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^{13}C Incorporation into Signature Fatty Acids as an Assay for Carbon Allocation in Arbuscular Mycorrhiza

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The ubiquitous arbuscular mycorrhizal fungi consume significant amounts of plant assimilated C, but this C flow has been difficult to quantify. The neutral lipid fatty acid 16:1ω5 is a quantitative signature for most arbuscular mycorrhizal fungi in roots and soil. We measured carbon transfer from four plant species to the arbuscular mycorrhizal fungus *Glomus intraradices* by estimating ^{13}C enrichment of 16:1ω5 and compared it with ^{13}C enrichment of total root and mycelial C. Carbon allocation to mycelia was detected within 1 day in monoxenic arbuscular mycorrhizal root cultures labeled with [^{13}C]glucose. The ^{13}C enrichment of neutral lipid fatty acid 16:1ω5 extracted from roots increased from 0.14% 1 day after labeling to 2.2% 7 days after labeling. The colonized roots usually were more enriched for ^{13}C in the arbuscular mycorrhizal fungal neutral lipid fatty acid 16:1ω5 than for the root specific neutral lipid fatty acid 18:2ω6,9. We labeled plant assimilates by using $^{13}CO_2$ in whole-plant experiments. The extraradical mycelium often was more enriched for ^{13}C than was the intraradical mycelium, suggesting rapid translocation of carbon to and more active growth by the extraradical mycelium. Since there was a good correlation between ^{13}C enrichment in neutral lipid fatty acid 16:1ω5 and total ^{13}C in extraradical mycelia in different systems ($r^2 = 0.94$), we propose that the total amount of labeled C in intraradical and extraradical mycelium can be calculated from the ^{13}C enrichment of 16:1ω5. The method described enables evaluation of C flow from plants to arbuscular mycorrhizal fungi to be made without extraction, purification and identification of fungal mycelia.

Arbuscular mycorrhizal (AM) fungal mycelia acquire hexoses released by the roots of their host (3, 39, 42, 43) and metabolize them to lipids, mainly neutral lipids, such as triacylglycerols (39). Neutral lipids are transported throughout the fungal mycelium (4), are metabolized through the glyoxalate cycle (3, 24), and probably provide the major fungal energy source. The mechanisms that regulate C transfer from plant to fungus are not well understood (21). However, AM fungal colonization affects plant C metabolism (13, 38, 51, 52) and the genes that regulate this metabolism (20, 40).

AM fungal neutral lipids usually are stored in intraradical vesicles or in spores and make up a large proportion of the AM fungal biomass (6, 22, 36). The fatty acids of these lipids have a characteristic and specific composition (7). In *Glomus intraradices*, 50 to 70% of the neutral lipids are the fatty acid 16:1ω5 (19, 35), which is uncommon in other groups of fungi (28, 32, 47) and can be used as an AM fungal signature (31, 37). ^{13}C nuclear magnetic resonance has been used to determine C-metabolic pathways after ^{13}C labeling of monoxenic root cultures (3). The incorporation and turnover of C in AM fungal mycelium can be difficult to measure because the hyphae are not easily extracted and separated from roots or soil.

^{13}C enrichment or ^{13}C dilution of signature compounds, such as the neutral lipid fatty acid (NLFA) 16:1ω5 can be quantified by gas chromatography-mass spectrometry, which enables the measurement of the C turnover in AM fungi in complex materials, roots and soil in particular (2). Extraction and purification of mycelia (see reference 44) is therefore not needed. However, all AM fungi do not contain the same amount of NLFA 16:1ω5 (19). The root lipid NLFA 18:1ω6,9 does not increase in roots after AM colonization and is rare in AM fungi, so it can be used to estimate turnover of plant lipids in AM-colonized plants (35). Similarly, some phospholipid fatty acids (PLFAs) are good indicators of soil bacteria (16). NLFAs are not as good for estimating bacterial biomass since bacteria usually do not store neutral lipids (32).

The objective of the present study was to determine whether ^{13}C labeling of plant assimilates, followed by compound specific isotope ratio mass spectrometry (IRMS), can be used to quantify the C transfer from plant to fungus in the AM symbiosis. We hypothesized (i) that ^{13}C incorporation into the AM fungal signature compound NLFA 16:1ω5 is correlated to the total ^{13}C incorporation in the fungal mycelium and (ii) that the transfer of C to the fungus would result in ^{13}C enrichment in the AM fungal signature fatty acid 16:1ω5 at a similar speed and quantity as in plant lipids. The advantages of the proposed method include that it is a relatively fast and easy way to estimate C transfer from plant to intraradical mycelium and that the estimates of C translocation to the extraradical mycelium are sensitive enough for the technique to be used in

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complicated substrates, such as soil. ^{13}C nuclear magnetic resonance and ^{14}C autoradiography also can be used to study C allocation, but neither of these techniques can be used for quantitative measurements.

