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Fungal Lipid Accumulation and Development of Mycelial Structures by Two Arbuscular Mycorrhizal Fungi

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We monitored the development of intraradical and extraradical mycelia of the arbuscular mycorrhizal (AM) fungi *Scutellospora calospora* and *Glomus intraradices* when colonizing *Plantago lanceolata*. The occurrence of arbuscules (branched hyphal structures) and vesicles (lipid storage organs) was compared with the amounts of signature fatty acids. The fatty acid 16:1 ω 5 was used as a signature for both AM fungal phospholipids (membrane constituents) and neutral lipids (energy storage) in roots (intraradical mycelium) and in soil (extraradical mycelium). The formation of arbuscules and the accumulation of AM fungal phospholipids in intraradical mycelium followed each other closely in both fungal species. In contrast, the neutral lipids of *G. intraradices* increased continuously in the intraradical mycelium, while vesicle occurrence decreased after initial rapid root colonization by the fungus. *S. calospora* does not form vesicles and accumulated more neutral lipids in extraradical than in intraradical mycelium, while the opposite pattern was found for *G. intraradices*. *G. intraradices* allocated more of its lipids to storage than did *S. calospora*. Thus, within a species, the fatty acid 16:1 ω 5 is a good indicator for AM fungal development. The phospholipid fatty acid 16:1 ω 5 is especially suitable for indicating the frequency of arbuscules in the symbiosis. We propose that the ratio of neutral lipids to phospholipids is more important than is the presence of vesicles in determining the storage status of AM fungi.

Fungi in the Glomeromycota are obligate symbionts, which colonize host plant roots from spores, extraradical hyphae, or previously colonized roots. Hyphae grow from colonized roots into the soil and form the extraradical mycelium. Lipid droplets in the hyphae accumulate in the developing spores (3, 7). The extraradical mycelium may spread along the root to form new entry points, but usually spreads out from the host root to form an extensive extraradical mycelium (9, 25). From the point of mycorrhizal colonization, intercellular (*Arum*-type colonization) or intracellular (*Paris*-type colonization) hyphae spread into the root and side branches of hyphae and produce arbuscules, finely branched hyphal structures surrounded by the host plasma membrane (30). In this way, a large area of contact with the host is created, increasing the area of the host plasma membrane up to 10-fold in colonized cells (31). Arbuscules are short-lived structures believed to have a turnover rate of 1 to 2 weeks (1) and probably are a critical site for nutrient transfer between the symbionts (30). At a later stage, the fungus may form vesicles, which are lipid-filled storage structures (7, 20) with a low turnover rate, in intercellular spaces. Typical for *Glomus intraradices* is that this fungus can also form intraradical spores, which may have a storage function as well. Lipids may, however, also be transported in lipid bodies to the extraradical mycelium within a few days (3, 11).

The amount of neutral lipids (for energy storage) is usually higher than that of phospholipids (membrane constituents) in arbuscular mycorrhizal (AM) fungi, since these fungi store a large proportion of their energy carbon as neutral lipids (24).

The intraradical mycelium takes up hexoses from the host, which are metabolized to yield neutral lipids (27). Neutral lipids thus have a central role in carbon metabolism and transport in AM fungi and are probably the main respiratory substrate in the extraradical mycelium (2). Triacylglycerols are the main type of neutral lipids found in large amounts in AM fungal spores and vesicles (4, 7, 19). In contrast, neutral lipids appear to be rare in arbuscules (20). The neutral lipids in glomalean fungi are enriched in 16C fatty acids compared with the phospholipids; the neutral lipid fatty acid (NLFA) 16:1 ω 5 is especially abundant (13, 24). This fatty acid is not normally found in other fungi (18) and has been used as a signature for AM fungal lipids in different growth systems (14, 16, 21). This fatty acid accumulates in roots during AM colonization (12, 13, 23), and the amount accumulated is correlated to microscopically estimated measures of total root colonization (22). Although the background levels of the phospholipid fatty acid (PLFA) 16:1 ω 5 in soil systems are rather high, the amount of NLFA 16:1 ω 5 is a sensitive indicator for the amount of AM mycelia (21).

