Biochemistry of ungerminated and germinated spores of the vesicular-arbuscular mycorrhizal fungus, *Glomus caledonius*: changes in neutral and polar lipids

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Abstract Neutral and polar spore lipids of the vesicular-arbuscular (VA) endophyte *Glomus caledonius*, were identified and quantitatively determined during spore germination, germ tube growth, and germ tube senescence. There are no previous reports detailing the spore lipid components of any member of the Endogenaceae, which is in the Zygomycotina. The fungus contained 45 to 72% total lipid depending upon its stage of growth. The concentration of neutral lipids decreased during germination while the polar lipids increased. Triacylglycerides were the most abundant neutral lipid, with lesser amounts of diacylglycerides, monoacylglycerides, free fatty acids, bound fatty acids, hydrocarbons, and sterols. The major fatty acids identified by gas–liquid chromatography and mass spectrometry were 16:1, 16:0, and 18:1. The minor fatty acids identified were n-3 and n-6 polyunsaturates. The n-3 polyunsaturated fatty acids have not been reported before in Zygomycetes. The fatty acid composition of the individual lipid classes was examined. The major phospholipids were phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine, with smaller amounts of diphosphatidylglycerol and phosphatidic acid. The free sterol fraction was in greater quantity than sterol esters during germination and germ tube elongation. The capacity to synthesize sterols was demonstrated. Approximate net rates of change in the different lipid components were calculated. During spore germination and early germ tube growth, there was a net synthesis of lipids, with a large production of free fatty acids, in the germinating spore. Later in the growth period there was a net degradation of lipid, characterized by a large conversion of free fatty acids to unidentified compounds. During this period net free sterol synthesis ceased and sterol ester synthesis continued using the existing free sterol.—**Beilby, J. P., and D. K. Kidby.** Biochemistry of ungerminated and germinated spores of the vesicular-arbuscular mycorrhizal fungus, *Glomus caledonius*: changes in neutral and polar lipids. *J. Lipid Res.* 1980. **21:** 739–750.

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There is now a growing interest throughout the world in vesicular-arbuscular (VA) endophytes, and the potentially important role they may play in agriculture, in particular in phosphorus-deficient soils (1–4). Many plants take up more phosphate and grow better in soils of low fertility when inoculated with VA endophytes (1). The physiology of VA endophytes has become of great interest from both the theoretical and applied points of view. Despite this wide interest, there have been no comprehensive biochemical studies on their spores. The spores formed by VA endophytes are almost certainly an important mechanism for survival and dispersal.

Most of the biochemical studies of VA endophytes are concerned with their mycorrhizal relationship with plants and only one study has been made of the mycelial lipids of these fungi (5). The composition of fungal lipids has been the subject of a number of recent reviews (6–8). Ho and Trappe demonstrated that ^{14}C-labeled photosynthate accumulated in the lipid rich chlamydospores of *Glomus mosseae* (9). Bevege, Bowen, and Skinner (10) also observed ^{14}C-labeled photosynthate in fungal hyphae external to the plant roots. MacDonald and Lewis (11) inferred from cytochemical evidence that *G. mosseae* possessed an Embden-Meyerhof-Parnas system, a tricarboxylic acid cycle and a hexose monophosphate shunt.

There are many reports of oil droplets being observed in spores and hyphae of VA endophytes (1, 5, 12, 13). Cooper and Lösel (5) made a study of the distribution, quantity and composition of

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

lipids in uninfected roots, of roots infected with *G. mosseae* and the external mycelium. The VA endophyte-infected root contained more total lipid than uninfected roots and the mycelium had high levels of neutral lipids, especially triacylglycerides, diacylglycerides, and free fatty acids. The phospholipid distribution of the hyphae was described.

Although it is not yet possible to grow these fungi in axenic culture, germination and limited germ tube growth can be obtained on agar (14–17). Some aspects of the physiology of germinating VA endophyte spores have been examined (18), but no detailed biochemical studies have been done on either ungerminated or germinated spores.

The purpose of this investigation was to gain insight into the early biochemical events of spore germination and germ tube growth. In addition, this knowledge may help to reveal how these spores are able to survive in their ecological environment.

This study describes the qualitative and quantitative lipid content of ungerminated spores, and details the changes which occur during germination and germ tube growth of the VA endophyte, *Glomus caledonius*.

MATERIALS AND METHODS

Spore production and isolation

Glomus caledonius spores were grown either in pot culture or under a field pasture of *Trifolium subterraneum* (18). Spores of *G. caledonius* were isolated from air-dried soil by centrifugation in 50% (w/v) sucrose solution and sterilized using a 5.0% (w/v) chloramine T solution (18).

Culture conditions

Soil extract agar media was prepared as previously described (19) except that it contained chloramphenicol at 100 μg/ml. Surface-sterilized spores were placed on type HA Millipore filter segments, (Millipore Corporation, Bedford, MA) overlaying the agar; this enabled easy harvesting of the spores. The inoculated Petri dishes were enclosed in plastic bags and incubated in the dark at 20 ± 2°C.

Spore harvest

G. caledonius spores were examined microscopically at a magnification of 200X and removed from the Millipore filters with a fine sable hair brush. Harvest times were after 7, 14, and 21 days of incubation. Spores to be weighed were dried in vacuo over KOH pellets for 24 hr prior to weighing.

Extraction of lipids

Total lipid was extracted from the spores according to the procedure of Folch, Lees, and Sloane Stanley (20) except for the following modifications. Immediately after harvest 500–1,000 *G. caledonius* spores were disrupted in a glass, screw-capped mortar and pestle, containing a small volume of methanol at 0°C. The contents were checked microscopically to ensure that all spores were fractured. Solvent volume was adjusted with methanol to 0.5 ml, the tube was shaken, and 1 ml of chloroform added. The tube was then shaken for 1 hr 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 volumes of saline (0.9% w/v NaCl). The aqueous phase was extracted twice with 40 volumes of chloroform–methanol–saline 86:14:1 (v/v). The pooled chloroform phases were evaporated to dryness under a stream of nitrogen in a Teflon-lined, screw-capped glass vial. Samples were stored in benzene at −20°C.

