Triacylglycerol Consumption During Spore Germination of Vesicular-Arbuscular Mycorrhizal Fungi

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Lipids and fatty acids of spores of the vesicular-arbuscular mycorrhizal fungus *Glomus versiforme* were identified and quantitatively determined at different times of germination. Triacylglycerols were, by far, the most abundant lipid (38% on a wet-weight basis). Phosphatidylethanolamine, together with minor quantities of other phospholipids, was the main polar lipid. Palmitoleic, palmitic and oleic acids were quantitatively the most important fatty acids in total lipids, and even more so in the triacylglycerol fraction. Minor percentages of fatty acids, identified as ω3 and ω6 polyunsaturated, completed the fatty acid spectra. Germination of *G. versiforme* spores evokes a continuous decrease of triacylglycerols and an increase of phospholipids. The balance of fatty acids during germination suggests either a degradation or a transference of fatty acids from triacylglycerols to phospholipids.

KEY WORDS: Fatty acids, fungi, germination, lipase activity, lipid analysis, neutral lipids, phospholipids, spores, triacylglycerol degradation, vesicular-arbuscular mycorrhiza.

Studies on the physiology of vesicular-arbuscular mycorrhiza (VAM) fungi are of interest from both theoretical and applied points of view because they play an important role in agriculture, mainly in phosphorus-deficient soils. Although VAM have high lipid content, the available information on the lipid and fatty acid compositions of these fungi is scanty. In early studies (1), the occurrence of high levels of neutral lipids was reported, especially triacylglycerols, in roots infected with *Glomus mosseae*, as well as in the external mycelium. Citrus roots infected with *G. mosseae* were shown to contain 16-, 18- and 20-carbon fatty acids, unusual in higher plant material, and were attributed to the fungus (2,3). The composition of sterols and fatty acids of ungerminated spores of *Acaulospora laevis* (4) was also reported. Results from lipid analysis of intra- and extramatrical structures of the closely related genera *Gigaspora* and *Glomus* were discussed in relation to taxonomic status (5). Much less is known about the dynamics of lipids in mycorrhiza; however, oscillating changes in neutral, polar and total lipids, as well as a continuous increase of sterols, were described by Beilby and Kidby (6,7) during the germination of *G. caledonium* spores.

We studied the lipid classes and fatty acid compositions in both ungerminated and germinated spores of the VAM fungus *G. versiforme*, a species that forms sporocarps and contains high proportions of triacylglycerols. The study focussed on the fate of triacylglycerols and aimed at providing information on their physiology and data of possible interest in lipase technology. The variations found in the triacylglycerols, phospholipids and fatty acids during the germination are described.

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MATERIALS AND METHODS

Isolation of spores. The species of vesicular-arbuscular mycorrhizal fungi used in these experiments was *G. versiforme* (Karsten) Berch. It was maintained on *Medicago sativa* L. and *Sorghum vulgare* L. Plants were grown in 500-mL open pots of 9:1 sand/soil steamed mixture. *Medicago sativa* seeds were sown in moistened sand, and after two weeks the seedlings were transplanted to the pots and grown at room temperature.

Spores were collected from the pot cultures by wet-sieving and decanting the soil (8). They were picked up under a dissecting microscope (20–70 ×) and removed from soil detritus with the aid of thin forceps. Spores were less than six-months-old when used.

In germination tests, spores were disinfected with 2% chloramine T plus 200 ppm streptomycin (9,10) for 20 min. The sterilant solution was drained off, and spores were rinsed with sterilized distilled water. They were incubated in the dark on petri dishes containing 2% water-agar at 25°C, in two or three replicates per experiment. Germinated spores were harvested at 5, 10, 15 and 20 d of incubation. Batches of spores were processed and analyzed in triplicate.

Extraction and analysis of lipids. Samples of germinated and ungerminated spores (1–2 mg wet cells) were finely disrupted in a small conic glass tube with isopropyl alcohol to inactivate the lipases and phospholipases before extracting the lipids with chloroform/methanol (2:1, vol/vol) by the technique of Folch *et al.* (11). The preparations were checked microscopically to ensure that cell walls had been fractured.