We have already described the development of root-colonizing structures of *Scutellospora calospora* and *Glomus intraradices* in *Plantago lanceolata* (34). In the present study, we investigated the relationship of the colonizing structures to the accumulation of phospholipids and neutral lipids in each fungus. We hypothesized that arbuscule occurrence is correlated to an increase in AM fungal phospholipids, while the occurrence of storage structures in the root (vesicles and intraradical spores) is correlated to the accumulation of neutral lipids in intraradical mycelia. The two fungi investigated differ morphologically in that *S. calospora* does not form vesicles or intraradical spores but forms auxiliary bodies on the extraradical mycelium (8). For both fungi, we studied the distribution of the

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TABLE 1. PLFA 16:1 ω 5 and NLFA 16:1 ω 5 in roots of *P. lanceolata* inoculated with *S. calospora* or *G. intraradices* or left uninoculated

Time (days)	Fatty acid concn (nmol g of root ⁻¹) ^a					
	PLFA 16:1 ω 5			NLFA 16:1 ω 5		
	Uninoculated	<i>Scutellospora</i>	<i>Glomus</i>	Uninoculated	<i>Scutellospora</i>	<i>Glomus</i>
20	ND	7 \pm 4	31 \pm 11	ND	110 \pm 56	440 \pm 66
32	2 \pm 1	12 \pm 4	170 \pm 32	22 \pm 17	180 \pm 62	15,000 \pm 5,000
53	4 \pm 0	46 \pm 10	290 \pm 18	45 \pm 5	260 \pm 24	30,000 \pm 4,700
67	2 \pm 0	19 \pm 3	150 \pm 13	60 \pm 10	230 \pm 44	43,000 \pm 5,200
80	7 \pm 3	43 \pm 8	240 \pm 44	96 \pm 9	410 \pm 94	52,000 \pm 2,700

^a For the amounts of both PLFA 16:1 ω 5 and NLFA 16:1 ω 5, significant effects were found for the fungal inoculum and the time of harvest, in addition to a significant interaction between these two experimental factors ($P < 0.001$, two-way ANOVA; $n = 3$; mean \pm standard error). ND, no detectable amount.

lipids between intraradical and extraradical mycelium as well as the relative allocation to storage lipids.

MATERIALS AND METHODS

The data used in this study were obtained from an experiment in which root-free sand compartments with different pH values were used to investigate the effect of pH on the growth of extraradical mycelium (34). In the present study, we used data only from the high-pH treatment, since this pH was similar to that of the soil-sand mixture of the root compartment. More than 90% of the extraradical mycelium was present in the root compartment. Thus, we do not consider the amount of fatty acids in the sand compartment. The data on percent root colonization and lipid content in the soil are those presented by van Aarle et al. (34), but the fatty acid data from roots and the fatty acid composition of extraradical mycelium have not been presented earlier.

Experimental setup. *Plantago lanceolata* L. was grown in a mesh bag (20- μ m-pore mesh) placed centrally in a pot and surrounded with 400 g of acid-washed quartz sand. pH was maintained at 6 with MES (2-[N-morpholino]ethanesulfonic acid) buffer (for further details, see reference 34). The mesh bags were filled with a mixture of moraine clay loam and river sand (1:1), which had been irradiated (10 kGy) to eliminate indigenous AM fungi. An inoculum (10-g soil-root mixture) of either *S. calospora* (Nicol. & Gerd.) Walker & Sanders (BEG 43) or *Glomus intraradices* Schenck & Smith (BEG 87) was mixed with 140 g of soil before it was added to the mesh bags. For nonmycorrhizal controls, 150 g of the soil without inoculum was used.

Seeds of *P. lanceolata* were surface sterilized and pregerminated as described previously (34). Four seedlings were transplanted into each mesh bag. The system was maintained at a water holding capacity of 60%. The plants were grown in growth propagators in a greenhouse (34).

We harvested 20, 32, 53, 67, and 80 days after transplantation from three replicate pots. Plants were washed clean of soil after collection. Subsamples of soil and roots were frozen for later determination of the fatty acid content. Other subsamples of the roots were used to determine mycorrhizal colonization microscopically. The total dry weights of roots were calculated by multiplying the total fresh weight with the dry weight proportion as determined on subsamples. Mycelium was extracted from the sand compartment at the last harvest in order to estimate the hyphal fatty acid composition.