MATERIALS AND METHODS

Monoxenic arbuscular mycorrhiza cultures. The AM fungus *Glomus intraradices* Schenck & Smith (DAOM 197198; Biosystematics Research Center, Ottawa, Canada) was grown monoxenically in mycorrhizal association with root-organ cultures of carrot (*Daucus carota* L.). The cultures were clones of carrot roots (line DC1) that had been transformed with the T-DNA of the root-inducing (Ri) plasmid from the bacterium *Agrobacterium rhizogenes* (5). The cultures were originally established as described by St-Arnaud et al. (48). AM-colonized cultures were maintained at 24°C in petri dishes on a minimal nutrient medium (MM) (5) containing sucrose at 10 g liter^{-1} as the C source and 35 μM P (KH_2PO_4 [4.8 mg liter^{-1}]) gelled with 0.3% Phytagel (Sigma Chemical Co., St. Louis, MO).

Monoxenic time course study. Monoxenic experimental plates were initiated by transferring a 14-mm diameter plug of solid MM from 4-month-old cultures, containing colonized roots, extraradical mycelium and spores, into the center of an 85-mm plate containing sterile solid MM. After 9 weeks of growth, the plates were supplied with sterile filtered D-[^{13}C]glucose solution (7 mg of glucose per dish, U-$^{13}C_6$ at 99% ^{13}C; Cambridge Isotope Laboratories, Andover, MD) in a ring, 5 mm outside the initial inoculum plug. The agar surface at this stage was completely covered by roots and mycelia. Plates were analyzed 1, 2, and 7 days after labeling (two replicate plates at each harvest). MM (ca. 15 ml) with only hyphae was cut out from the plates and dissolved in 250 ml of 10 mM sodium citrate (12) by being mixed on a magnetic stirrer for 1 h at low speed at 20°C to leave extraradical mycelia and spores. The mycelia (0.56 ± 0.11 mg of dry mycelium per plate) were collected by filtration on a 25-μm-pore-size nylon mesh and washed in deionized water in a 500-ml glass beaker. Roots were collected with forceps from the plates and washed in 10 mM sodium citrate to remove any remaining solid medium. Mycelia and roots were stored at −20°C until analyzed for ^{13}C enrichment and fatty acid content and composition. Two plates with axenic root cultures (without AM fungus) were included to measure the fatty acid content in noncolonized roots. These cultures were not ^{13}C labeled.

Monoxenic P experiment. For a detailed description of this experiment, see experiment 2 in Olsson et al. (36). The data from this experiment are already published (36), but here we use the data from three different inorganic P treatments to calculate a conversion factor for the estimation of total C flow based on C flow to the signature compound NLFA 16:1ω5c. Briefly, plugs of solid MM containing carrot roots, mycelia, and spores of *G. intraradices* were transferred at the start of the experiment from a 3- to 4-month-old culture to fresh medium in one compartment of a two-compartment petri dish (27). Liquid media were added to the second compartment 54 days after the inoculation of the first (root) compartment. The liquid medium contained one of three P levels: (i) no P (P-free), (ii) 25 μM P as KH_2PO_4 (low-P), and (iii) 2.5 mM P as KH_2PO_4 (high-P). Root compartments were labeled with D-[^{13}C]glucose (10 mg of glucose per dish) 30 days after the addition of the liquid medium. The aqueous [^{13}C]glucose solution (100 μl, 100 mg ml^{-1}, filter sterilized) was pipetted onto the solid medium 0.5 cm inside the barrier to the liquid compartment. Cultures were harvested 7 days after labeling. Mycelia were collected by using forceps, and the few roots (roots had been trimmed once during the growth period) growing together with the mycelia in the liquid compartment were removed under a dissecting microscope by using forceps. Mycelia were stored at −20°C until freeze-dried for the measurement of ^{13}C and lipids. The solid medium of the root compartment was dissolved in 250 ml of 10 mM sodium citrate by being mixed on a magnetic stirrer for 1 h at low speed at 20°C. These roots also were collected, freeze-dried, weighed, and analyzed, as were the roots from the liquid media.