Total lipids were analyzed by thin-layer chromatography (TLC) on high-performance TLC Silicagel 60 plates (Merck, Darmstadt, Germany) with hexane/ether/acetic acid (80:20:2, vol/vol/vol) for neutral lipid separation and with chloroform/methanol/acetic acid/water (65:25:4:4, by vol) or chloroform/methanol/28% aqueous ammonia (65:35:5, vol/vol/vol) for phospholipids. The spots were localized by exposing the plates to iodine vapor. Lipids were identified by comparison of their R_f values with corresponding standards run on the same plates and by means of color reactions on the plates. The selective spray reagents were: molybdenum blue for phospholipids and phosphonolipids, chloro-benzidine for ceramide groups, ninhydrin for amino-containing lipids, α-naftol for glycolipids and sulfuric-acetic acid for sterols.

Quantitative determination of the lipid classes was performed by TLC coupled to a flame-ionization detector (FID) (12) in a Iatroscan apparatus model TH 10 (Iatron Laboratories, Tokyo, Japan), after their separation on chromarods type S-III and triple development as previously described (13). Neutral and polar lipid classes were quantitated by comparison with known amounts of standards run under the same conditions and with monoacylglycerol as internal standard (12). Total lipids were calculated by the summation of individual lipid weights. The signals from the FID were registered on a Hewlett-Packard (Palo

TABLE 1

Lipid Classes from Ungerminated Spores of the Vesicular-Arbuscular Mycorrhiza Fungus *Glomus versiforme*

Lipids[a]	μg Lipid/100 μg cell	Percent wt/lipid wt
Total lipids	47.2 ± 5.4	100.0
Total neutral lipids	42.6 ± 3.2	90.3
Total polar lipids	4.6 ± 1.0	9.7
Triacylglycerols	37.9 ± 6.2	80.4
Free fatty acids	4.2 ± 1.3	8.9
Free sterols	0.5 ± 0.2	1.0
Phosphatidylethanolamine	3.1 ± 0.9	6.5
Other phospholipid	1.5 ± 1.2	3.2

[a]Lipids were analyzed by high-performance thin-layer chromatography (TLC) and identified on the plates. Quantitation was done by TLC-flame-ionization detection. Results are the average of four determinations ± SD.

Alto, CA) model HP-3396 A integrator. Statistical analyses were performed by means of Student's *t*-test.

Fatty acid analysis. Aliquots of the extracts of total lipids and triacylglycerols were saponified with 10% potassium hydroxide in ethanol at 80°C under nitrogen for 45 min. The unsaponifiables were extracted with petroleum ether. The fatty acids were extracted with petroleum ether after acidification and esterified with 3N HCl in methanol. Fatty acid methyl esters were analyzed by gas-liquid chromatography (GLC) in a Hewlett-Packard 5840 A apparatus equipped with an FID. A column packed with 10% SP-2330 on Chromosorb WAW was used. Temperature was programmed for a linear increase of 3°C per min from 140 to 220°C (14). The chromatographic peaks were tentatively identified by comparison of their retention times with those of standards chromatographed under the same conditions. The chainlength of the fatty acids was determined by rechromatographing the samples after hydrogenation (15). The number of double bonds of the unsaturated fatty acids was confirmed by separation of methyl esters by TLC-$AgNO_3$, with the technique proposed by Dudley and Anderson (16). Each fraction was eluted and analyzed again by GLC, as described above.

RESULTS

Lipids of ungerminated spores. The total lipid content on a wet-weight basis of the ungerminated spores and their lipid classes are shown in Table 1. Neutral lipids were the most significant fraction, and their principal characteristic was the high content of triacylglycerols, which represented 80% of total extracted lipids.

The polar lipid fraction was constituted mainly of phosphatidylethanolamine. In addition to this common phospholipid, a more polar amino phosphatide, whose R_f differed greatly from that of phosphatidylserine, was also present. Neither glycolipids nor sphingolipids were found in this species.

Percent compositions of the main fatty acids from total lipids, determined by GLC, are shown in Table 2. Fatty acid methyl esters were tentatively recognized on the basis of their relative retention times; carbon chainlengths and number of double bonds were confirmed by auxiliary techniques. Monoenoic acids of 16 and 18 carbons predominated over saturated acids. Minor quantities of α and γ isomers of linolenic acid, as well as other polyunsaturated fatty acids of the ω6 and ω3 series, complete the fatty acid spectra.

Fatty acids from the isolated triacylglycerol fraction were also analyzed (results not shown). There were significant quantities of oleic, palmitoleic and palmitic acids in this lipid fraction.

Quantitative variations along the spore germination. Hyphae were produced by spores on the agar surface. The hyphal elongation was noticeable within the first ten days and continued after spore germination up to twenty days.