AM fungal root colonization assessment. A modification of the procedure of Phillips and Hayman (28) was used to stain root samples with trypan blue. Roots were heated at 80°C in 10% KOH until sufficiently cleared. Roots were stained at room temperature overnight in 0.1% trypan blue in lactic acid, glycerol, and water (1:1:1 [vol/vol/vol]). The magnified intersections method, as described by McGonigle et al. (17), was used to determine the total AM fungal root colonization and the frequency of arbuscules and vesicles. *G. intraradices* also formed some intraradical spores. These were included in the vesicular occurrence, since both are storage structures for lipids and since it was often difficult to differentiate between these two types of structures. We will use the term "vesicular occurrence," since the vesicles clearly were the dominating structure.

Lipid extraction and analysis of AM fungal signature fatty acids. Lipids were extracted from freeze-dried soil (extraradical mycelium) and roots (intraradical mycelium), and at the last harvest, lipids were also extracted from mycelium in the sand compartment. Roots were ball milled in iron beakers except for the roots from 20 days. At 20 days, there were too few roots for ball milling; instead, these root samples were homogenized with pestles and sand in 1.5-ml microcentrifuge tubes. Extracted mycelia were shaken in 50-ml Teflon tubes with iron balls (7-mm diameter). The lipids from soil, roots, and mycelia were extracted as

described by Bligh and Dyer (6). Briefly, samples were vortexed (15 s) in a one-phase mixture of citrate buffer, methanol, and chloroform (0.8:2:1 [vol/vol/vol], pH 4.0). Lipid extracts were separated from the pellets after centrifugation at 3,000 \times g for 10 min. Lipids were fractionated into neutral lipids, glycolipids, and phospholipids on prepacked silica columns (100-mg sorbent mass; Varian, Harbor City, Calif.) by elution with 1.5 ml of chloroform, 6 ml of acetone, and 1.5 ml of methanol. The fatty acid residues of the neutral lipids (NLFAs) and phospholipids (PLFAs) were transformed into free fatty acid methyl esters, which were identified and quantified by gas chromatography (10).

Calculations based on signature fatty acids. PLFA 16:1 ω 5 was used as an indicator of AM fungal biomass (24), and NLFA 16:1 ω 5 was used as an indicator of AM fungal storage lipids (13, 24). Background levels from the nonmycorrhizal controls were subtracted in all cases except for the results given in Table 1. The ratio between neutral lipid and phospholipid content was used as an indication of allocation to energy storage in AM fungi (21). We calculated this ratio based on the fatty acid 16:1 ω 5 after values from nonmycorrhizal controls had been subtracted.

Statistics. Data on fatty acids in roots were subjected to two-way analysis of variance (ANOVA) to identify differences due to fungal inoculum and time of harvest. The relationship between AM colonization and fatty acid accumulation was described by regression analysis, and the significance of the correlation coefficient was determined by the method of Sokal and Rolf (32).

RESULTS

Accumulation of AM fungal lipids upon fungal root colonization. Colonization with either of the fungi increased the content of the AM fungal signature PLFA and NLFA 16:1 ω 5 in the roots of *P. lanceolata* (Table 1). The NLFA content was higher than the PLFA content in both fungi. *G. intraradices* accumulated much higher content of neutral lipids than *S. calospora*, and the root colonization also was more intensive with this fungus. The roots of *P. lanceolata* grew throughout the experimental period, and AM colonization did not alter root growth (34). Neither were the concentrations of the two most common PLFAs in the *P. lanceolata* nonmycorrhizal roots (16:0 and 18:2 ω 6,9) influenced by the AM fungal root colonization. These concentrations were about 10 times those of the AM fungal signature PLFA in colonized plants. The concentration of the two root PLFAs decreased with time in both mycorrhizal and nonmycorrhizal plants, whereas the concentration of the AM fungal signature PLFA increased mainly at the start of the experiment.