Whole-plant study with three different host species. *Allium porrum* L. (12 per pot), *Plantago lanceolata* L. (9 per pot), or *Trifolium subterraneum* L. (5 per pot) were planted in 0.5-liter pots filled with 500 g of a mixture containing 80% acid-washed sand, 10% sterilized sandy loam soil (see reference 18 for soil details), and a 10% pot culture inoculum of *G. intraradices* (BEG 87) or *Gigaspora margarita* (MAFF520054; MAFF GenBank, National Institute of Agribiological Sciences, Tsukuba, Japan). Seeds were surface sterilized (5% Ca-hypochlorite, 15 min), pregerminated in petri dishes, and transplanted into the pots. Two replicate pots for each combination of plant species and fungus were placed in a greenhouse with average 22°C day and 18°C night temperatures, a minimum of 270 μmol m^2 s^{-1} photosynthetic photon flux density supplemented with 400 W OSRAM light bulbs as required, and an 18-h photoperiod. Plants were watered weekly with a low-P nutrient solution (50) per pot (60 ml in all were added during the growth period to each pot). Additional N, as NH_4NO_3, was provided with 1.2 mg of N per pot for *Plantago* and *Trifolium* and 0.5 mg of N per pot for *Allium* during the growth period. Two pots of two-month-old plants were labeled in a closed chamber (volume, 4.2 dm^3) with 25 ml of ^{13}C-CO_2 (99% $^{13}CO_2$; Larodan Fine Chemicals, Malmö, Sweden) injected with a gas-tight syringe through a septum. Removing the lid of the chamber interrupted the 3-h pulse period. Approximately 6.6 mg of ^{13}C were assimilated per pot, assuming that all of the 25 ml of added ^{13}C-CO_2 were assimilated. At 7 days after labeling, plants were harvested, and root samples were collected and rinsed carefully with water on a sieve before further processing. Soil samples free of roots were collected for fatty acid analysis. Roots were stained with trypan blue and AM fungal colonization was determined microscopically (50). Fatty acid composition and ^{13}C enrichment of lipids extracted from roots and from soil were determined on samples that had been stored frozen (−20°C).

Extraction of intraradical mycelium. Intraradical mycelium was collected after enzymatic digestion of the remaining roots from the whole-plant study. Fresh root samples (cleaned from debris, extraradical mycelium, and spores) were kept on ice, cut into pieces 5 mm in length, and placed in a freshly prepared, filtered enzyme digestion solution as described by Saito (41). The digestion solution contained 2% cellulase (from *Trichoderma harzianum*, 11.6 U mg^{-1}; Sigma) and 1% pectinase (from *Rhizopus* sp. 0.60 U mg^{-1}; Sigma). The samples were placed under vacuum (created by water suction) for 10 to 15 min to remove air from the roots and then covered with Parafilm and incubated at 30°C for 2 h on a shaker at 120 rpm. Digested root samples were collected on a 25-μm-pore-size nylon mesh, washed clean with washing buffer (41), and kept on ice. The intraradical mycelium was separated by hand from the stele and cortical cells under a dissecting microscope by using needles and forceps and then transferred to fresh washing buffer. Almost pure intraradical mycelia could be extracted from *Allium* roots but not from the other two plant species. Samples from *Plantago* and *Trifolium* are therefore best regarded as enriched for intraradical mycelia. For *Allium*, we collected root pieces from which the intraradical mycelium had been removed to use as fungus-free samples of cortex and stele. For *Plantago* and *Trifolium*, we collected root pieces that lacked AM fungal colonization on the basis of dissecting microscope observations. Total ^{13}C enrichment was determined in the collected intraradical mycelia, in samples enriched for intraradical mycelia, and in the mycelium-free root samples.

Whole-plant study with mycelium in root-free sand compartments. Gavito and Olsson (18) provide a detailed description of this experiment. The results for NLFA 16:1ω5 from this experiment were used previously to calculate C flow to AM fungal lipids (17) in response to amendment treatments ([i] organic matter [dry yeast], [ii] a full nutrient solution without P, or [iii] a solution containing only P). Here we present the ^{13}C-enrichment data (%) of NLFA 16:1ω5, 18:2ω6,9, and solid tissue. Rectangular polyvinyl chloride boxes (22-cm length by 11-cm width by 9.5-cm height) were divided into three compartments with two screens of 25-μm-pore-size nylon mesh. AM fungal hyphae can pass through this nylon mesh, but the roots cannot (37). The central compartment (5.5-cm width) was filled with nonsterilized soil (18) serving both as the growth substrate and as AM fungal inoculum for the host plants. One of the two side hyphal compartments received the amendment treatment, and the other side was left unamended as a control. The two side compartments were equal in size and were filled with 750 g of autoclaved quartz sand (Ahlsell-Mineral, Malmö, Sweden) that had been washed five times in deionized water. There were five complete randomized blocks.