The results obtained after analyzing the lipids during spore germination on water-agar are shown in Figures 1 and 2. Total lipids diminished progressively during 16-d germination (from 47 to 38 μg/100 μg wet cells; $P < 0.02$). Neutral lipids decreased significantly; values fell down to about one-half of the initial levels in the first ten days ($P < 0.01$). This variation in neutral lipids was the consequence of a marked decrease in triacylglycerols (from 38 to 6.3 μg/100 μg wet cells; $P < 0.01$). In contrast, the free fatty acids and the phospholipids ($P < 0.02$) showed a continuous increase.

The fatty acid spectrum of total lipids during spore germination was also determined. Results shown in Figure

TABLE 2

Major Fatty Acids from Total Lipids of Ungerminated Spores of the VAM Fungus *Glomus versiforme*[a]

Fatty acids	Percent w/w
Palmitic (16:0)	11.9 ± 0.2
Palmitoleic (16:1ω7)	53.2 ± 0.8
Stearic (18:0)	3.6 ± 0.1
Oleic (18:1ω9)	12.0 ± 0.3
Linoleic (18:2ω6)	1.8 ± 0.2
PUFA of 18, 20 and 22°C[b]	5.6 ± 0.3

[a]Minor chromatographic peaks make for 100%. VAM, vesicular-arbuscular mycorrhiza. Fatty acids were analyzed as methyl esters by GLC, and identified by auxiliary techniques.
[b]Includes: 18:3ω6, 18:3ω3, 20:3ω6, 20:4ω6, 20:5ω3, 22:3ω6, 22:3ω3 and 22:4ω6. PUFA, polyunsaturated fatty acids.

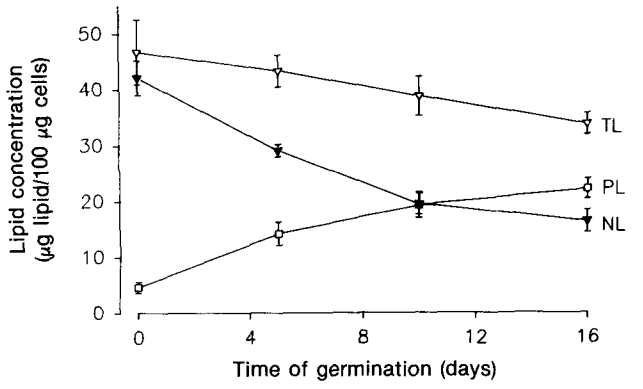

FIG. 1. Variations in total, neutral and polar lipid concentrations during the germination of spores of the vesicular-arbuscular mycorrhiza fungus. TL, total lipids; NL, neutral lipids; PL, polar lipids. Error bars indicate standard deviation n-1.

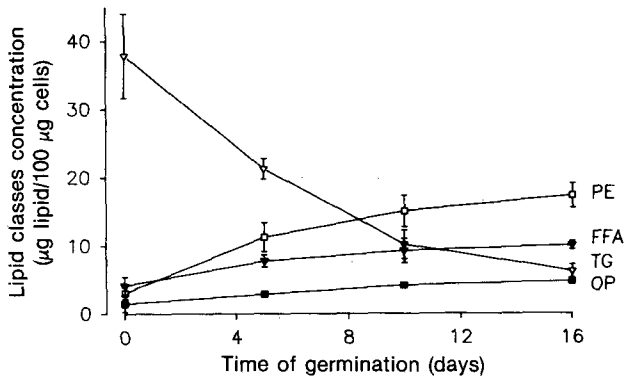

FIG. 2. Effect of germination times on lipid classes in *Glomus versiforme* spores. TG, triacylglycerols; FFA, free fatty acids; PE, phosphatidylethanolamine; OP, other phospholipid. Same notations as in Figure 1.

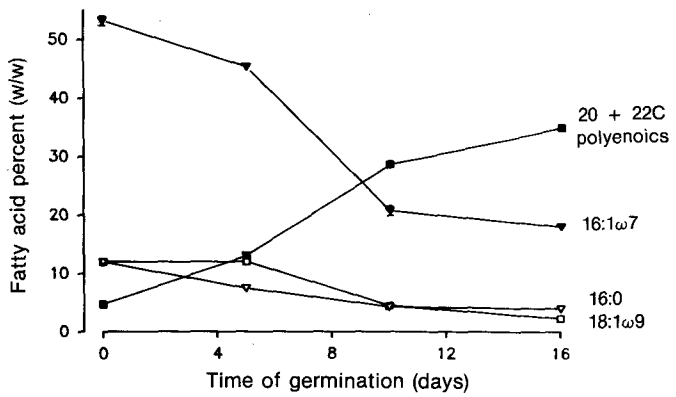

FIG. 3. Quantitative changes in fatty acids from total lipids during spore germination. Same notations as in Figure 1.