Total root colonization was related to the AM fungal signature fatty acids (Fig. 1). The concentrations of both NLFA and PLFA 16:1 ω 5 increased with increasing root colonization. This correlation was significant for both fungi. However, when samples harvested after 20 days were excluded from the analysis of the *G. intraradices* data, there was no significant relationship

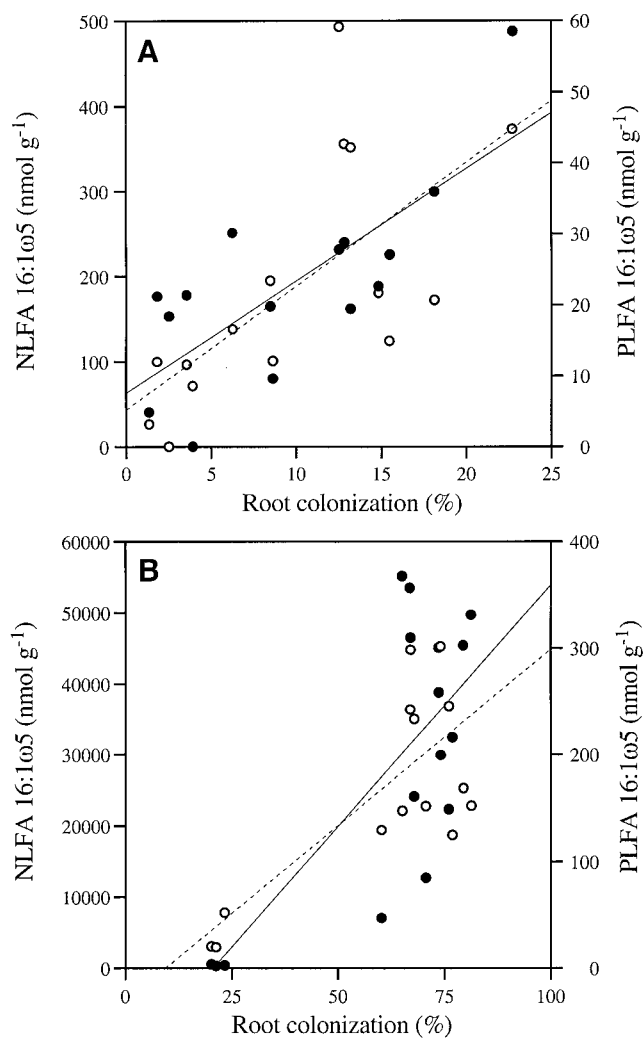


FIG. 1. Relationship between total AM fungal root colonization (percent) and the concentration (nanomoles per gram of root) of the NLFA 16:1 ω 5 and the PLFA 16:1 ω 5 in roots. Amounts from nonmycorrhizal control roots were subtracted (Table 1) ($n = 15$). Solid lines and symbols represent NLFA; dotted lines and open symbols represent PLFA. (A) NLFA 16:1 ω 5 ($y = 13x + 64$; $r^2 = 0.57$) and PLFA 16:1 ω 5 ($y = 1.7x + 5.2$; $r^2 = 0.44$) in roots colonized by *S. calospora*. (B) NLFA 16:1 ω 5 ($y = 680x - 14,000$; $r^2 = 0.53$) and PLFA 16:1 ω 5 ($y = 3.3x - 31$; $r^2 = 0.54$) in roots colonized by *G. intraradices*.

between fatty acid accumulation and AM colonization ($r^2 = 0.00$ for PLFA 16:1 ω 5, and $r^2 = 0.05$ for NLFA 16:1 ω 5).

Relationship between morphological structures and AM fungal lipids. The frequency of arbuscular occurrence and accumulation of the AM fungal PLFA 16:1 ω 5 by *S. calospora* were closely related (Fig. 2A). A decrease at around 60 days was recorded for both parameters, after which they increased again. The occurrence of arbuscules and level of PLFA 16:1 ω 5 also were related in *G. intraradices* (Fig. 2B), and again, both parameters decreased at approximately 60 days and then increased again. These temporary decreases were associated with a period of rapid root growth. The content of AM fungal neutral lipids increased in *Scutellospora*-colonized roots during the experimental period, but as expected, no vesicles or intraradical spores were formed by

