:2 cis 9, 12 per total fatty acids, with age, whereas 18:1 cis 9 increased. This is the opposite of results found in the present study; here the percentage of composition of 18:2 cis 9, 12 increased with increasing age of mycelium, while the percentage of composition of 18:1 cis 9 and 18:0 decreased with mycelial age. Unsaturated fatty acid levels are reported to increase in organisms grown at suboptimal temperatures, whereas saturated fatty acid levels increase at higher growth temperatures (2). However, the optimal growth temperature for *R. solani* AG-1 IA has been reported at 28 C (12,19). Therefore, in the present study, the increase in unsaturation with age was not due to suboptimal growth temperatures.

Of the 12 fatty acids identified and quantified, only nine were used in creating the library entry. Fatty acids must have a low variance-to-mean ratio to be a used peak in the library entry. In this study, we distinguished between the 10 isolates of *R. solani* AG-1 IA and were able to correctly identify isolates when used as unknowns against a 10 isolate library.

In conclusion, fatty acid analysis of *R. solani*, using the MIS, is possible with modification of the original MIS aerobe method. Future work will attempt to develop library entries for each anastomosis and intraspecific group, which may result in a rapid and cost-effective alternative to labor-intensive anastomosis testing currently used.

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