Fatty Acid and Sterol Composition of Ungerminated Spores of the Vesicular-Arbuscular Mycorrhizal Fungus, *Acaulospora laevis*

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ABSTRACT

The fatty acids and sterols of ungerminated chlamydospores of the vesicular-arbuscular (VA) endophyte *Acaulospora laevis* were examined by gas chromatography and mass spectrometry. The total lipid content of the spores was 45.5% of the spore dry weight. Predominant fatty acids were palmitoleic (52.5%), palmitic (25.5%) and oleic (7.4%). Minor fatty acids consisted of a range of (n-3) and (n-6) polyunsaturated acids. The occurrence of (n-3) polyunsaturated fatty acids is rare in fungi of the order Mucorales. Three sterols were identified as 24-ethylcholesterol (79.9%), cholesterol (11.0%) and 24-methylcholesterol (9.2%). No ergosterol was detected. Lipids of the chlamydospores of *A. laevis* are compared with those of *Glomus caledonius*.

INTRODUCTION

The biology of the vesicular-arbuscular (VA) mycorrhizal plant relationships has stimulated a lot of interest because of their potential importance in the uptake of nutrients by higher plants.

There have been few biochemical studies on the spores of VA endophytes. However, there have been many reports of lipid droplets in the spores and hyphae of the fungi (1-4). A study of the distribution, quantity and composition of the lipids in uninfected roots, of roots infected with *Glomus mosseae* and the external mycelium revealed that the VA endophyte-infected roots contained more total lipid than uninfected roots and that the mycelium had high levels of neutral lipids (2).

Although spores of *Acaulospora laevis* cannot be grown in axenic culture, spores can be grown in pot culture in sufficient quantity for chemical analysis. This paper reports the fatty acid and sterol composition of ungerminated spores of the VA endophyte *A. laevis*, and compares the results with those previously reported for the lipids of the VA endophyte *G. caledonius* (5,6).

EXPERIMENTAL PROCEDURES

A. laevis spores were grown on the roots of *Trifolium subterraneum*, in soil which had been steam-treated (7). The spores were separated from air-dried soil, as previously described (7). Spores were dried in vacuo over KOH pellets for 24 hr prior to weighing. Immediately after separation, 600-1,000 *A. laevis* spores were disrupted in a screw-capped glass mortar and pestle, containing a small vol of methanol at 0 C. The preparation was checked microscopically to ensure that all spores were fractured. The disrupted spores were then extracted by shaking on a wrist shaker for 1 hr in 2 ml chloroform/methanol (2:1) at 0 C. This extraction process was repeated twice and the third extraction was done at 40 C. The chloroform/methanol extracts were washed with 0.2 vol of saline (0.9% w/v NaCl), and the aqueous phase was extracted by the Folch et al. method (8) as previously reported (5). The pooled chloroform phases were concentrated under nitrogen.

Fatty acids were analyzed as their methyl esters, which were prepared by transesterification of the neutral lipid extract using methanol containing 3% H_2SO_4 (9). Free sterols and sterol esters were isolated by 2-dimensional thin layer chromatography (TLC) (10). Sterol esters were hydrolyzed (9) and were acetylated together with the free sterols in pyridine/acetic anhydride (2:1) overnight at room temperature. The residue of the chloroform/methanol-extracted spores was hydrolyzed by refluxing with 20% aqueous KOH for 2 hr. Bound sterols were extracted into petroleum ether (bp 30-60 C) and bound fatty acids were recovered in petroleum ether after acidification with 6 N HCl (9). Both sterols and fatty acids were derivatized as before.

Fatty acids and sterols were analyzed by gas liquid chromatography (GLC), using a Varian Aerograph 2700 gas chromatograph equipped with a flame-ionization detector. Fatty acid methyl esters were separated on 1.8 m x 3.4 mm glass columns packed with 10% EGSS-X on Supelcoport 100/120 mesh at 200 C and with 10% BDS (butanediol succinate polyester) on Supelcoport 100/120 mesh at 170 C. Fatty acid analysis was carried out isothermally; the injector and detector temperatures for all GLC

analyses were 270 C. Chain length and the degree of unsaturation of the fatty acids were verified before and after hydrogenation in methanol with platinum catalyst (9) by comparison of their relative retention times with authentic samples, and/or by graphic determination of equivalent-chain-length (ECL) values (11). Sterols were identified by comparison of their relative retention times with authentic samples (12). The amounts of individual sterols and fatty acid methyl esters were determined by comparison of peak areas to the internal standards, stigmasterol and methyl heptadecanoate, respectively. Peak area was determined by triangulation. The identity of major fatty acids and sterols was confirmed by mass spectroscopy (MS) with a Varian Matt 311 mass spectrometer, using an ionization potential of 70 eV. Results expressed are the means of 3 batches of spores grown in pot culture.