Total lipid was determined by the method of Marsh and Weinstein (21). Total phosphorus and phospholipid phosphorus were determined by the method of Rouser, Fleischer, and Yamamoto (22).

Thin-layer chromatography

The identification of lipids was made by co-chromatography with the following standards: phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, sphingomyelin, diphosphatidylglycerol, phosphatidic acid, tristearin, palmitic acid, cholesterol, cholesteryl palmitate, n-tricosane, methyl palmitate, 1,2;1,3-distearin, and monostearin (Sigma Chemical Company, St. Louis, MO).

Two-dimensional TLC on sodium carbonate-impregnated silica gel H (E. Merck, Darmstadt, Germany) on glass plates 70 × 70 mm was used to resolve the total lipids (23). Lipids were detected on silica gel plates impregnated with 2,5-bis[5′-*tert*-butylbenzoxazolyl-(2′)]thiophene (BBOT) or by spraying with 2,7-dichlorofluorescein.

Phospholipids were recovered from silica gel by washing twice with chloroform–methanol 2:1 (v/v), once with chloroform–methanol 1:1 (v/v) and finally with methanol. Micro-TLC separation of phospholipids was performed as described by Neuhoff (24).

One-dimensional TLC was carried out on microplates (48 × 24 mm) developed with chloroform–

methanol–acetone–acetic acid–water 60:26:6:10:6 (v/v). Two-dimensional TLC was performed on microplates (40 × 28 mm) using chloroform–methanol–conc NH_4OH 60:25:4 (v/v) in the first dimension, and chloroform–methanol–acetone–acetic acid–water 75:15:30:15:7.5 (v/v) in the second dimension.

In addition to co-chromatography, phospholipids were identified with the following spray reagents: ammonium molybdate reagent for phospholipids (25), α-naphthol for glycolipids, and ninhydrin reagent for amino-containing phospholipids (26); 50% H_2SO_4 was used for non-specific location of lipids by charring.

Preparation of lipids for analysis

Sterol esters were hydrolyzed (26) and derivatized, as were the free sterols, either in N,O-bis-(trimethylsilyl)-acetamide (BSA) or pyridine–acetic anhydride 2:1 (v/v). Fatty acids were transmethylated (26). Bound fatty acids and bound sterols were recovered from the chloroform–methanol-extracted spores by refluxing with 20% aqueous KOH for 2 hr. Bound sterols and fatty acids were extracted and derivatized for GLC analysis as before (26).

Gas liquid chromatography

Fatty acid methyl esters were analyzed by GLC on 1.8 m × 3.4 mm glass columns packed with *a*) 10% EGGS-X on Supelcoport 100/120 mesh (Supelco, Inc., Bellefonte, PA) at 200°C and *b*) 10% butanediol succinate polyester on Supelcoport 100/120 mesh at 170°C using a Varian 2700 gas chromatograph, equipped with a flame ionization detector. Chain length and the degree of unsaturation of the fatty acids were verified before and after hydrogenation in methanol with platinum catalyst (26), by comparison of their relative retention times with authentic samples, and/or by graphical determination of equivalent chain length (ECL) values (27). The identity of the major fatty acids was confirmed by mass spectroscopy, with a Varian Matt 311 mass spectrometer, using an ionization potential of 70eV.

Hydrocarbons were analyzed on a glass column 1.8 m × 3.4 mm packed with 3% SE-30 on Supelcoport 100/120 mesh using temperature programming from 100 to 300°C at 4°C/min and then held isothermally. The amounts of individual fatty acids and hydrocarbons were determined by comparison of peak areas to those of relevant internal standards, n-tricosane for hydrocarbons and methyl heptadecanoate for fatty acid methyl esters. Peak area was determined by triangulation. No attempt was made to identify individual hydrocarbon species.

TABLE 1. Dry weight and total lipid of spores of *G. caledonius* from plants grown under two regimes

Spore Source	Total Lipid[a]	Spore Weight[a]
Pot culture	46.5 ± 1.7%	0.99 ± 0.1 μg/spore
Pasture soil	70.0 ± 2.5%	2.06 ± 0.2 μg/spore

[a] Values are the means with standard deviations of three determinations each of 1,000 spores.

Spores were produced on *Trifolium subterraneum* either in pots of steam-treated soil in a glass house, or under a field pasture. Total lipid was determined by the method of Marsh and Weinstein (21).

Glassware was chromic acid-washed, and rinsed exhaustively with double glass-distilled deionized water before use. Solvents were AR grade but were double-distilled in glass (26) prior to use and stored in glass-stoppered bottles. High purity nitrogen was bubbled through all solvents prior to lipid extraction. Extraction procedures were carried out at 0°C. Parallel blank extractions were run through the entire procedure. All experiments were repeated three times and the results are the means of each set, for spores grown in pot cultures.

RESULTS

The total lipid content of ungerminated spores of *Glomus caledonius* varied with the spore weight (**Table 1**). Individual spores grown in pot cultures were about half the weight of spores grown under pasture and their lipid contents were 46% and 70%, respectively. Spores incubated on soil extract agar had germinated by day 7, and their germ tubes reached a maximum length of approximately 1.0–1.5 mm by day 21. The dry weight of germinating spores reached a maximum at day 3, showing an uptake of nutrients from the medium (**Fig. 1**) and decreased to a minimum by day 14. The following lipid changes in *G. caledonius* spores occurred during germination.

Total lipid

The total lipid content of the spores increased by 34% to a maximum by day 7 with the largest increase in total lipid occurring within the first 7 days (Fig. 1).