Seven pregerminated seeds of *P. lanceolata* were planted in a row across the root compartment to create a line of plants equidistant from the two side compartments. *Plantago lanceolata* shoots in three polyvinyl chloride boxes at a time were labeled with $^{13}CO_2$ (99% $^{13}CO_2$; Larodan Fine Chemicals) in a closed chamber (volume, 33 dm^3). The transparent lid of a greenhouse box was altered, with a fan and gas sampling tubing connected to an infrared gas analyzer. The initial CO_2 concentration was recorded. $^{13}CO_2$ (125 ml) was injected with a gas tight syringe through a septum. The CO_2 concentration inside the box increased from 240 to 270 ppm to 500 to 650 ppm after injection. The labeling period lasted until the CO_2 concentration inside the box decreased to its initial level (between 60 and 90 min). Approximately 20 mg of ^{13}C was assimilated per pot. Plants were harvested 1 week after labeling, and soil and root samples were collected. ^{13}C-enrichment was determined in fatty acids extracted from mycelium collected from the sand compartment by wet sieving (18).

Lipid analysis. Mycelial samples were milled with stainless steel balls (7-mm diameter) by shaking by hand for 15 s in 50-ml Teflon tubes, and roots (100 mg) were ball milled (15 s, 300 strokes min^{-1}) in stainless steel beakers. The lipids from the mycelia (0.5 to 1 mg [dry mass]), roots (15 to 30 mg), and soil (10 g)

were extracted by vortex mixing (1 min) in a one-phase mixture of citrate buffer, methanol, and chloroform (0.8:2:1 [vol/vol/vol]; pH 4.0) (8). The lipids were fractionated into neutral lipids, glycolipids, and phospholipids on prepacked silica columns (100 mg of sorbent mass; Varian Medical Systems, Palo Alto, CA) by elution with 1.5 ml of chloroform, 6 ml of acetone, and 1.5 ml of methanol, respectively. The fatty acid residues in the neutral lipids and the phospholipids were transformed into free fatty acid methyl esters that were analyzed by gas chromatography using a 50-m HP5 capillary fused silica column (Hewlett Packard, Palo Alto, CA) with H_2 as the carrier gas (for further details, see reference 17). The fatty acids were identified from their retention times relative to that of the internal standard (fatty acid methyl ester 19:0). These were compared to those earlier determined by gas chromatography-mass spectrometry.

Signature fatty acids. NLFA 16:1ω5 is a sensitive signature of AM fungi in both roots and soil (19, 34). PLFA 16:1ω5 is a constituent of AM fungal membranes, with rather low specificity as a signature due to relatively low content in AM fungi and a high background in soil originating from bacteria (34). NLFA 16:0 is a general NLFA that often increases in concentration in plant roots after AM colonization due to the high content of storage lipids in AM fungi (34). NLFA 18:2ω6,9 is present in plant storage lipids. It also is the dominant fatty acid in most fungi but occurs at very low levels in AM fungi (19, 35). PLFAs i15:0, a15:0, and cy19:0 are three bacterium-specific PLFAs that can be used as indicators of bacterial biomass (16).

Determination of ^{13}C enrichment in crude tissue samples and fatty acids. Freeze-dried mycelia (ca. 20 μg) or ball-milled root material (ca. 100 μg) were placed in tin capsules (crude tissue samples) and analyzed by continuous-flow IRMS with an ANCA-NT 20-20 stable isotope analyzer interfaced to a solid-liquid preparation module (PDZ Europa Scientific Instruments, Crewe, United Kingdom). The $^{13}C/^{12}C$ ratios of CO_2 of the combusted samples (total C) were determined with a 0.01% precision. The data were expressed as ^{13}C atom% with reference to a sucrose (BDH Laboratory Supplies, Poole, United Kingdom) standard, calibrated against the Pee Dee Belemite standard (the limestone fossil *Belemnitella americana* from the Cretaceous Pee Dee formation in South Carolina; International Atomic Energy Agency [11]).

The ^{13}C enrichment in fatty acid methyl esters was determined in the 20-20 IRMS interfaced with a Hewlett-Packard 6890 gas chromatograph equipped with a split/splitless injector, a flame ionization detector, a high-temperature reaction furnace mounted, and a CTC Combi Prep and Load System (Crelab Instruments, Stockholm, Sweden). The chromatographic conditions were as described for the lipid analysis except that He was used as the carrier gas at a constant pressure of 36 lb/in^2. The effluent from the capillary column passed through an Al tube with CuO wires at 860°C, where the fatty acids were converted to CO_2. Cogenerated water vapor was removed via a Nafion membrane (PDZ Europa Scientific Instruments), and the purified CO_2 was released into the 20-20 IRMS.