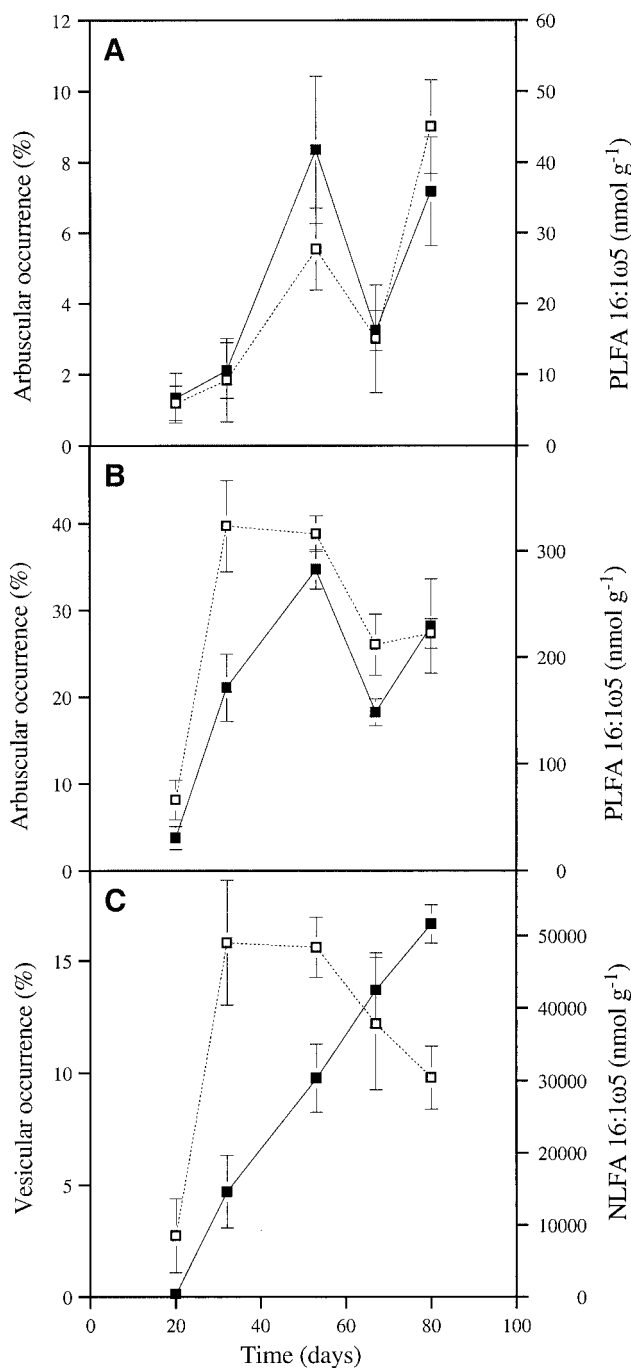


FIG. 2. Occurrence (percent) of arbuscules (A and B) and vesicles (C) in *S. calospora* (A) and *G. intraradices* (B and C) over time compared with the concentrations (nanomoles per gram) of PLFA and NLFA 16:1 ω 5 in the roots. Open symbols, arbuscular or vesicular occurrence; solid symbols, PLFA or NLFA 16:1 ω 5. *S. calospora* does not form vesicles. Fatty acid concentrations in nonmycorrhizal control roots were subtracted before calculation of the concentrations originating from the AM fungi (Table 1). The error bars show the standard error ($n = 3$).

this fungus. The content of AM fungal neutral lipids also increased continuously in *Glomus*-colonized roots, but the frequency of vesicles peaked after 32 days and then decreased (Fig. 2C). Vesicles of *G. intraradices* were found at

TABLE 2. NLFA composition and total amount of NLFAs in the extraradical mycelium of two arbuscular mycorrhizal fungi^a

Fungus	NLFA (%)						Total NLFA (nmol mg ⁻¹)
	16:1 ω 5	16:0	18:2 ω 6,9	18:1 ω 9	18:1 ω 7	18:0	
<i>S. calospora</i>	27	50	2	7	10	4	74
<i>G. intraradices</i>	37	47	3	5	4	3	20

^a The mycelium in the sand compartment was sampled at 80 days. The fatty acid nomenclature is given according to Tunlid and White (33).

the first harvest, even though very little of the AM fungal signature NLFA was detected at this time.

Partitioning of lipids between the intraradical and extraradical mycelia. The total NLFA concentration of the extraradical mycelia extracted from the sand compartment was about three times higher in *S. calospora* than in *G. intraradices* (Table 2). The PLFA content of the extracted extraradical mycelia from the sand was too low to be estimated.

