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Lipid and fatty acid composition of hyphae and spores of arbuscular mycorrhizal fungi at different growth stages

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The lipid and fatty acid compositions of *Glomus intraradices* and *G. claroideum* mycelia, extracted from quartz sand in a compartmentalized growth system, were analysed. The fungi were grown in association with *Cucumis sativus* and *Trifolium subterraneum*, respectively. For both fungi, the fatty acids 16:1 ω 5 and 16:0 dominated in the neutral lipid fraction, and 18:1 ω 7 made up a significant part of the phospholipids. The fatty acids were used as estimators of the amount of neutral lipids and phospholipids of AM fungi as well as to calculate the biomass of different parts of their mycelium. The phospholipid content was higher in hyphae than in spores, whereas the opposite was observed for neutral lipids. In 3-mo-old *G. intraradices* mycelium, spores accounted for 90% of the external biomass, and calculations indicated that about 20% of the spore biomass consisted of neutral lipids. In both fungi the fatty acid compositions of hyphae and spores were similar regardless of the age of the mycelium.

Using the signature fatty acid 16:1 ω 5 to calculate the distribution of AM biomass for a 2-mo-old mycelium of *G. claroideum*, we found that the fungal biomass was equally distributed between the external mycelium and the internal mycelium in the host root.

INTRODUCTION

Internal and external arbuscular mycorrhizal (AM) mycelium is usually rich in lipids and contains visible lipid droplets in all tissues (Cooper & Lösel, 1978). Beilby & Kidby (1980) estimated that lipids made up 54–72% of the spore biomass of *Glomus caledonium*, and Jabaji-Hare, Deschene & Kendrick (1984) found similar proportions of lipids in AM fungal vesicles. Triacylglycerides were the most common type of neutral lipids in vesicles and spores (Jabaji-Hare *et al.*, 1984). Fatty acids are major constituents of both phospholipids and neutral lipids, and AM fungi have a characteristic fatty acid composition that distinguish them from other fungi (Beilby & Kidby, 1980; Beilby, 1980; Nordby, Nemec & Nagy, 1981). AM fungi typically contain high contents of the fatty acids 16:1 ω 5 and 18:1 ω 7 as well as polyunsaturated 20-carbon fatty acids.

Analysis of the fatty acid composition of micro-organisms can be used to differentiate between species and has been employed for taxonomic purposes, mainly in bacteria (Lechevalier & Lechevalier, 1988). Recently this approach has also been used in phylogenetic studies of AM fungi (Bentivenga & Morton, 1996). Furthermore, certain fatty acids in AM fungal phospholipids can be used for estimating the

biomass of the AM mycelium in soil and roots (Olsson *et al.*, 1995). By measuring the contents of specific fatty acids in both the phospholipid and neutral lipid fractions, the relation between the biomass of mycelium and that of storage structures can be estimated, which in turn may reflect the degree of carbon allocation to storage structures in the AM fungi (Peng *et al.*, 1993; Olsson, Bååth & Jakobsen, 1997).

By allowing the AM mycelium to grow from a colonised root into clean quartz sand, a relatively pure mycelium can be extracted and used for physiological studies (Johansen, Finlay & Olsson, 1996). The purity of mycelium extracted from sand was assessed by analysing the fatty acid composition of the extracted *G. intraradices* mycelium. This work showed that the amounts of bacteria and saprotrophic fungi in the samples were insignificant. Thus the composition and distribution of fatty acids in AM fungi can be studied in greater detail by using mycelium extracted from sand instead of mycelium in soil systems. Such studies are necessary for evaluating the usability of signature fatty acids in soil.

The following questions were addressed in the present study: (i) How does the type of lipid extraction procedure influence lipid recovery from AM mycelia? (ii) As AM mycelium ages, does its fatty acid composition change? (iii) Does the fatty acid composition of phospholipids (membrane compounds) differ from that of neutral lipids (storage compounds)? (iv) What are the lipid contents of AM spores and hyphae? (v) How are the lipids and biomass distributed between the internal and external phases of the AM mycelium?