The ^{13}C atom% values were calculated based on atom ^{13}C of the reference CO_2 gas, injected three times at the beginning and end of a chromatographic run. The reference CO_2 was standardized with the Pee Dee Belemite standard by using a solid-liquid preparation module. Integration for each peak was checked and corrected manually. The ^{13}C enrichment of fatty acids was calculated after correction for the C added in the methanolysis step of the fatty acid analysis procedure.

RESULTS

Monoxenic time course study. ^{13}C enrichment was observed in roots and hyphae 1 day after application of [^{13}C]glucose to monoxenic carrot roots cultures with *G. intraradices* (Fig. 1). Rapid ^{13}C enrichment in the AM fungal fatty acid 16:1ω5 showed that new lipids were being synthesized. The background levels of fatty acid 16:1ω5 in noncolonized carrot roots were low. The content of PLFA 16:1ω5 increased from 5 nmol g^{-1} in noncolonized roots to 140 nmol g^{-1} in AM-colonized roots, whereas NLFA 16:1ω5 increased from 80 nmol g^{-1} to 7,600 nmol g^{-1} and NLFA 16:0 from 1.0 to 6.6 nmol g^{-1}. Thus, the majority of this PLFA and these NLFAs were mainly of AM fungal origin in the colonized roots (as expected from previous studies [33-36]), while the content of the PLFAs 16:0 and 18:1ω6,9 and NLFA 18:2ω6,9 did not increase due to AM colonization and can be considered as primarily plant root signatures. At 7 days after labeling, the root PLFAs, 16:0 and 18:2ω6,9 were enriched for ^{13}C in a manner similar to that for total root C, but the PLFA 16:1ω5 had a higher ^{13}C enrichment than did the root material (Fig. 1A). The ^{13}C-enrichment of the NLFAs 16:0 and 16:1ω5 (Fig. 1B) in AM colonized roots was higher than in the root lipids (represented by NLFA 18:2ω6,9). ^{13}C enrichment was slightly higher in intraradical my-

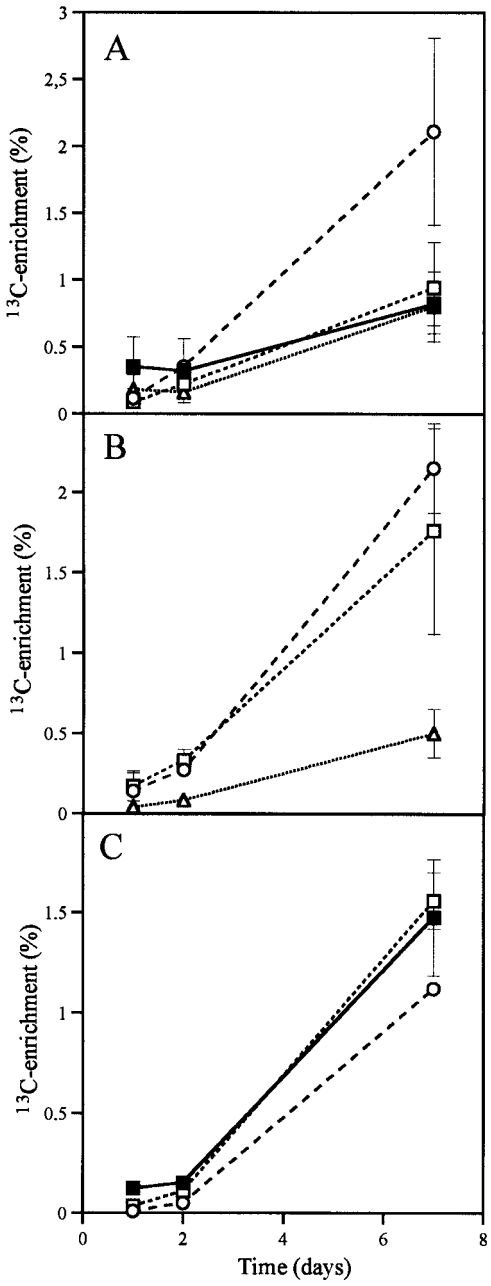

FIG. 1. Time course study of monoxenic AM carrot root system. (A) ^{13}C enrichment of total root C and PLFAs within roots. (B) ^{13}C-enrichment of NLFAs in roots. (C) ^{13}C enrichment of total C and NLFAs within extraradical mycelium. Symbols: ■, total C; ○, fatty acid 16:1ω5; □, fatty acid 16:0; △, fatty acid 18:1ω6,9. ^{13}C enrichments of roots and AM mycelium were estimated at different times after labeling (n = 2, ± the standard error [SE]). Where no error bars can be seen, the SE is smaller than the symbol.