Neutral lipids

The percentage of neutral lipids decreased to a minimum by day 14 and then increased slightly (**Table 2**). Analysis of the neutral lipid total fatty acids showed a distribution of fatty acids ranging from 14:0 to 20:5 (**Table 3**). The maximum degree of unsaturation of the total fatty acids was in the ungerminated

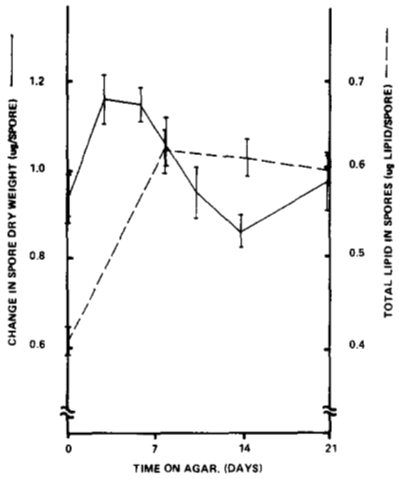


Fig. 1. Changes in the total lipid and dry weight of the spores of *G. caledonius* during germination and germ tube growth on nutrient agar. Values are the means with standard deviations of three determinations each of 1,000 spores.

spores. The most abundant fatty acid was 16:1 with other predominant fatty acids being 16:0 and 18:1. The 14:0 fatty acid showed a large increase during germ tube growth from days 14 to 21. Of the minor fatty acids, 20:4 (n-3) showed a 15-fold increase by day 7. The percentage of saturated and polyene fatty acids increased, whereas the monoene fatty acids decreased during the growth period.

Triacylglycerols

Triacylglycerols were a large proportion of the dry weight of ungerminated spores (25%), and also were the predominant neutral lipid during germination and germ tube growth, except at day 7 when the free fatty acid fraction was greater (Fig. 2). The major fatty acids in the triacylglycerols of the ungerminated and germinated spores were 16:0 and 18:1 (**Table 4**) and the concentrations of these fatty acids fluctuated during germination. There was an 80-fold increase in the concentration of 20:4 (n-3) by day 7, and it then declined rapidly. The saturated fatty acids increased during germination and germ tube growth. The monoene fatty acids decreased by day 7, then increased and plateaued, while the polyene fatty acid peaked at day 7, reflecting the change in 20:4 (n-3).

Free fatty acids

The fatty acid fraction increased by almost 50% at day 7 and thereafter decreased to less than half its initial value (Fig. 2). The major free fatty acids were 16:1, 16:0 and 18:1 (Table 3). The predominant fatty acid in the ungerminated spore was 16:1 and its concentration generally increased with time. The percentage of saturated free fatty acids tended to increase during germination and germ tube growth, the monoene fatty acids fluctuated, and the polyene fatty acids increased slightly, except 20:5 (n-3) which increased 4-fold.

1,3-diacylglycerols

Levels of 1,3-diacylglycerols steadily increased throughout germination and germ tube growth more than 8-fold. The major fatty acid of the 1,3-diacylglycerols throughout germination and germ tube growth was 16:1 except on day 7 where this was exceeded by 18:2 (n-6) (Table 4). Other major fatty acids were 16:0 and 18:1. By day 7, 20:4 (n-3) had increased 80-fold. This large increase was reflected in the degree of unsaturation which reached a maximum by day 7. The polyene fatty acids also increased rapidly by day 7, then decreased and plateaued, whereas the monoene fatty acids decreased by day 7, then increased and plateaued.

1,2-diacylglycerols

The 1,2-diacylglycerols showed little change to day 7 and then doubled by day 14, finally declining to near the level in the ungerminated spores (**Fig. 2**). The major fatty acids in the 1,2-diacylglycerols

TABLE 2. Changes in lipid composition of spores of *G. caledonius* during germination and germ tube growth

Lipid Classes	Percentage of Lipids Time on Agar (Days)			
	0[a]	7[b]	14[c]	21[d]
Total lipid (% dry weight)	45.2 ± 2.9	55.4 ± 5.9	71.8 ± 8.2	60.8 ± 7.4
Neutral lipid (% total lipids)	95.3 ± 4.0	87.4 ± 5.1	81.6 ± 4.5	89.4 ± 3.3
Phospholipids (% total lipids)	3.5 ± 0.4	8.5 ± 0.7	12.1 ± 0.9	8.2 ± 1.0
Polar lipids (% total lipids)	4.7	12.6	18.4	10.6

Each harvest time represents the mean ± SD value obtained from three batches of spores which totaled (*a*) 3,000, (*b*) 2,800, (*c*) 2,400, and (*d*) 1,500 spores.

Total lipid, neutral lipid, and phospholipids were determined as described under "Methods". The polar lipid values represent the difference between total lipids and neutral lipids.

TABLE 3. Changes in fatty acid composition of neutral lipid fractions of the spores of *G. caledonius* during germination and germ tube growth

Fatty Acid	Total Neutral Lipid Fatty Acids				Triacylglycerols				Free Fatty Acids			
	Day 0	Day 7	Day 14	Day 21	Day 0	Day 7	Day 14	Day 21	Day 0	Day 7	Day 14	Day 21
14:0[a]	1.0[b] ± 0.2	1.1	13.5	11.6	t[c]	1.3	0.5	0.2	0.6 ± 0.1	0.4	0.6	1.4
16:0	26.0 ± 0.6	26.0	24.8	26.4	26.3 ± 0.5	29.0	28.4	33.0	27.4 ± 0.3	26.2	34.2	26.4
16:1	47.7 ± 0.9	47.4	34.3	43.3	56.6 ± 1.0	40.5	50.6	56.7	42.0 ± 0.9	59.1	42.4	49.9
18:0	1.0 ± 0.1	3.5	2.0	1.0	0.1 ± 0.1	3.9	0.3	0.1	0.8 ± 0.1	0.7	2.0	1.3
18:1	15.4 ± 0.5	9.9	12.2	7.1	11.3 ± 0.2	9.3	12.9	6.3	23.3 ± 0.5	11.1	13.0	12.7
18:2(n-6)	2.5 ± 0.1	2.3	1.8	1.1	1.2 ± 0.1	2.4	1.1	0.5	0.8 ± 0.2	0.5	1.2	0.7
18:3(n-6)	0.9 ± 0.2	0.8	0.9	1.3	0.5 ± 0.1	1.3	0.7	0.2	0.3 ± 0.1	t	1.0	0.7
18:3(n-3)	0.4 ± 0.1	0.4	0.3	0.3	0.3 ± 0.1	0.7	0.2	0.2	0.5 ± 0.1	t	t	0.4
20:2(n-6)	0.2 ± 0.1	0.7	0.8	0.6	t	1.1	0.1	0.1	0.2 ± 0.1	0.1	1.0	0.6
20:3(n-6)	0.1 ± 0.1	0.1	0.4	0.3	0.1 ± 0.1	0.2	t	t	t	t	t	t
20:3(n-3)	2.7 ± 0.4	0.7	2.6	3.9	1.6 ± 0.2	0.2	2.4	1.0	2.7 ± 0.3	0.6	0.8	1.4
20:4(n-3)	0.3 ± 0.1	4.5	2.3	1.1	0.1 ± 0.1	8.2	0.2	t	0.6 ± 0.1	0.1	0.7	0.1
20:5(n-3)	1.9 ± 0.3	2.4	4.1	1.9	2.0 ± 0.3	2.2	2.7	1.9	1.0 ± 0.2	1.0	2.5	4.4
Saturates[d]	28.0	30.6	40.3	39.0	26.4	34.2	29.2	33.3	28.8	27.3	36.8	29.1
Monoenes[e]	63.1	57.3	46.5	50.4	67.9	49.8	63.5	63.0	65.3	70.2	55.4	62.6
Polyenes[f]	9.0	11.9	13.2	10.5	5.8	16.3	7.4	3.9	6.1	2.3	7.2	8.3