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MATERIALS AND METHODS

Biological material and experimental set-up

Cucumber (*Cucumis sativus*) was grown in nylon-mesh (20 µm) bags inoculated with approx. 500 surface-sterilized (5% Chloramin T) spores of *Glomus intraradices* (BEG 87), and subterranean clover (*Trifolium subterraneum*) was grown similarly with *G. claroideum* (BEG 14). The growth medium in the mesh bags consisted of a 400 g 1:1 mixture of soil (with properties as described by Jakobsen & Nielsen, 1983) and quartz sand. The mesh bags were placed centrally in 1.5 l pots lined with plastic bags, and the space outside each mesh bag was filled with washed quartz sand (1400 g, particle size distribution: 90% 0.2–0.6 mm and 10% 0.06–0.2 mm) to serve as a root-free hyphal compartment. All growth media were irradiated (10 kGy, electron beam) to suppress the growth of micro-organisms other than the inoculated fungi. The hyphal compartment was covered with non-transparent plastic to prevent algal growth. Plants were kept in a growth chamber with a 16/8 h light/dark cycle, a temperature of 15/24 °C (night/day), and an average photon flux density of 350 µmol m⁻² s⁻¹ PAR. The plants were watered daily to maintain the soil moisture at 70% of the water holding capacity of the sand. To obtain mycelia of different ages, *G. intraradices* mycelium was obtained from growth systems that were 1 or 3 mo old, while *G. claroideum* was obtained from 1 or 2 mo old growth systems.

Mycelium and root extraction

Mycelium was extracted from the sand by wet sieving (mesh size 100 µm). External mycelium was transferred to a Petri dish and freed from sand by repeated washings and transferring the mycelium to new water. Fatty acid analysis could then be performed on freeze-dried and weighed mycelium. Roots were separated from the soil by wet sieving.

Spores from old mycelium of both fungi were separated from the hyphae by repeatedly mixing them around in water and removing the released spores. The separation work was carried out until all spores had been removed from the hyphae. Spores could not be removed from the hyphae of young mycelia because they were connected too firmly to the hyphae.

Lipid extraction and fatty acid analysis

To extract lipids from freeze-dried mycelium, spores or roots, milled mycelia and spores were shaken in 50-ml Teflon tubes with iron balls (7 mm diam.), and roots were ball-milled in iron beakers. The lipids from fungal and plant material were then extracted according to the procedure described by Bligh & Dyer (1959), which includes extraction of samples by vortex mixing (1 min) in a one-phase mixture of citrate buffer, methanol and chloroform (0.8:2:1, v/v/v, pH 4.0, the B & D mixture). Phospholipids and neutral lipids were purified by silica column fractionation, and the fatty acid residues in both types of lipids were transformed to free fatty acid methyl esters and analysed by gas chromatography as described by Frostegård, Tunlid & Bååth (1993).

Various techniques for extracting lipids from 3-mo-old mycelium were evaluated by comparing amounts of the fatty acid 16:1ω5 extracted from the different lipid fractions. The extraction procedures evaluated were (i) B & D extraction only; (ii) vortex mixing with 3 g sand; (iii) freeze drying and (iv) the ordinary extraction procedure used in this study which consisted of freeze-drying and ball milling. In all cases the lipids were finally extracted from samples by vortex mixing with the one-phase mixture of citrate buffer, methanol and chloroform (B & D extraction).

Biomass calculations

The lipid contents of spores and hyphae were calculated on the basis of the estimated fatty acid concentration. The sum of all fatty acids in the samples was used for calculating the total lipid mass. Triglycerides, the most common type of neutral lipids in AM fungi, consist of three fatty acid residues on a glyceride backbone. Thus 3 mol fatty acids are equivalent to 1 mol triglycerides. Tripalmitin is a triglyceride with three 16:0 (palmitic acid) fatty acid residues. The molar weight of tripalmitin (807 g mol⁻¹) was used to calculate the mass of neutral lipids from the molar amount of fatty acids.

In a similar way, the mean weights of three phospholipids common in AM fungi (phosphatidyl ethanolamine, phosphatidyl serine and phosphatidyl choline, assuming similar amounts of these three lipids in AM fungi) were used to calculate the molar weight of the phospholipids (781 g mol⁻¹, assuming one 16:0 and one 18:1 fatty acid residue in each phospholipid). Two mol fatty acids are equivalent to 1 mol phospholipids.

The biomass of the internal mycelium was calculated using the amount of phospholipid fatty acid (PLFA) 16:1ω5 when values from non-mycorrhizal plants had been subtracted (see Table 5). It was assumed that the relation between PLFA 16:1ω5 and biomass in the internal mycelium was the same as that in the external mycelium. Thus, the content of PLFA 16:1ω5 in the external mycelium was used to calculate the biomass of the internal mycelium.