S. calospora accumulated similar amounts of PLFAs in intraradical and extraradical mycelia (estimated from root and

soil fatty acid analyses, respectively) until the last harvest, when most of the PLFAs were found in the extraradical mycelium (Fig. 3A). Most of the NLFAs also were present in the extraradical mycelia (Fig. 3C). For *G. intraradices*, the pattern was reversed, and most of the AM fungal lipids were found in the intraradical mycelia (Fig. 3B and D). The amount of NLFAs in the extraradical mycelia appeared to have reached a maximum by the end of the experiment, but they were still accumulating at a rapid rate in the intraradical mycelia (Fig. 3D).

Relative allocation to storage lipids. The ratio between NLFAs and PLFAs (using fatty acid 16:1 ω 5 as the indicator) was difficult to calculate for the extraradical mycelia due to high background levels of the PLFA 16:1 ω 5 in soils with non-mycorrhizal control plants and was not meaningful for the early harvests. In those instances in which the ratio could be calculated, it was clearly higher for *G. intraradices* than for *S. calospora* (Fig. 4). For *S. calospora*, the ratio was higher in the extraradical mycelia than in the intraradical, although not statistically significant, and there were no changes in the ratio over time (Fig. 4A). For *G. intraradices*, the ratio for the

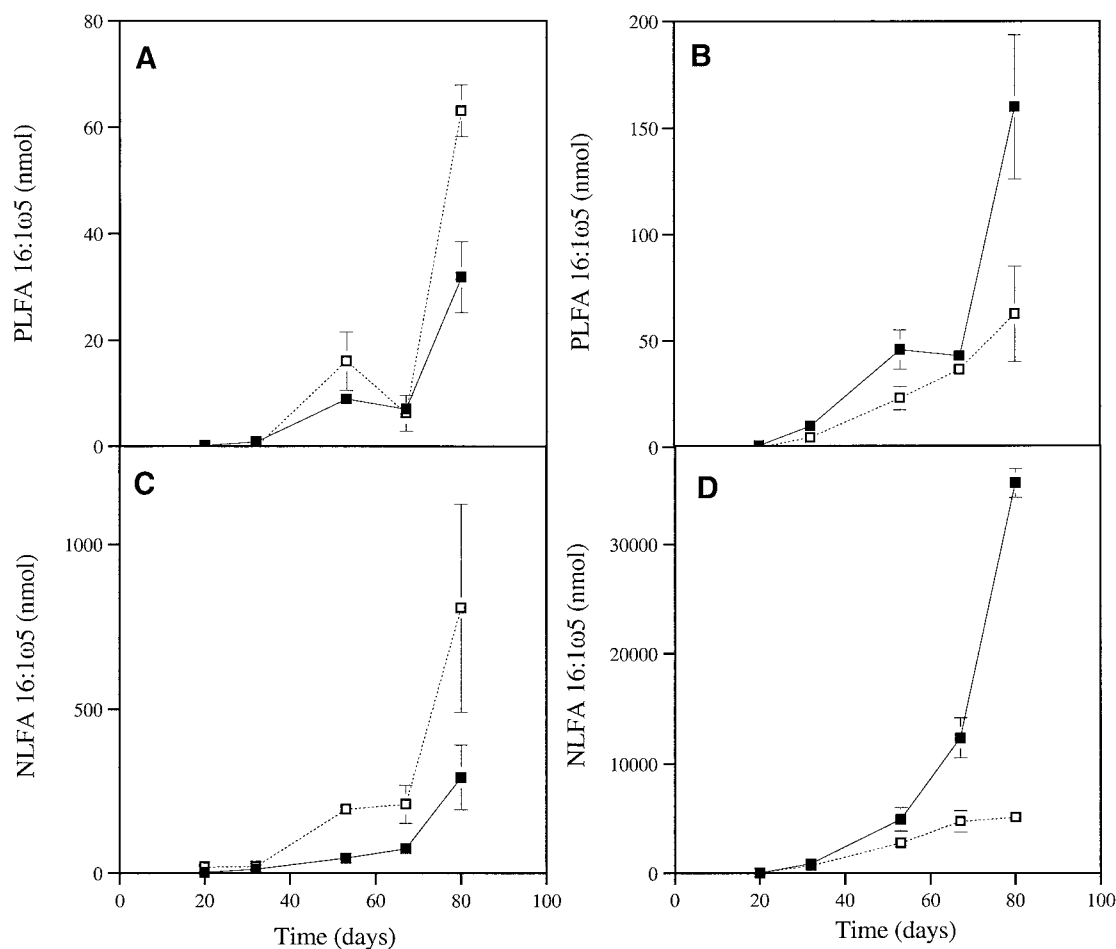


FIG. 3. Comparison of the total amount (nanomoles) of AM fungal PLFA (A and B) and NLFA (C and D) of *S. calospora* (A and C) and *G. intraradices* (B and D) in intraradical (solid symbols) and extraradical mycelia (open symbols). The amounts of fatty acids in nonmycorrhizal control roots and soil were subtracted before calculation of the amount originating from the AM fungi. The error bars show the standard error, and where no error bars can be seen, the standard error was smaller than the symbol ($n = 3$).

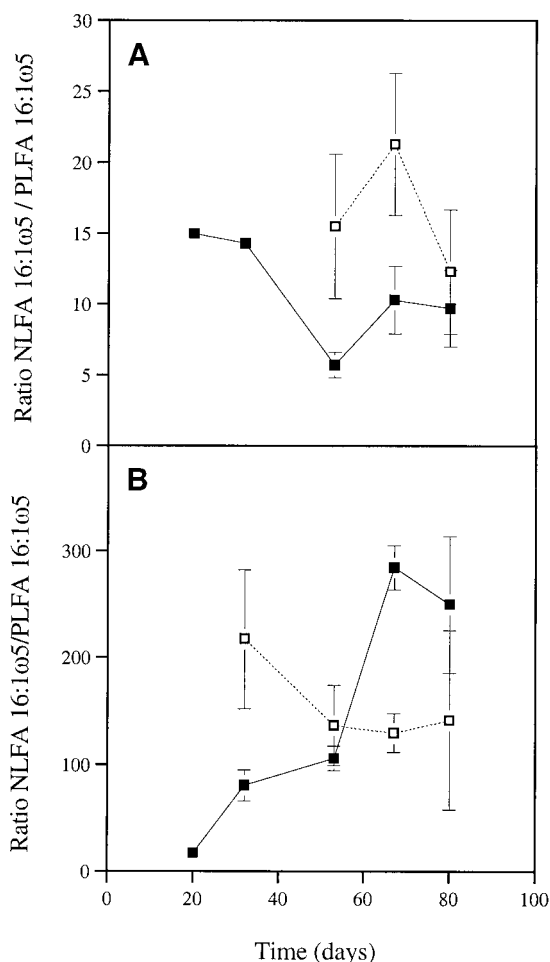