[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] Mean relative percentage of fatty acids ± SD, from three batches of spores.
[c] t, trace < 0.1%.
[d] Percentage of saturated fatty acids.
[e] Percentage of monoene fatty acids.
[f] Percentage of polyene fatty acids.
Fatty acids were determined as described under Methods, using gas–liquid chromatography and mass spectrometry.

of the ungerminated spores were 16:0, 16:1, and 18:1 (Table 4). By day 7, 20:4 (n-3) increased 52-fold and the concentration of 18:0 increased 23-fold; 16:0 decreased 5-fold and 18:1 decreased approximately 3-fold. The major fatty acids by day 21 were 16:1, 16:0, 20:4 (n-3), and 18:1. The level of saturated fatty acids declined during germination and germ tube growth, and the polyene and monoene fatty acid levels fluctuated during this time.

Monoacylglycerols

The monoacylglycerol fraction increased approximately twofold by day 14 and then dropped to almost half its initial value (Fig. 2). In the monoacylglycerols

TABLE 4. Changes in fatty acid composition of neutral lipid fractions of the spores of *G. caledonius* during germination and germ tube growth

Fatty Acid	1,2-Diacylglycerols				1,3-Diacylglycerols				Monoacylglycerols			
	Day 0	Day 7	Day 14	Day 21	Day 0	Day 7	Day 14	Day 21	Day 0	Day 7	Day 14	Day 21
14:0[a]	1.7[b] ± 0.2	4.1	1.1	2.9	t[c]	2.2	t	0.7	0.3 ± 0.1	t	3.1	0.8
16:0	40.0 ± 0.5	8.2	32.9	24.4	31.4 ± 0.5	18.5	29.4	28.0	7.8 ± 0.3	24.5	15.8	24.2
16:1	39.1 ± 0.6	20.4	32.0	40.0	41.4 ± 0.7	18.8	42.8	43.5	4.3 ± 0.2	28.0	22.5	35.7
18:0	0.9 ± 0.2	20.4	0.8	1.1	1.3 ± 0.2	7.3	1.8	0.6	14.5 ± 0.5	0.2	0.5	1.8
18:1	14.3 ± 0.4	5.0	14.1	12.2	20.3 ± 0.4	3.4	11.6	13.8	21.1 ± 0.6	15.0	22.7	8.4
18:2(n-6)	0.9 ± 0.2	9.6	4.0	0.7	2.5 ± 0.1	18.9	2.7	0.2	26.9 ± 0.8	10.9	10.0	3.9
18:3(n-6)	0.8 ± 0.2	1.6	0.4	1.5	0.2 ± 0.1	3.9	0.8	1.7	15.6 ± 0.6	2.5	2.5	1.1
18:3(n-3)	0.1 ± 0.1	ND[g]	0.2	0.3	0.2 ± 0.1	0.2	0.1	0.1	1.1 ± 0.2	1.1	1.5	0.2
20:2(n-6)	0.3 ± 0.1	3.1	1.1	1.3	0.5 ± 0.1	5.6	0.3	0.2	1.9 ± 0.3	1.9	1.5	1.1
20:3(n-6)	1.1 ± 0.3	0.5	0.4	0.6	0.7 ± 0.2	2.2	1.3	2.1	1.2 ± 0.4	0.6	1.5	2.4
20:3(n-3)	0.2 ± 0.1	1.7	0.8	1.1	1.3 ± 0.3	3.2	0.3	0.2	2.3 ± 0.4	2.2	2.5	0.1
20:4(n-3)	0.4 ± 0.1	24.8	10.9	14.1	0.2 ± 0.1	16.0	9.1	8.9	ND	3.7	0.9	0.6
20:5(n-3)	0.2 ± 0.1	0.8	0.4	ND	0.2 ± 0.1	ND	ND	ND	3.0 ± 0.3	9.5	16.7	19.7
Saturates[d]	42.6	32.7	34.8	28.4	32.7	28.0	31.2	29.3	22.6	24.7	19.4	26.8
Monoenes[e]	53.4	25.4	46.1	52.2	61.7	22.2	54.4	57.3	25.4	43.0	45.2	44.1
Polyenes[f]	4.0	42.1	18.2	19.6	5.8	50.0	14.6	13	52.0	32.4	37.1	29.1

Footnotes [a] to [f] as listed on Table 3.
[g] ND, not detected.