RESULTS

Lipid extraction from 3-mo-old AM mycelium

Based on the amounts of the PLFA 16:1ω5 extracted, it was concluded that the only extraction treatment that yielded more lipids from mycelium than the B & D extraction alone was ball milling (Table 1). As indicated by yields of the neutral lipid fatty acid (NLFA) 16:1ω5, ball milling enhanced the yield of neutral lipids by more than 10-fold compared with the other methods. Freeze-drying also increased the yield of neutral lipids compared with B & D extraction alone. The NLFA/PLFA ratio was 276 in the lipids extracted from 3-mo-old mycelium using the method including ball milling. The NLFA/PLFA of lipids extracted by B & D was 10 times lower, reflecting the fact that most PLFAs, but no NLFAs, are easily removed from mature spores by B & D extraction. Sand together with the B & D mixture did not enhance lipid extraction.

Table 1. Estimated efficiencies of different extraction methods in extracting lipids from 3-mo-old mycelium of *Glomus intraradices*.

	PLFA 16:1ω5 (nmol mg ⁻¹)	NLFA 16:1ω5 (nmol mg ⁻¹)	NLFA 16:1ω5/ PLFA 16:1ω5 (ratio)
B & D	0.082 ^{ab}	2.2 ^a	27 ^a
Sand + B & D	0.114 ^a	2.9 ^a	25 ^a
Freeze dry + B & D	0.070 ^b	5.4 ^b	79 ^b
Freeze dry + ball mill + B & D	0.166 ^c	45 ^c	276 ^c
ANOVA (<i>P</i> -value)	0.001	< 0.001	< 0.001

All extractions were made on freeze-stored mycelium (−20°). Fatty acid 16:1ω5 was used as an indicator of both phospholipids (as PLFA) and neutral lipids (as NLFA). Differences between extraction procedures were tested by ANOVA (*n* = 2, except for the ball mill treatment where *n* = 6). The ANOVAs on NLFA 16:1ω5 and the PLFA/NLFA 16:1ω5 ratios were made on log-transformed data due to differences in the variance. Values in any column with the same suffix letter are not significantly different (*P* = 0.05).

Fatty acid composition of hyphae and spores

For both fungi the dominating fatty acids in phospholipids were 16:1ω5, 16:0 and 18:1ω7, and the proportion of 18:1ω7 was higher in phospholipids than in neutral lipids, whereas the proportion of 16:1ω5 was higher in the neutral lipids (Table 2).

There were no major differences in fatty acid composition between hyphae and spores. Hyphae of both species had a higher concentration of PLFAs compared with spores. Spores of both fungi had a higher NLFA content compared with hyphae. The NLFA/PLFA ratio further emphasized the differences in fatty acid composition between the different lipid classes: The highest NLFA/PLFA ratio was observed for

Table 3. Proportion of dry mass, phospholipids and neutral lipids in a 3-mo-old mycelium of *Glomus intraradices*. The mass of lipids was calculated as the number of moles of fatty acids and molar weights of the different lipids.

	Weight (μg)		Proportion (%)	
	Hyphae	Spores	Hyphae	Spores
Dry mass	500	4900	9.3	90.7
Phospholipids	1.9	9.6	16.5	83.5
Neutral lipids	7.7	972	0.8	99.2

16:1ω5 in both fungi. The total NLFA/PLFA ratio was highest for spores, and an especially high value was obtained for the 3-mo-old mycelium of *G. intraradices*.

A subsample of the 3-mo-old *G. intraradices* mycelium was used for separating spores and hyphae. The hyphae accounted for less than 10% of the total weight of mycelium. Spores constituted 90% of the biomass, and calculations based on the fatty acid data indicated that 20% of the spore biomass was in the form of neutral lipids (see Table 3). The proportion of PLFAs was highest in hyphae where they constituted 0.4% of the hyphal biomass.

Fatty acid composition of mycelium of different ages

There were no general differences in fatty acid composition between 1- and 2-mo-old mycelium of *G. claroideum*, although the oldest mycelium seemed to contain a higher proportion of the PLFA 16:1ω5 (Table 4). Nor were any large differences in fatty acid composition found between 1- and 3-mo-old mycelium of *G. intraradices* (obtained from different experiments, data not shown). The content of NLFAs was more than

Table 2. All detectable and identified fatty acids in extracted mycelium and spores of extraradical mycelium of 3-mo-old *Glomus intraradices* and 2-mo-old *G. claroideum* mycelium.