FIG. 4. Ratio between AM fungal PLFA and NLFA 16:1 ω 5 in the roots (intraradical mycelia [solid symbols]) and soil (extraradical mycelia [open symbols]) for *S. calospora* (A) and *G. intraradices* (B) over time. Ratios were calculated after the amounts present in nonmycorrhizal control roots had been subtracted. The error bars show the standard error, and where no error bars can be seen, the standard error was smaller than the symbol ($n = 3$).

intraradical mycelia increased throughout the experimental period, but no major changes were observed for the extraradical mycelia (Fig. 4B). At the end of the experimental period, the ratio was higher (but not statistically different from) in the intraradical mycelia than in the extraradical mycelia of *G. intraradices*.

DISCUSSION

Signature fatty acids are an objective tool for evaluating the amount of microbial biomass (33) and can be used to compare the development of intraradical and extraradical mycelia of AM fungi (21). *G. intraradices* had higher intensity of colonization under the present experimental conditions and partitioned most of its storage lipids and biomass to the intraradical mycelia, whereas *S. calospora* partitioned most to the extraradical mycelia.

Accumulation of lipids by the AM fungi in colonized roots is a significant carbon cost to the plant (26), but the higher

carbon demand of *G. intraradices* did not alter the growth of *P. lanceolata*. We found 50 μ mol of *G. intraradices* NLFA 16:1 ω 5 per g of root at the last harvest (Table 1). This level corresponds to approximately 30 μ mol of triacylglycerols per g of root in intraradical mycelium (24). Assuming an average triacylglycerol molar weight similar to that of tripalmitin (807 g mol⁻¹), we estimate that there was 26 mg of AM fungal triacylglycerols per g of root in *P. lanceolata* colonized with *G. intraradices*. Thus, approximately 3% of the root weight was fungal storage lipids. This lipid reserve is probably sequestered in structures with a low turnover rate (e.g., intraradical spores and vesicles), but also provides a source of lipids for translocation to the extraradical mycelia (3, 27).