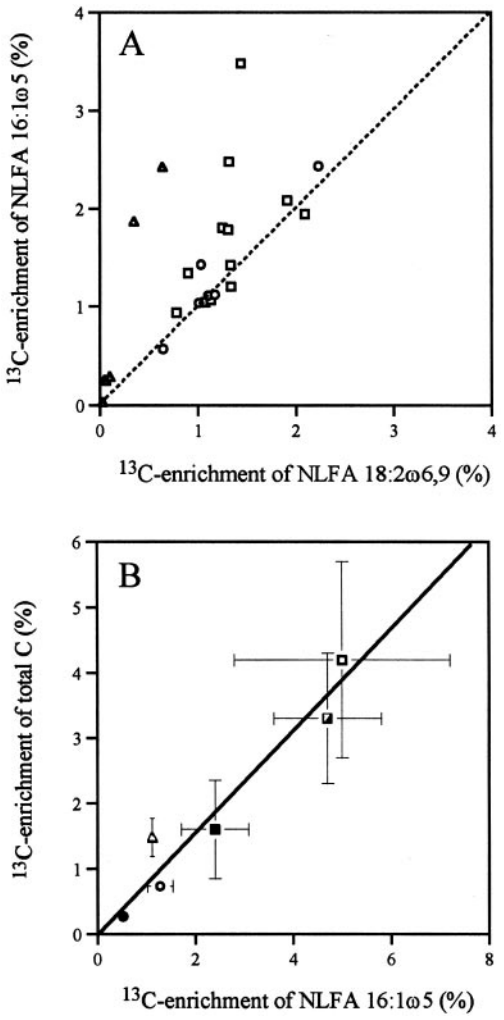

FIG. 2. Comparisons of ^{13}C enrichment of NLFA 16:1ω5 to enrichment of NLFA 18:2ω6,9 and total C. (A) Plant roots studied in three experiments where the dotted line represents $k = 1$. (B) AM fungal mycelium across four experiments ($y = 0.77x$, $r^2 = 0.94$, $P < 0.01$). Triangles indicate monoxenic time course study, squares indicate monoxenic P experiment (open squares denotes no P, half-filled squares indicate low-P, and filled squares indicate high-P), open circles represent experiments with multiple plant hosts (extracted intraradical mycelium was used for determination of total hyphal ^{13}C in panel B), and closed circles represent the sand compartment study. Means ($n = 2$ for the monoxenic time course study, $n = 12$ for the monoxenic P experiment, 6 for experiment with multiple plant hosts and 30 for sand compartment study) ± the SE; where no error bars can be seen, the SE is smaller than the symbol.

celium (Fig. 1B) than in extraradical mycelium (Fig. 1C), as indicated by the signature NLFAs 16:1ω5 and 16:0.

Monoxenic P experiment. Available phosphorus reduced ^{13}C enrichment in the extraradical mycelia, and the ^{13}C enrichment of NLFA 16:1ω5 was significantly higher in extraradical mycelia than in intraradical mycelia (4.0% ± 0.84% compared to 1.7% ± 0.21%, $P < 0.05$ [paired t test, $n = 12$]). The PLFA with the greatest enrichment in the extraradical mycelia was 16:1ω5 (3.9% ± 0.40%, mean over all P treatments), whereas PLFAs 16:0 and 18:2ω6,9 had similar enrichment (3.2% ± 0.24% and 3.0% ± 0.28%, respectively). The ^{13}C enrichment was slightly higher in NLFA 16:1ω5 than in NLFA 18:2ω6,9 (Fig. 2A). The lowest ^{13}C enrichment in extraradical mycelia was observed in the high-P treatment, but the relation between ^{13}C enrichment in total C and in NLFA 16:1ω5 was similar for all three P treatments (Fig. 2B). This experiment demonstrated that ^{13}C was translocated since the labeled substrate was applied to one compartment, and the ^{13}C enrichment was measured in extraradical mycelia collected from a second compartment.

Whole-plant study with three different host species. *Glomus intraradices* colonized all three host plants at a rate ranging from 41% (*Plantago*) to 56% (*Trifolium*) of the root length (Table 1). *Glomus intraradices* colonization increased the content of both PLFA and NLFA 16:1ω5 in the host roots and in the soil due to growth of extraradical mycorrhizal mycelia. The neutral lipid fraction of *G. intraradices* colonized roots was dominated in particular by NLFAs 16:1ω5 and 16:0 (Fig. 3A). In the roots, PLFA 16:1ω5, NLFA 16:1ω5, and NLFA 16:0 were mainly of AM fungal origin (Table 1). NLFA 18:2ω6,9 was mainly of root origin because it was present at similar levels in control and AM-colonized roots. The content of NLFA 18:2ω6,9 varied between the different plant species but did not increase due to AM colonization.