	<i>G. intraradices</i>						<i>G. claroideum</i>					
	PLFAs (%)		NLFAs (%)		NLFA/PLFA (nmol g ⁻¹ /nmol g ⁻¹)		PLFAs (%)		NLFAs (%)		NLFA/PLFA (nmol g ⁻¹ /nmol g ⁻¹)	
	Hyphae	Spores	Hyphae	Spores	Hyphae	Spores	Hyphae	Spores	Hyphae	Spores	Hyphae	Spores
16:1ω7	1.1	2.3	0.2	2.5	12.3	40.2	2.7	—	3.3	—	28.9	—
16:1ω5	18.2	12.7	38.8	33.8	15.6	287.9	15.4	26.7	42.6	49.0	65.4	98.3
16:0	42.8	50.7	35.8	41.6	4.8	116.5	62.9	60.4	40.4	39.6	15.2	35.2
18:×1	—	—	0.4	0.1	—	—	—	—	0.1	—	—	—
18:×2	—	—	1.4	0.6	—	—	—	—	0.2	0.4	—	—
18:2ω6, 9	1.3	5.0	0.2	2.3	2.4	23.5	—	—	2.5	—	—	—
18:1ω9	3.6	4.5	1.3	4.9	6.5	53.6	1.8	—	4.8	1.1	61.8	—
18:1ω7	17.3	15.9	4.0	4.3	1.8	38.2	9.5	7.4	2.9	3.8	7.1	27.9
18:1	0.2	—	0.5	0.3	—	—	—	—	0.4	0.6	—	—
18:0	3.5	6.3	0.2	2.7	2.6	7.4	7.7	5.5	0.9	0.6	2.9	5.8
20:5	—	—	0.5	0.1	—	—	—	—	—	—	—	—
20:×1	—	—	0.3	0.1	—	—	—	—	—	—	—	—
20:×2	8.0	0.9	15.8	5.4	16.1	318.3	—	—	1.7	4.8	—	—
20:2	—	—	0.1	—	—	—	—	—	—	—	—	—
20:1	—	—	0.2	0.0	—	—	—	—	0.2	—	—	—
20:3	—	—	0.2	0.0	—	—	—	—	—	—	—	—
Total (nmol mg ⁻¹)	9.8	5.0	57	738	5.8	146.8	7.3	5.4	172	287	24	53

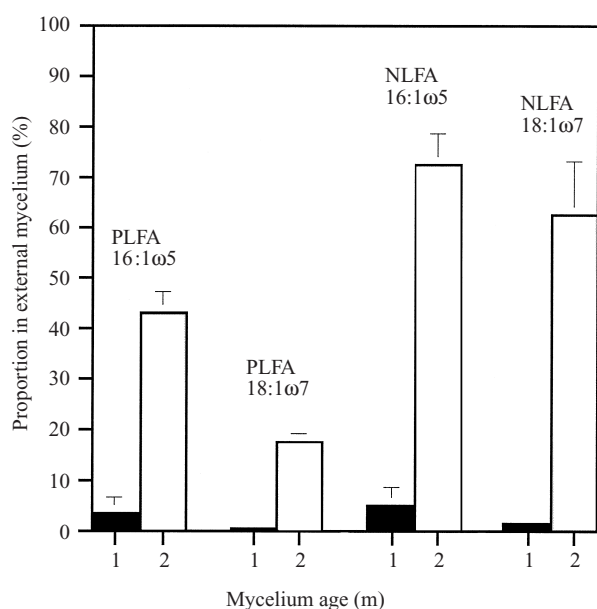
— denotes not detectable.

Table 4. Fatty acid distribution in 1- and 2-mo-old *Glomus claroideum* mycelium growing in symbiosis with *Trifolium subterraneum* ($n = 2$). Distributions of both phospholipids (PLFAs) and neutral lipids (NLFAs) are given.