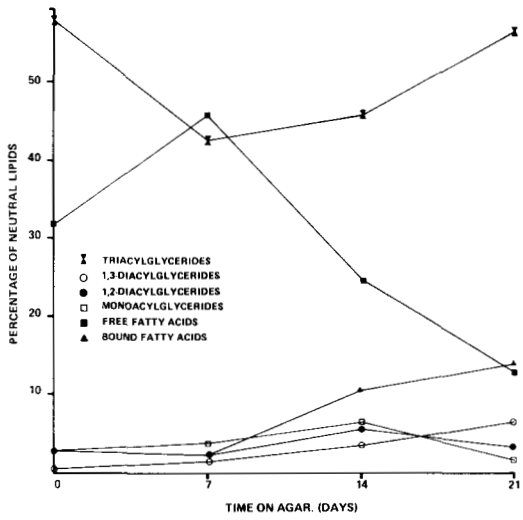


Fig. 2. Changes in the composition of fatty acid containing neutral lipids in the spores of *G. caledonius* during germination and germ tube growth. Lipid components were isolated by chloroform–methanol 2:1 (v/v) extraction and micro-thin-layer chromatography as described under Methods. Qualitative and quantitative fatty acid analysis was carried out by gas–liquid chromatography in combination with mass spectroscopy. Mean ± SD from three batches of spores.

of the ungerminated spores the most abundant fatty acids were 18:2 (n-6), 18:1, 18:3 (n-6), and 18:0 (Table 4). This fatty acid pattern changed during germination with 16:1 being the major fatty acid at day 7, followed by 16:0, 18:1 and 18:2 (n-6). By day 21, 16:1 and 16:0 comprised 60% of the fatty acids in the monoacylglycerol fraction and 20:5 (n-3) made up a further 20%. The polyene fatty acids decreased during germination and germ tube growth to almost half that of ungerminated spores, whereas the monoene fatty acids increased rapidly to day 7 then remained constant.

Bound fatty acids

Bound fatty acids slightly decreased by day 7 and then increased 7-fold. Bound fatty acids of the ungerminated spores showed 20:3 (n-3) to be the major fatty acid with 18:2 (n-6), 14:0, and 16:0 in lesser amounts (**Table 5**). By day 7, 20:5 (n-3) was the predominant acid along with 16:0 and 14:0. Between days 7 and 21, 14:0 and 20:3 (n-3) increased 4-fold and 3-fold, respectively, while 20:5 (n-3) decreased and was not detectable by day 21. The amount of saturation doubled throughout germination and germ tube growth, while the polyene content decreased by half and the monoene fatty acids remained constant on days 7 and 14, and then decreased to almost the initial value by day 21.

Fatty acid methyl esters

No naturally occurring fatty acid methyl esters were detected as has been reported in other fungi (6). A small amount of fatty acid methylation occurred when lipids were extracted in chloroform–methanol 2:1(v/v). However, when extracted in petroleum ether, no methylated fatty acids were observed.

Sterols

The total sterol content of *G. caledonius* during germ tube development showed a steady increase. The sterol fraction comprised free sterols, sterol esters, and bound sterols. Free sterols constituted the

TABLE 5. Changes in fatty acid composition of neutral lipid fractions of the spores of *G. caledonius* during germination and germ tube growth

	Bound Fatty Acids				Sterol Ester Fatty Acids			
Fatty Acid	Day 0	Day 7	Day 14	Day 21	Day 0	Day 7	Day 14	Day 21
14:0[a]	16.1[b] ± 0.4	13.4	37.9	52.9	19.1 ± 0.3	2.3	61.1	67.3
16:0	9.7 ± 0.3	14.8	8.7	6.1	24.4 ± 0.4	23.2	12.0	10.7
16:1	1.6 ± 0.2	3.4	0.9	1.1	18.7 ± 0.3	12.7	4.5	3.4
18:0	2.5 ± 0.3	6.0	5.5	3.8	10.5 ± 0.2	36.8	5.3	3.4
18:1	2.5 ± 0.2	4.0	6.3	3.4	16.0 ± 0.3	11.6	8.4	0.3
18:2(n-6)	24.7 ± 0.6	9.4	2.4	2.2	5.1 ± 0.2	0.9	0.5	6.2
18:3(n-6)	0.6 ± 0.2	4.2	0.5	6.0	0.7 ± 0.1	1.4	0.8	0.9
18:3(n-3)	0.5 ± 0.1	ND[g]	1.0	0.3	1.3 ± 0.1	0.2	0.1	1.7
20:2(n-6)	0.2 ± 0.1	0.6	1.4	2.3	1.2 ± 0.3	0.5	0.7	1.9
20:3(n-6)	t[c]	0.6	1.6	ND	1.2 ± 0.2	0.2	0.6	1.1
20:3(n-3)	27.6 ± 0.5	7.7	12.7	21.9	0.9 ± 0.1	1.9	0.2	1.6
20:4(n-3)	1.3 ± 0.3	1.3	3.9	ND	0.9 ± 0.1	8.3	5.1	1.5
20:5(n-3)	12.7 ± 0.4	34.6	17.2	ND	ND	ND	ND	ND
Saturates[d]	28.3	34.2	52.1	62.8	54.0	62.3	78.4	81.4
Monoenes[e]	4.1	7.4	7.2	4.5	34.7	24.3	12.9	3.7
Polyenes[f]	67.6	58.4	40.7	32.7	11.3	13.5	8.9	14.9

Footnotes [a] to [f] as listed on Table 3.
[g] ND, not detected.

major group up to day 14 and thereafter the bound sterols predominated (**Fig. 3**). The free sterol content increased 4-fold by day 14 and then decreased to approximately double the original value. The sterol ester content was halved by day 7, but then increased 7-fold and the bound sterols increased 6-fold between days 14 and 21.

Hydrocarbons

Hydrocarbons, the smallest lipid class of the ungerminated spores, increased 10-fold during germination and germ tube growth (Fig. 3).

Sterol ester fatty acids

The major fatty acids of the sterol ester fraction of the ungerminated spores were 16:0, 14:0, 16:1, and 18:1 (Table 5). The fatty acid profile changed by day 7 when 18:0 was predominant, followed by 16:0 and 16:1. During this time there was a 9-fold increase in the amount of 20:4 (n-3). Between 7 and 14 days, 14:0 increased 27-fold becoming the most abundant fatty acid followed by 16:0 and 18:1. By day 21, 14:0, 16:0, and 18:2 (n-6) were dominant. The concentration of saturated fatty acids increased throughout germination and germ tube growth, while the monoene content declined, and the polyene fraction remained approximately constant.