RESULTS AND DISCUSSION

The total lipid content of *A. laevis* chlamydospores was 45.5% of the spore weight, which is high compared to most fungal spores (13). *G. caledonius*, the only other VA endophyte spore which has been studied, displayed a similar lipid content of 45.5%, and this increased during germination to 70% of the spore weight (5). From the small amount of work that has been done on fungal spore lipids from the order Mucorales, it is apparent that they generally have lower lipid contents than either *A. laevis* or *G. caledonius* (13). High total lipid levels have been observed in rust spores, such as aeciospores of *Cronartium ribicola* and basidiospores of *C. fusiforme* with 18 and 31%, respectively (14,15).

The fatty acids of *A. laevis* spores were similar to those of other fungi and plants in that a large proportion of the total fatty acids, 90.6% were made up of 14:0, 16:0, 16:1, 18:0, 18:1, 18:2 and 18:3. However, the fatty acids of *A. laevis* and *G. caledonius* apparently are distinguished from most other fungi by the presence of a range of polyunsaturated fatty acids (5). *A. laevis* contains 9.4% of its total fatty acids as polyunsaturates (Table I). The C_{20}-polyenoic fatty acids comprise 8.5% of the total fatty acids, whereas the longer chain length fatty acids made up the remaining 0.7%. There were 2 odd-chain-length fatty acids identified, 23:3(n-6) and 25:3(n-6). However, they were present only in trace amounts. Fatty acids, as methyl esters, were identified by comparison of the retention times with those of authentic standards and also with fatty acid methyl esters previously identified by GLC-MS

TABLE I

Fatty Acids of *Acaulospora laevis*

Fatty acids	Neutral lipid fatty acids (%)	Bound fatty acids (%)
14:0[a]	0.6 ± 0.2[b]	
16:0	25.5 ± 0.5	2.3 ± 0.4
16:1(n-7)	52.5 ± 1.0	23.4 ± 0.6
18:0	0.9 ± 0.3	14.8 ± 0.9
18:1(n-9)	7.4 ± 0.3	25.3 ± 1.1
18:2(n-6)	1.7 ± 0.2	10.5 ± 0.8
18:3(n-6)	0.3 ± 0.1	6.9 ± 0.4
18:3(n-3)	0.3 ± 0.2	6.3 ± 0.5
20:2(n-6)	2.3 ± 0.4	3.9 ± 0.4
20:3(n-6)	2.0 ± 0.3	0.7 ± 0.2
20:3(n-3)	1.5 ± 0.2	5.9 ± 0.6
20:4(n-6)	1.1 ± 0.2	
20:5(n-3)	1.4 ± 0.3	
22:4(n-6)	0.3 ± 0.2	
22:5(n-6)	0.2 ± 0.1	
23:3(n-6)	t[c]	
24:1(n-9)	0.2 ± 0.1	
25:3(n-6)	t	
26:3(n-6)	t	

[a]Number of carbon atoms in acid:number of double bonds; n represents the number of carbon atoms between the terminal double bond and the methyl end of the molecule. Double bond position provisionally identified only.

[b]Relative percentage of fatty acids ± SD, from 3 batches of spores.

[c]t = trace, < 0.1%.

prepared from *G. caledonius* spores (5). Also, minor polyunsaturated fatty acids were identified by ECL values, before and after hydrogenation. The fatty acids identified in *A. laevis* are very similar to those identified in *G. caledonius*. However, the range of polyunsaturated acids was greater in *A. laevis*. The predominant fatty acids of *A. laevis* and *G. caledonius* were 16:1 52.9% and 47.7%, 16:0 25.6% and 26.0%, 18:1 7.7% and 15.4%, and 18:2(n-6) 1.9% and 2.5%, respectively. The only difference seen in the fatty acids identified in both spores was that *A. laevis* contained 20:4(n-6) whereas *G. caledonius* contained 20:4(n-3).