	Fatty acids (%)									Total (nmol mg ⁻¹)
	16:1 ω 7	16:1 ω 5	16:0	18:2 ω 6, 9	18:1 ω 9	18:1 ω 7	18:1	18:0	20: \times 2	
PLFAs										
1 mo	0	11.6	63.5	1.4	1.8	11.6	0	10.0	0	4.2 \pm 2.2
2 mo	0.5	20.1	66.3	0	1.1	8.6	0.2	3.3	0	7.6 \pm 0.6
NLFAs										
1 mo	2.4	39.1	48.1	0.8	3.0	2.8	0.1	2.6	1.1	37 \pm 26
2 mo	0.4	49.9	41.3	0.4	1.3	3.3	0.5	0.3	2.4	167 \pm 52

Table 5. Fatty acid composition in non-mycorrhizal (NM) roots of 1-mo-old *Trifolium subterraneum* and roots colonized by *Glomus claroideum* (AM). Contents of both phospholipids (PLFAs) and neutral lipids (NLFAs) are given ($n = 2$).

	Fatty acids (pmol mg ⁻¹ root D.W.)									Total fatty acids
	15:0	16:1 ω 7	16:1 ω 5	16:0	17:0	18:2 ω 6, 9	18:1 ω 9	18:1 \times	18:1 ω 7	
PLFAs										
NM	38	30	8	956	28	569	58	269	0	1950
AM	45	22	100	1432	28	791	74	295	171	2958
NLFAs										
NM	200	137	6	1900	56	1420	163	758	0	4640
AM	118	97	2542	3760	34	1000	152	553	202	8460

**Fig. 1.** Distribution of lipids between external and internal mycelium of *G. claroideum* as indicated by the fatty acids 16:1 ω 5 and 18:1 ω 7 ($n = 2$, \pm S.E.).

four-fold higher in the 3-mo-old mycelium compared with the 1-mo-old material.

Distribution of fatty acids and biomass between external and internal AM mycelium

Roots of *T. subterraneum* showed particular high content of PLFAs 16:1 ω 5 and 18:1 ω 7 when colonized by *G. claroideum* (Table 5). The distribution of phospholipids and neutral lipids in internal and external mycelium of *G. claroideum* was studied using these two AM fungal fatty acids (Fig. 1). Measured levels of both fatty acids indicated that the proportion of neutral lipids in old AM was higher in external mycelium than in internal mycelium. Only a small amount of mycelium in 1-mo-old growth systems had grown into the hyphal compartment; thus only a low proportion of the fatty acids was found in external mycelium.

The biomasses of roots and external mycelium in the sand compartment were measured gravimetrically, and the biomass of the internal mycelium was estimated by using a conversion factor for PLFA 16:1 ω 5 obtained from the external mycelium (see Table 4). In the old mycelium amounts in the sand

Table 6. Biomass (D.W.) distribution in AM formed by *Trifolium subterranean* and *Glomus claroideum*. Two replicate systems (\pm S.E.) containing mycelium 1-mo and 2-mo old were used. External mycelium was extracted from sand compartments.

Age of growth system (mo)	Total biomass (mg)			AM biomass distribution (%)		
	Plant root	Mycelium		Plant root	Mycelium	
		Internal	External		Internal	External
1	339 \pm 22	19.0 \pm 7.6	1.6 \pm 0.1	94.3	5.3	0.4
2	886 \pm 68	32.2 \pm 0.3	27.2 \pm 1.8	93.7	3.4	2.9

compartment were similar to those in the roots (Table 6). The total estimated AM fungal biomass accounted for 6.3% of the total root and fungal biomass in the 2-mo-old system. The amount of AM mycelium in the root compartment soil (400 g) was not estimated. If the amount of external mycelium present in the soil compartment was the same as that present in the sand compartment, the biomass of the external mycelium would have been 52% of the total AM fungal biomass.

DISCUSSION

This study shows that spores and hyphae of AM fungi are similar in terms of their fatty acid composition and that this composition does not change much as the mycelium ages. The fatty acid composition differed in phospholipids and neutral lipids in similar ways for both fungi studied, in accordance to what has been found earlier for other AM fungi (Olsson *et al.*, 1995; Johansen *et al.*, 1996). Furthermore, we showed that the spores appear to contain most of the neutral lipids in the AM mycelium and that the amount of external biomass formed by AM fungi tends to exceed the amount of internal mycelial biomass.

Fatty acid profiles of AM fungi have been shown to be consistent within species regardless of the storage time, plant host species or mycelium generation (Bentivenga & Morton, 1994). In the present study the amount of PLFA 16:1 ω 5 per unit biomass of the two AM species remained rather constant as the mycelium aged (Table 4) and its distribution between spores and hyphae was highly consistent (Table 2), which makes it a suitable biomass indicator. Furthermore, PLFA 16:1 ω 5 is a good signature compound since it represents a very small portion of the fatty acids in most other fungi (Dembitsky, Rezanka & Shubina, 1993a–c; Müller, Kantola & Kitunen, 1994; Larsen, Olsson & Jakobsen, 1998). For example, the PLFA 16:1 ω 5 has proved suitable for estimating the biomass of AM mycelium in soil in laboratory systems (Olsson *et al.*, 1995, 1998).