Polar lipids

In contrast to the neutral lipids, the polar lipids increased to a maximum at day 14 and then declined (Table 2). It is not known what compounds represented the difference between the polar lipids and the phospholipids of the spores.

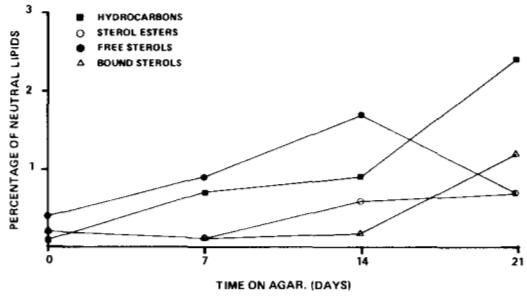


Fig. 3. Changes in the composition of non-fatty acid containing neutral lipids in the spores of *G. caledonius* during germination and germ tube growth. Lipid components were isolated by chloroform–methanol 2:1 (v/v) extraction and micro-thin-layer chromatography as described under Methods. Qualitative and quantitative fatty acid analysis was carried out by gas–liquid chromatography in combination with mass spectroscopy. Mean ± SD from three batches of spores.

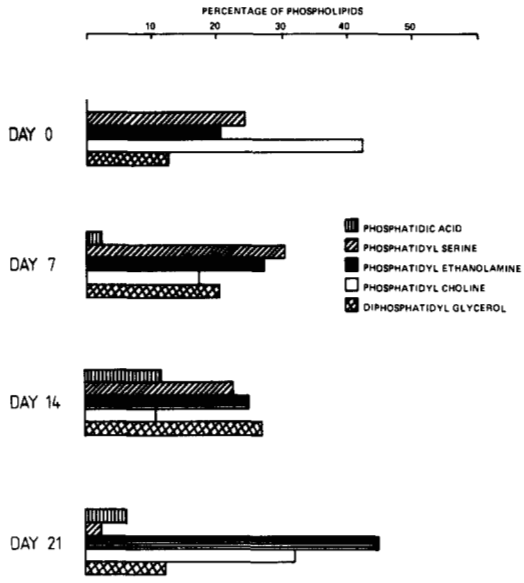


Fig. 4. Changes in the phospholipid composition of the spores of *G. caledonius* during germination and germ tube growth. Phospholipids were isolated by extraction with chloroform–methanol 2:1 (v/v) and micro TLC. Phospholipids were identified by cochromatography with standards and analyzed by determining lipid phosphorus content. Mean ± SD from three batches of spores.

Phospholipids

Phosphatidylethanolamine was the major component with phosphatidylcholine, diphosphatidylglycerol, phosphatidic acid, and phosphatidylserine making up the minor fractions after 21 days of incubation (**Fig. 4**). No phosphatidylinositol or other glycolipids were detected.

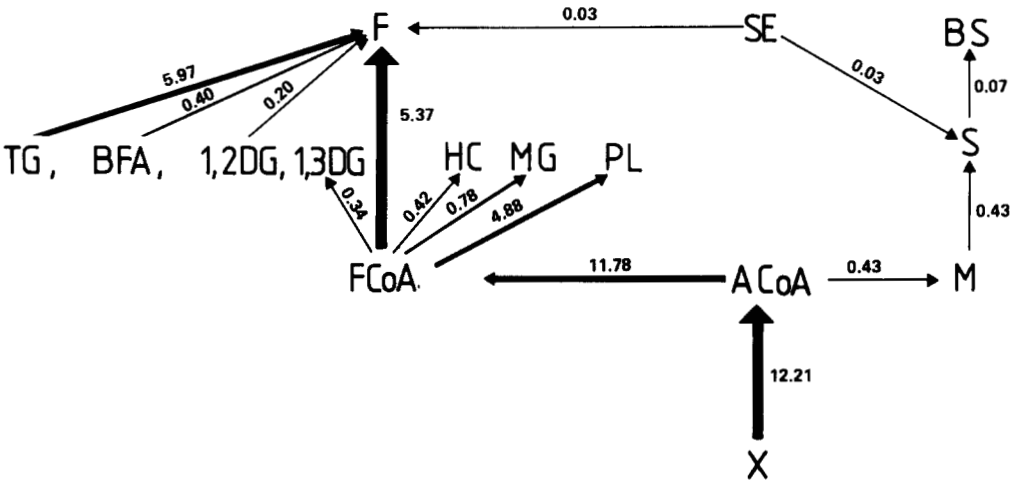
Phosphatidic acid and diphosphatidylglycerol both increased up to day 14. Phosphatidyl serine decreased 10-fold between days 14 and 21. While phosphatidylcholine went from the largest phospholipid component in the ungerminated spore to the smallest component on day 14, and then increased. Phosphatidylethanolamine increased throughout germination and germ tube growth.

DISCUSSION

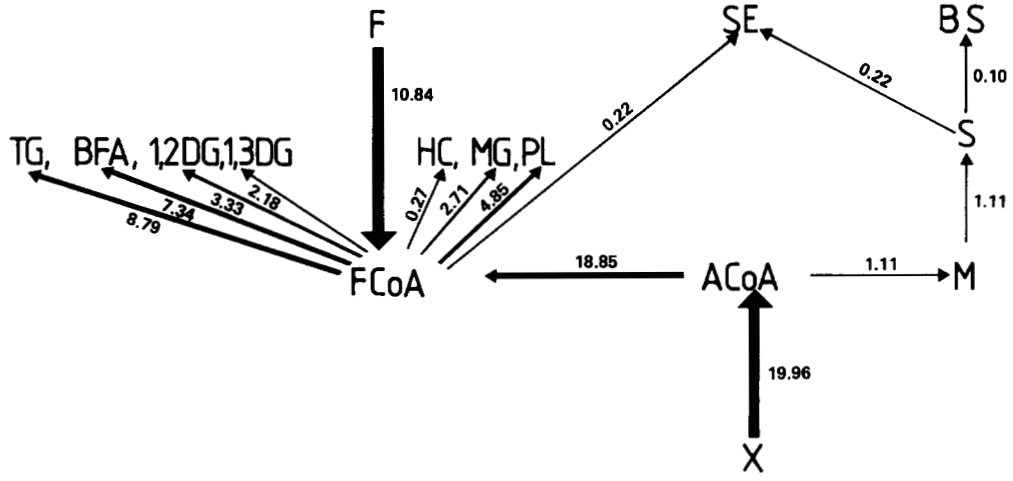
Spores of *Glomus caledonius* contain a range of polyunsaturated fatty acids, which are synthesized during germ tube growth. This is the only fungus examined of the order Mucorales, to contain (n-3) polyunsaturated fatty acids (7).[1]