The major fatty acids of diatoms have been shown to be 16:1 and C_{20}-polyenoic acids (16); the presence of the polyunsaturated fatty acids 20:4 and 20:5 in the nonphotosynthetic diatom *Nitzschia alba* was characteristic of photosynthetic diatoms (17). Such acids may be involved in the function of the photosynthetic apparatus in algae (17). Similar inferences may be drawn for *A. laevis* and *G. caledonius*, i.e., perhaps these fungi once had photosynthetic capabilities but have since lost them. However, on the other hand, the poly-

TABLE II

Sterols of *Acaulospora laevis*

Sterols	Total sterols[a] (μg sterols/mg dry weight)	Free sterols (%)[b]	Sterol esters (%)	Bound sterols (%)
Cholesterol	0.18 ± 0.02	20.0 ± 2.4	0.5 ± 0.1	3.4 ± 0.5
24-Methyl-cholesterol	0.15 ± 0.03	10.3 ± 1.6	10.4 ± 1.2	1.5 ± 0.3
24-Ethyl-cholesterol	1.31 ± 0.2	69.6 ± 9.2	89.1 ± 12.3	95.1 ± 15.0

[a]The figures are the average μg sterol/mg dry weight of relative peak area ± SD obtained from 3 batches of spores.

[b]The figures are the average percentages of relative peak area ± SD obtained from 3 batches of spores.

unsaturated fatty acids may be membrane components. Polyunsaturated fatty acids guarantee high flexibility of membranes at low temperatures and are less susceptible to photooxidation at high daytime temperatures (18). Because of the ubiquitous nature of VA endophytes, polyunsaturated fatty acids may play an important role in helping maintain the viability of these organisms.

Bound fatty acid in *A. laevis* represented 1.2% of the total fatty acid and their range was less than that of the total fatty acids (Table I). Also, the distribution was quite different: 18:1(n-9), 16:1(n-7), 18:0 and 18:2(n-6) were the major fatty acids.

Sterols were extracted from spores of *A. laevis*, fractionated by TLC, acetylated and separated by GLC, using a 3% SE-30 column (1.8 m × 3.4 mm). Tentative identifications based on retention data of the 3 sterols resolved were cholesterol, 24-methylcholesterol and 24-ethylcholesterol (Table II) (12). The major sterol that was separated represented 79.9% of the total sterols in the ungerminated spores and gave a GLC retention time that corresponded to 24-ethylcholesterol (12). The mass spectrum of this sterol acetate showed ion peaks at m/e 396 [M^+-(acetate)], 381 [M^+-(CH_3 + acetate)], 255 [M^+-(side chain + acetate)] and 213 [M^+-(side chain + 42 + acetate)], indicating a monounsaturated C_{29} sterol acetate with the double bond in the steroid nucleus, thereby confirming the structure to be 24-ethylcholesterol.

The second most abundant sterol acetate represented 11.0% of the total sterols and had a GLC retention time that corresponded to cholesterol acetate (12). The mass spectrum showed ion peaks at m/e 368 [M^+-(acetate)], 353 [M^+-(CH_3 + acetate)], 255 [M^+-(side chain + acetate)] and 247 [M^+-(acetate + C_9H_{13})], confirming the structure of this sterol acetate to be that of cholesterol. The smallest sterol component isolated comprised 9.2% of the total sterols and had a GLC retention time that corresponded to 24-methylcholesterol (12). The mass spectrum of this sterol acetate showed ion peaks at m/e 382 [M^+-(acetate)], 367 [M^+-(CH_3 + acetate)] 255 [M^+-(side chain + acetate)] and 213 [M^+-(side chain + 42 + acetate)], indicating a monounsaturated C_{28} sterol acetate with one double bond in the steroid nucleus, thus confirming the structure to be 24-methylcholesterol.

The total sterol content for spores of *A. laevis* was 0.16% of the spore weight. This is lower than the 0.39% sterol content reported for *G. caledonius* (6), but is still higher than the range reported for some fungi in the order Mucorales, which ranged from undetectable to 0.025% of mycelium dry weight (19). The total sterol content of *A. laevis* consisted of 49.8% free sterols, 34.4% sterol esters and 15.8% bound sterols. The major sterol in all fractions studied was 24-ethylcholesterol (Table II), followed by cholesterol in the free and bound sterols, and 24-methylcholesterol in the sterol esters. Ergosterol is considered to be the major fungal sterol, and has been more frequently reported in the Mucorales than has cholesterol (13). However, no ergosterol was detected in *A. laevis*. The lipids in spores of *G. caledonius* and *A. laevis* have similar total lipid contents: both contain a large range of fatty acids with (n-3) and (n-6) polyunsaturated fatty acids, and cholesterol, 24-methylcholesterol and 24-ethylcholesterol.

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