Smith et al. (29) found that *S. calospora* forms a denser extraradical mycelium close to the plant roots than does *Glomus caledonium* when associated with *Medicago truncatula*. Thus, the growth strategies of *Glomus* and *Scutellospora* are different in terms of soil exploration and energy storage. In the present study, *G. intraradices* accumulated more total lipids than *S. calospora* and had a clearly higher allocation to storage lipids. The NLFA/PLFA ratio was low in *S. calospora*, and the ratio in extraradical mycelia was not significantly different from that in the intraradical mycelia. This similarity suggests a low neutral lipid content in the auxiliary cells, which constitute a substantial part of the biomass of *S. calospora*.

Spores and vesicles are storage organs for neutral lipids (3, 7, 15, 19). We found that *S. calospora*, which does not form vesicles or intraradical spores, accumulated very little neutral lipids in the roots. For *G. intraradices*, the formation of vesicles does not coincide with the accumulation of neutral lipids. Similar results were found by Graham et al. (13), who observed that lipid accumulation by *G. intraradices* and another *Glomus* isolate occurred after vesicle colonization in citrus roots. This timing results in a low correlation between vesicle occurrence and the amount of the fatty acid 16:1 ω 5. Graham et al. (12) also showed that the fatty acid 16:1 ω 5 continued to accumulate due to colonization by *G. intraradices* after 70 days of plant growth, at which time, the number of vesicles no longer increased. We hypothesize that vesicles are formed before lipid accumulation and are only later filled with lipids. Such a process is consistent with the low correlation between neutral lipid concentration and vesicular occurrence in our experiment. Even if the correlation between vesicles and neutral lipids over time is weak, the high NLFA/PLFA ratio in the intraradical and the extraradical mycelia of *G. intraradices*, compared to the much lower ratio for *S. calospora*, is a clear indication of the accumulation of neutral lipids in both vesicles and spores.

Arbuscules, in contrast to spores and vesicles, have a high turnover rate. They are short-lived structures that in most cases completed a cycle within 16 days (1). The area of contact between fungus and plant increases when arbuscules are formed (1, 31). Phospholipids are correlated to the membrane area of microorganisms (33), and the PLFA content provides a measure of the contact area of intraradical structures between AM fungi and plants. Analysis of PLFAs is a quantitative technique and should provide a good measure of fungal membrane surface area. However, PLFAs can be used as an indicator for AM fungal biomass only when an appropriate non-mycorrhizal control is available for the background levels in soil or in plant roots. In the present study, the presence of

arbuscules and PLFA content tracked each other closely. Furthermore, whenever there was a temporary decrease in arbuscular occurrence (e.g., associated with a period of rapid root growth), we also observed a decrease in PLFA concentration. This pattern indicates that fewer new arbuscules per unit of root length were formed during this period.

We found a positive correlation between total root colonization and AM fungal signature fatty acids similar to that observed earlier by Olsson et al. (22). We consider this correlation to be merely a general trend. Neither of the parameters should be considered a definitive estimator for total fungal biomass, since the correlation fails when the total root colonization exceeds 50% (Fig. 1B). The AM fungal signature NLFA was weakly correlated to vesicle occurrence with time, although the NLFA/PLFA ratio indicated the accumulation of storage lipids in vesicles and intraradical spores. However, care should be taken when comparing the lipid accumulation of different species, especially since taxonomically related differences in fatty acid composition may affect the results. For example, both *Glomus* and the *Scutellospora* species have a high proportion of fatty acid 16:1 ω 5, while the proportion of this fatty acid is normally low in *Gigaspora* species (5).

In this study, we showed that the concentration of AM fungal signature PLFA is correlated with the occurrence of arbuscules within a species and that quantification of vesicular colonization does not necessarily reflect the quantity of neutral lipid accumulation in *Glomus* species. These results are consistent with the hypothesis that neutral lipids are produced not only to be stored, but also to support the translocation of carbon within the mycelium (3). Phospholipids, on the other hand, are membrane components and probably remain at the place in which they were synthesized.

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