[1] The subdivisions Mastigomycotina and Zygomycotina were previously known as the Phycomycete. The taxonomic classifications followed here are those used in reference 28.

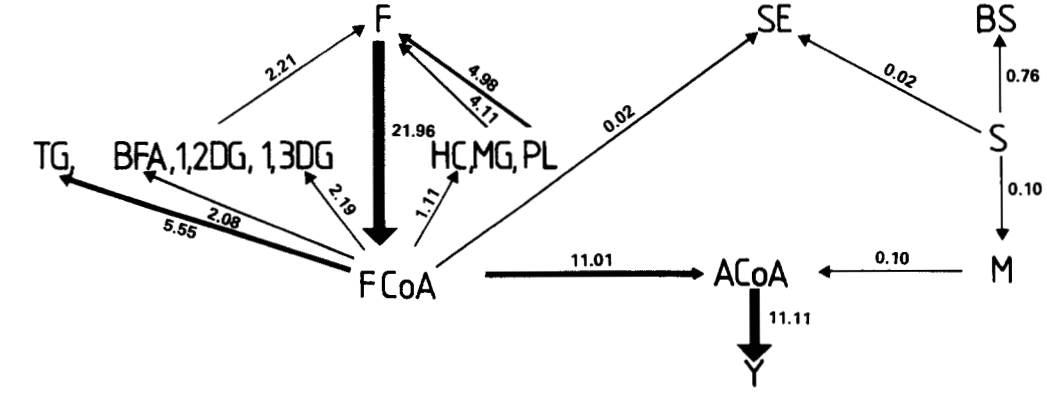
Days 0–7



Days 7–14



Days 14–21



Changes in environmental conditions, such as temperature, affect the lipid content of fungi (29, 30) and the conditions under which the spores of *G. caledonius* were formed had a large effect on spore size and total lipid content (Table 1). However, the total lipid and relative proportion of lipid classes are reported for specific growth conditions.

There is only a limited amount of published information available on lipid changes during fungal spore germination. The total lipid content of *G. caledonius* increased by 27% of the total spore dry weight, from zero time until day 14, while the germinated spore weight reached a minimum on day 14. In the Oomycete *Pythium ultimum* Trow, the total lipid varied from 3 to 48% depending on its stage of growth (31). Also in *P. ultimum*, lipid accumulated continuously throughout the growth period, reaching a maximum concentration when the net increase in mycelial weight had ceased.

The most abundant lipid class of *G. caledonius* was the triacylglycerol which represented 58% of the neutral lipids in the ungerminated spores. Triacylglycerols are also the predominant lipid fraction in a number of other fungi (6). The general trends of the neutral lipid fractions of spores of *G. caledonius* during germination and germ tube growth showed an increase in content of 1,2- and 1,3-diacylglycerols, monoacylglycerols, bound fatty acids, hydrocarbons, and total sterols, while triacylglycerols and free fatty acids decreased. Spores of other obligate plant parasitic fungi have also been reported to synthesize lipids during germination. *Uromyces phaseoli* increased in phospholipids and sterol content (32, 33), *Melampsora lini* increased in lipid phosphate content (34), and *Ustilago maydis* increased in sterols, phospholipid, and diacylglycerols (35).

Germinating spores of *G. caledonius* take up nutrients from the incubation medium as shown by a 21% increase in weight after 3 days on nutrient agar. It seems probable that these exogenous nutrients provided the energy requirements for germination and germ tube growth since the lipid content of the spores increased continuously up to day 7. Other biotrophs have also been shown to utilize nutrients from growth media (36, 41).

The total neutral lipid fatty acid composition of ungerminated spores of *G. caledonius* was characterized by a high content of the unsaturated fatty acids 16:1 and 18:1, which comprised 63% of the total fatty acids. Such high levels of 16:1 are unusual in fungi (6, 7). by day 21 the levels of 16:1 and 18:1 in *G. caledonius* had dropped, with a subsequent increase in the degree of saturation of the total fatty acids. The fatty acids of ungerminated spores of *Rhizopus arrhizus* have also been reported to be more unsaturated than those of the mycelia (6).

Within the Chytridomycetes and Oomycetes, there is a greater potential for producing long chain polyunsaturated fatty acids (C > 18:3), than in the Zygomycetes (7). *G. caledonius*, which is considered to be a Zygomycete, produced 18:3 (n-6) and 18:3 (n-3) like other species in that class of the Mucorales (7).

The amount of 20:4 (n-3) in germinating spores of *G. caledonius* showed a large increase in the monoacylglycerols, 1,2- and 1,3-diacylglycerols, triacylglycerols, and sterol ester fatty acids fractions up to day 7. Similarly 20:5 (n-3) increased 3-fold in the bound fatty acids and monoacylglycerol fractions over the same period. There appear to be no reports of Zygomycetes that synthesize the (n-3) polyenoic fatty acids, which are reported here for *G. caledonius*. Some members of the Oomycetes produce the polyunsaturated fatty acid 20:5 (n-3) (7). However, there were no polyunsaturated fatty acids longer than 20:5 detected in *G. caledonius* as have been found in the Oomycetes and Chytridomycetes (7).