^{13}C enrichment of total C was higher in intraradical mycelium than in root pieces from the same root system but devoid of fungal structures (Table 1). ^{13}C enrichment also was higher in NLFA 16:1ω5 than in total C of the extracted intraradical mycelium (Table 1). The ^{13}C enrichment was slightly higher in NLFA 16:1ω5 than in NLFA 18:2ω6,9, and their ^{13}C enrichment was correlated ($r^2 = 0.92$, $P < 0.001$; Fig. 3A).

The background level of NLFA 16:1ω5 in the soil was very low (Table 1), and >99% of this fatty acid was estimated to be from extraradical fungal mycelia of *G. intraradices*. The same fatty acid had higher ^{13}C enrichment in the soil than did three bacterial PLFAs (Table 2). Of the analyzed PLFAs, 16:1ω5 was most enriched with ^{13}C. The extraradical mycelia of *G. intraradices* contributed 30 to 50% of the PLFA 16:1ω5 in the soil (Table 1), assuming that the background amounts came from other microorganisms. ^{13}C enrichment also was found in the bacterial PLFAs i15:0, a15:0, and cy19:0 (Table 2, Fig. 3B). The ^{13}C enrichment in PLFAs a15:0 and cy19:0 was significantly higher in soil with *G. intraradices* than without (Table 2). In the soil without *G. intraradices* the enrichment of PLFA 16:1ω5 and the bacterial PLFAs was similar.

Whole-plant study with mycelium in root free sand compartments. ^{13}C enrichment of the NLFA 16:1ω5 in extraradical mycelia varied between 0.16 and 0.84% and was similar to that measured in the experiment with multiple plant hosts. The addition of nutrient medium to the sand compartments did not alter the ^{13}C enrichment in the NLFA 16:1ω5 of the extraradical mycelium (data not shown). In the present experiment we used a field inoculum of AM fungi in which the fungi present had not been identified. The relation between ^{13}C enrichment in NLFA 16:1ω5-C and total C was similar to that found in defined systems.

DISCUSSION

We developed a new methodology to quantify the flow of plant assimilated C to microorganisms. We use a signature

TABLE 1. Root colonization of three plant species inoculated with *Glomus intraradices*, the content of fatty acid 16:1ω5 in roots and soil, and ^{13}C enrichment in the experiment with multiple plant hosts

	Background FA[a] (mean ± SE)	Mean result for *Glomus*-colonized roots ± SE[b]		
		Allium	*Plantago*	*Trifolium*
Colonization (% root length)				
Total		55 ± 3.2	41 ± 0.2	56 ± 1.4
Arbuscular		32 ± 9.0	20 ± 0.3	32 ± 9.8
Vesicles		20 ± 2.5	11 ± 1.0	15 ± 0.3
Root FA (nmol g^{-1})				
PLFA 16:1ω5	12 ± 2.3	350 ± 180	120 ± 33	170 ± 10
NLFA 16:1ω5	71 ± 21	17000 ± 3500	24000 ± 910	41000 ± 3500
NLFA 16:0	2200 ± 820	13000 ± 3800	11000 ± 1300	21000 ± 90
NLFA 18:2ω6,9	3400 ± 570	8100 ± 2100	2300 ± 120	1800 ± 270
Soil FA (nmol g^{-1})				
PLFA 16:1ω5	0.29 ± 0.02	0.57 ± 0.10	0.43 ± 0.09	0.51 ± 0.21
NLFA 16:1ω5	0.23 ± 0.04	50 ± 4.2	39 ± 8.0	33 ± 18
^{13}C enrichment[c] (%)				
Roots (total C)		0.61 ± 0.13	0.46 ± 0.18	0.40 ± 0.06
Intraradical mycelium (total C)		0.96 ± 0.48	0.56 ± 0.02	0.68 ± 0.04
Root NLFA 16:1ω5		1.9 ± 0.51	0.80 ± 0.23	1.1 ± 0.004

[a] Background of fatty acids (FA, $n = 6$) was estimated in roots of all three plant species colonized by *Gigaspora margarita*, which has 18:1ω9 instead of 16:1ω5 as the major fatty acid (19, 26).
[b] $n = 2$.
[c] ^{13}C enrichment denotes the excess ^{13}C when the natural abundance (1.15%) has been subtracted.