3 revealed a decrease in the content of palmitoleic, oleic and palmitic acids and a parallel increase in the polyenoic acids of 20 and 22 carbons.

DISCUSSION

Based on results from the lipid analysis, we may consider the VAM species studied as an oleaginous fungus. The ungerminated spores contain a significant proportion of triacylglycerols as expected because of their suggested role as storage compounds (17). The occurrence of a high concentration of triacylglycerols was also reported in other species of *Glomus* as well as in *A. laevis* and *Gigaspora margarita* (3-6). Nevertheless, in *G. caledonium* and *A. laevis*, the triacylglycerol proportions are comparatively lower than those found in the species studied in the present work. Other related microorganisms also contain substantial amounts of triacylglycerols; yeasts and molds both accumulate triacylglycerols, which occur in discrete droplets (18) and resemble the oil globules within the spores of VAM fungi.

Polar lipids are, in our case, surprisingly abundant (9% of the total lipids) when compared to the low levels found in ungerminated spores of other VAM species, where phospholipids represent less than 2% of the total lipids (5). It is also remarkable that phosphatidylethanolamine was the principal phospholipid in *G. versiforme*. This phospholipid is the main component of the unit membrane surrounding the lipid droplets in yeasts (18), and it might also play a similar role in the VAM lipid droplets. It is difficult to explain the lack of phosphatidylcholine, the typical and main constituent of the lipid bilayers in biological membranes. Perhaps this deserves further study concerning some of the physicochemical properties derived from such particular structures of the VAM membranes.

The spectra of fatty acids in the species examined show patterns similar to those previously reported for other members of Glomales (4,5,19), and seems to be a constant for this group of fungi. In this respect, the high content of palmitoleic acid, the detection of both isomers of linolenic acid (α and γ) and the long-chain fatty acids link these fungi with protoctista rather than with true fungi (20).

The importance of the triacylglycerol accumulation in ungerminated spores of this VAM fungus must be emphasized from the point of view of their functional role. Because most cells use triacylglycerols for energy storage, their high concentration in ungerminated spores would indicate that this accumulation is to provide energy-rich material for subsequent consumption during germination. However, the possible role of triacylglycerols as carbon sources for the synthesis of other lipids should also be considered.

Triacylglycerol degradation during spore germination is well demonstrated from our present results. After ten days of germination, the triacylglycerols were reduced to nearly one-fourth of their initial concentration. Such degradation implies the action of hydrolytic enzymes of the lipase type. This lipase activity was corroborated by *in vitro* assays performed with *G. versiforme* spore homogenates and labeled triacylglycerol as substrate (results not shown).

Phospholipids triple their concentration during the first five days of germination, and they continue to increase at least for two weeks. Because they are considered structural molecules with important functions as membrane components, the increase may be related to the need for new materials to form the germ tube that emerges through the spore wall and may be correlated to the length of hyphae produced by spores on the agar surface. Taking the germination medium characteristics into account,

the uptake of extraneous material by spores is probably minor. In consequence, the carbon (fatty acids) for phospholipid synthesis would have to arise from triacylglycerol.

As saturated and monoenoic fatty acids predominate in the triacylglycerol fraction, variation of the fatty acid composition from total lipids during germination seems to be a reflex of those changes produced in the composition of lipid classes. So, the relative increase of polyenoic fatty acids of both series may be correlated to the increment in the phospholipid concentration, and the decrease of monoenoic and saturated acids may be attributed to their consumption as triacylglycerol components. A balance of carbons among the lipid classes during the experimental germination reveals that a significant portion of the fatty acids produced after triacylglycerol hydrolysis disappears. Based on the suggested role of triacylglycerols as fuel reserve, their main fatty acids (palmitoleic and oleic) may be used, in part, for energy supply during spore germination. As such, these two fatty acids should be taken into account in further studies dealing with the fatty acid degradative metabolism in VAM fungi.

The relevant amounts of lipids in the spores of VAM fungi and, by contrast, their low content of trehalose (21,22), are evident. This fact, together with the results from the present study, reinforces Harley's (23) suggestion that the plentiful lipids of these fungi play the role of fungal carbohydrates in others. In addition to providing information on the physiological role, studies on VAM lipases might be of interest for technological purposes; research in this respect is in progress to obtain further information on the kinetics and specificity of these fungi enzymes.

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