The spores of *G. caledonius* had a low percentage of phospholipids throughout germination and germ tube growth because of the large neutral lipid content and, perhaps, because of the short length of germ tube produced. However, the percentage of phospholipid did increase up to day 14.

The large total lipid content of the spores of G. caledonius is unlike the lipid contents of many other fungi but like phylogenetically lower orders of plants such as the nonphotosynthetic diatom *Nitzochian alba*

Fig. 5. Directions of net metabolic flow between lipid components of the spores of *G. caledonius* during germination and germ tube growth. TG, triacylglycerols; BFA, bound fatty acids; 1,2DG, 1,2-diacylglycerols; 1,3DG, 1,3-diacylglycerols; F, free fatty acids; HC, hydrocarbons; MG, monoacylglycerols; PL, phospholipids; SE, sterol esters; S, free sterols; BS, bound sterols; FCoA, fatty acyl CoA; ACoA, acetyl CoA; M, mevalonic acid; X, unidentified precursors of acetyl CoA;Y, unidentified products. The numbers are the calculated rates which represent the mean net flow between lipid components expressed as μg/mg spore dry weight/day. FCoA, ACoA, and M intermediates were assumed to show no net accumulation during the course of the experiment. Germ tubes appeared on day 7, and maximum germ tube elongation was reached by day 14.

(37) and ungerminated spores of the fern *Anemia phyllitiolis L* (38), both of which have large triacylglyceride contents, of the order of 86% of the total lipid, and very small relative phospholipid contents, of the order of 5% of the total lipid.

Initially phosphatidylcholine followed by phosphatidylethanolamine were the major phospholipids of the ungerminated spores of *G. caledonius*. By day 21 the relative abundance of these two phospholipids had reversed, with phosphatidylethanolamine being the major compound. No phosphatidylinositol or glycolipids were detected in *G. caledonius*, or in the mycelium of *G. mosseae* (5), the other VA endophyte studied; however phosphatidic acid was found in low concentration in *G. caledonius* but was not detected in *G. mosseae* (5). Phosphatidylglycerol, which is rare in fungi (8), was found in the plant root associated hyphae of *G. mosseae* (8) but not in the ungerminated or germinating spore of *G. caledonius*. The phospholipid content of the hyphae of *G. mosseae* (5) and another Zygomycetes, *Phycomyces blakesleeanus* (39) both show phosphatidylethanolamine to be the predominant phospholipid followed by phosphatidylcholine. In both these cases the phosphotidylserine level was higher than that found in the day 21 *G. caledonius* spores.

The average sterol content of fungal organisms ranges from 0.70 to 1.0% of the dry tissue (6). McCorkindale et al. (40) found the freely extractable sterol content for 25 Mastigomycotina and Zygomycotina species to range from zero to 0.25%, which is lower than the average for fungi. However, hyphae of eleven species of Mucorales contained only 0.005 to 0.25% sterols as a percentage of the dry weight. The "freely extractable" sterols of *G. caledonius* ranged from 0.42% dry weight in the ungerminated spore to 0.85% dry weight in the 21 day old culture. Whereas the total sterol content ranged from 0.46% to 1.5% dry weight from the ungerminated spores to day 21. Individual sterol changes will be discussed elsewhere. The germinating spores of *G. caledonius* were shown to synthesize sterols and sterol esters throughout incubation in the experiment reported here. However the free sterol content decreased significantly between days 14 and 21, with the onset of senescence. It is probable that the free sterol fraction contributed to the large increase in the bound sterols observed by day 21.

Germ tubes of spores of *G. caledonius* incubated on soil extract agar did not increase in length between 14 and 21 days, and changes in the lipid composition may indicate the germ tubes are senescing after 14 days on agar. Bound sterols have been shown to increase greatly with the onset of senescence in mycelium of other fungi (41, 42). In germinated spores of *G. caledonius* there was a 6-fold increase in bound sterols between 14 and 21 days. In yeasts, diphosphatidylglycerol has almost exclusively been associated with mitochondrial membranes, and the amount present was closely correlated with the state of development of the mitochondria (43). If this is so for *G. caledonius*, the marked net decrease in content of diphosphatidylglycerol may reflect a loss of mitochondrial membrane components in the spores by day 21.

The earliest major change observed in the lipid fraction of the germinating spores, days 0 to 7, was a rise in free fatty acid content and the decline of triacylglycerols (Fig. 2). This observation implies a period of increased lipase activity. There are a number of reports of increased lipase activity in germinating fungal spores (6).

Mean net rates of change of the lipid components are shown in **Fig. 5;** these were calculated by dividing the changes in lipid components (μg/mg dry weight) by the time intervals between analysis. A net loss was apparent from days 14 to 21. The figures listed in Fig. 5 relate only to general trends for the time intervals examined, and represent net changes, which are a guide to the net metabolic flow among the lipid components. It is assumed that no unusual metabolic pathways are operative and relatively small amounts of intermediates (coenzyme A derivatives and mevalonic acid) exist. During days 0 to 7 there was a net flow of compounds into the lipid system, from precursors, with a synthesis of free sterols, bound sterols, phospholipids, monoacylglycerols, hydrocarbons, and 1,3-diacylglycerols and a breakdown of bound fatty acids, triacylglycerols, 1,2-diacylglycerols, and sterol esters. During days 7 to 14 there was an increased net flow of compounds into the system with a net synthesis of all lipid components examined, except for the free fatty acids, which were broken down. By days 14 to 21 this net flow had reversed and free sterols and fatty acids were being removed from the lipid system through unknown products Y. However, during this time lipids such as sterol esters, bound sterols, bound fatty acids, hydrocarbons, 1,3-diacylglycerols, and triacylglycerols were being synthesized.

The inability of the spores to produce extended germ tube growth on nutrient agar, does not appear to be due to the lack of synthesis of certain essential lipids, such as sterols, as has been shown in species of *Pythium* and *Phytophthora*. During germination and germ tube growth, spores of *G. caledonius* synthesized lipids, including sterols and (n-3) and (n-6) polyunsaturated fatty acids.

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