Fungal lipid accumulation and development of mycelial structures by two arbuscular mycorrhizal fungi

van Aarle, Ingrid; Olsson, Pål Axel

Published in:
Applied and Environmental Microbiology

DOI:
10.1128/AEM.69.11.6762-6767.2003

2003

Link to publication

Citation for published version (APA):

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.
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 16:1ω5 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 16:1ω5 is a good indicator for AM fungal development. The phospholipid fatty acid 16:1ω5 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.
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-µm pore mesh) placed centrally in a pot and surrounded with 400 g of acid-washed moraine clay loam and river sand (1:1), which had been irradiated 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 for 10 min and subsequently 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, the root samples were homogenized with pestles and sand in 1.5-ml microcentrifuge tubes. Extracted mycelium 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 (0.82:1 [vol/vol/vol], pH 4.0). Lipid extracts were separated from the pellets after centrifugation at 3,000 × 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 16:1ω5 and NLFA 16:1ω5 was used as an indicator of AM fungal biomass (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 16:1ω5 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 16:1ω5 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 18:2ω6,9) 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 16:1ω5 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

![Table 1](image.png)

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Uninoculated</th>
<th>GLomus</th>
<th>Scutellomospora</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>ND</td>
<td>7 ± 4</td>
<td>31 ± 11</td>
</tr>
<tr>
<td>32</td>
<td>2 ± 1</td>
<td>12 ± 4</td>
<td>170 ± 32</td>
</tr>
<tr>
<td>53</td>
<td>4 ± 0</td>
<td>46 ± 10</td>
<td>290 ± 18</td>
</tr>
<tr>
<td>67</td>
<td>2 ± 0</td>
<td>19 ± 3</td>
<td>150 ± 13</td>
</tr>
<tr>
<td>80</td>
<td>7 ± 3</td>
<td>43 ± 8</td>
<td>240 ± 44</td>
</tr>
</tbody>
</table>

*ND,* no detectable amount.
between fatty acid accumulation and AM colonization \( (r^2 = 0.00 \text{ for PLFA } 16:1 \omega 5, \text{ and } r^2 = 0.05 \text{ for NLFA } 16:1 \omega 5) \).

**Relationship between morphological structures and AM fungal lipids.** The frequency of arbuscular occurrence and accumulation of the AM fungal PLFA 16:1ω5 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 16:1ω5 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 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

![FIG. 1. Relationship between total AM fungal root colonization (percent) and the concentration (nanomoles per gram of root) of the NLFA 16:1ω5 and the PLFA 16:1ω5 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 16:1ω5 \( (y = 13x + 64; r^2 = 0.57) \) and PLFA 16:1ω5 \( (y = 1.7x + 3.2; r^2 = 0.44) \) in roots colonized by *S. calospora*. (B) NLFA 16:1ω5 \( (y = 680x - 14,000; r^2 = 0.53) \) and PLFA 16:1ω5 \( (y = 3.3x - 31; r^2 = 0.54) \) in roots colonized by *G. intraradices*.](image1.png)

![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 16:1ω5 in the roots. Open symbols, arbuscular or vesicular occurrence; solid symbols, PLFA or NLFA 16:1ω5. *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 \( (n = 3) \).](image2.png)
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 16:1ω5 as the indicator) was difficult to calculate for the extraradical mycelia due to high background levels of the PLFA 16:1ω5 in soils with nonmycorrhizal 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

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

<table>
<thead>
<tr>
<th>Fungus</th>
<th>NLFA (%)</th>
<th>Total NLFA (nmol mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16:1ω5</td>
<td>16:0</td>
</tr>
<tr>
<td><em>S. calospora</em></td>
<td>27</td>
<td>50</td>
</tr>
<tr>
<td><em>G. intraradices</em></td>
<td>37</td>
<td>47</td>
</tr>
</tbody>
</table>

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

![FIG. 3](image-url) 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).
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 µmol of G. intraradices NLFA 16:1ω5 per g of root at the last harvest (Table 1). This level corresponds to approximately 30 µmol 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 16:1ω5. Graham et al. (12) also showed that the fatty acid 16:1ω5 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 nonmycorrhizal 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 intraradial 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 16:1ω5, 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.

ACKNOWLEDGMENTS

The Swedish Council for Forestry and Agricultural Research and The Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning supported this work.

We thank Iver Jakobsen for the inoculum of S. calospora.

REFERENCES