The amount of fatty acids per unit biomass in the mycelium varied greatly for NLFAs but not for PLFAs owing to the different factors tested in this study. This demonstrates that PLFA 16:1 ω 5 is a better biomass indicator than NLFA 16:1 ω 5. In many different substrates, however, the background level of NLFA 16:1 ω 5 is usually lower than that of PLFA 16:1 ω 5. The bacterial community accounts for most of this background (Olsson *et al.*, 1995, 1998). This makes NLFA 16:1 ω 5 generally useful in the detection of AM mycelium. Graham, Hodge & Morton (1995) determined the fatty acid composition in a variety of *Glomus* species and found that their content of fatty acid 16:1 ω 5 was often around 60%. This is similar to the proportion of NLFAs accounted for by 16:1 ω 5 in the present study. The proportion of 16:1 ω 5 in phospholipids was much lower, but since the phospholipid fraction is only a small part of the total lipids, estimates of the latter by Graham *et al.* (1995) probably mainly reflect neutral lipids.

In both fungi neutral lipids seemed to accumulate in old mycelium. Previously, it has been shown that the content of

NLFA 16:1 ω 5 is highly correlated with the number of AM fungal spores in soil (Olsson *et al.*, 1997), and here we show that in the 3-mo-old mycelium of *G. intraradices*, the spores were lipid rich, whereas the hyphae seemed to be low in neutral lipids. In a study where field inoculum from sand dunes was used to create mycorrhizal *Plantago lanceolata* and *Festuca rubra*, it was shown that hyphal accumulation of neutral lipids occurred before sporulation (Olsson *et al.*, 1998). One could hypothesize that energy stores in the mycelium originate mainly in the root and that successful root colonization is a prerequisite for accumulation of energy storage products in the external mycelium. This may explain why neutral lipids accumulate in old mycelium once the mycorrhizal symbiosis has become well established. It seems as if investments in storage products are higher in external mycelium than in the internal mycelium (see Fig. 1).

The proportion of external biomass of the AM fungus was calculated based on the assumption that the amount of PLFA 16:1 ω 5 per unit biomass is the same for external and internal mycelium. This assumption may not be totally correct since the amount of phospholipid containing membranes may differ in arbuscules, vesicles, spores and hyphae. Instead PLFA 16:1 ω 5 may be a more precise estimator of membrane area of the AM fungus since the PLFA patterns are usually rather stable within a species (Lechevalier & Lechevalier, 1988; Morton & Bentivenga, 1994). The calculated proportion of external mycelium in this study should be considered to reflect a minimum value since it is difficult to obtain a 100% recovery when extracting mycelium from sand compartments. Furthermore, the amount of external mycelium can be expected to be higher in soil systems than in our sand system since higher amount of external mycelium is formed in soil compared to in sand (Ravnkov *et al.*, 1999). Quantification based on fatty acid signatures from a laboratory system with cucumber (Olsson *et al.*, 1995, 1997) indicated that external mycelium accounted for around 70% of the AM fungal biomass. In that system, external mycelium was estimated in hyphal compartments which made up only a small portion of the total soil volume. Thus, data had to be extrapolated to get estimates for the whole system, thereby decreasing the reliability of the results obtained. Using chitin as a signature compound for AM fungal biomass, Bethlenfalvay, Brown & Pacovsky (1982) found that 88% of the biomass was external for *Glomus fasciculatum* in 4-wk-old pot cultures with soybean. This result thus supports the findings obtained using signature fatty acids which indicate that a large proportion of the AM fungal biomass is located outside of the root. In view of the fact that AM fungal biomass can account for up to 16% of the root weight (Hepper, 1977; Toth *et al.*, 1991) we suggest that the biomass of external mycelium could be as large as the plant root biomass.

This study confirms the usefulness of PLFA 16:1 ω 5 as an indicator of AM fungal biomass, and by using it here we were able to show that in some cases the biomass of external mycelium can exceed that of the internal mycelium. The signature fatty acid 16:1 ω 5 provides a means of studying how the amount of AM mycelium as well as its energy stores vary depending on various environmental factors and the species of host plant.

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