lipid, such as NLFA 16:1ω5, to quantify the amount of C transferred in complex media with higher sensitivity and specificity than is possible through measurement of total ^{13}C in roots and soil. AM fungal ^{13}C can also be measured in purified extraradical mycorrhizal mycelia, but only after laborious picking and subjective identification (44). Staddon et al. (45) obtained a good signal in shoots after 4 h of pulse-labeling with $^{13}CO_2$ in field vegetation but had a very low signal in roots (maximum 0.1% enrichment) and almost no signal in soil, illustrating the low sensitivity that results unless a specific compound with a high turnover rate is targeted. The same methodology can be used to study C from plants to other rhizosphere microorganisms such as pathogenic fungi and bacteria, which have signature fatty acids that differ from those in roots and AM fungi (16, 25). Carbon that is sequestered by the fungus can be separated from that metabolized by the plant to yield lipids and thus allow a test of the hypothesis that C transferred from one host plant to the fungal mycelium is retained there and not returned or moved to new plants (14). It is now easy to acquire pure extraradical mycelium from monoxenic mycorrhizal cultures. Such monoxenic cultures provide a simple, easy-to-handle system to study carbon transfer in pure cultures (4, 39). Thus far, at least 27 AM fungal species have been cultured monoxenically (15), most commonly with

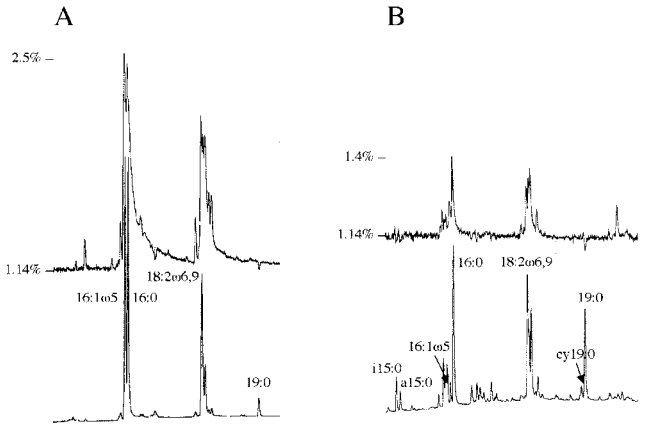

FIG. 3. Parts of fatty acid chromatograms (fatty acid methyl esters) from the experiment with multiple plant hosts shows the total amount of each fatty acid (lower chromatograms) and the ^{13}C enrichment as the $^{13}C/^{12}C$ ratio (upper chromatograms; the baseline represents natural abundance $^{13}C/^{12}C$ ratio). (A) Neutral lipid fatty acids from *Allium porrum* roots colonized by *Glomus intraradices*; (B) phospholipid fatty acids in a soil sample from a pot culture with *Allium* colonized by *G. intraradices*. The identities of the fatty acids are indicated. Fatty acid methyl ester 19:0 was added as an internal standard. Relative retention times compared to 19:0 were 0.699 (i15:0), 0.706 (a15:0), 0.788 (16:1ω5), 0.794 (16:0), 0.913 (18:2ω6,9), and 0.995 (cy19:0).

TABLE 2. The ^{13}C enrichment in NLFAs and PLFAs in soil from pots of *Allium*, *Plantago*, or *Trifolium* grown with or without (control) the AM fungus *Glomus intraradices* in the multiple host experiment

Fatty acid	% ^{13}C enrichment[a] (mean ± SE)	
	Control	*Glomus*
Glomus signatures		
NLFA 16:1ω5	0.43 ± 0.14	0.81 ± 0.15
PLFA 16:1ω5	0.083 ± 0.027	0.26 ± 0.06*
Bacterial signatures		
PLFA i15:0	0.042 ± 0.009	0.047 ± 0.006
PLFA a15:0	0.035 ± 0.009	0.077 ± 0.014*
PLFA cy19:0	0.064 ± 0.019	0.12 ± 0.010*

[a] Natural abundance of ^{13}C was subtracted ($n = 6$). *, Significantly different values between control and AM ($P < 0.05$, t test).

TABLE 3. Calculation of C flow to the intraradical mycelium in roots of three plant species

Plant type	NLFA 16:1ω5[a] (μmol g^{-1})	NLFA 16:1ω5 (mg of C g^{-1})	^{13}C enrichment in NLFA 16:1ω5 (%)	C flow to NLFA 16:1ω5 (μg g^{-1})	Total C flow to AM fungal mycelium[b] (μg g^{-1})
Allium	17	3.3	1.9	63	163
Plantago	24	4.6	0.80	37	96
Trifolium	41	7.9	1.1	87	226

[a] Data for the conversion of NLFA 16:1ω5 and its ^{13}C enrichment are taken from Table 1.

[b] The C flow to NLFA 16:1ω5 is calculated from equation 1, and the total C flow to AM fungal mycelium was calculated by multiplying that value with the conversion factor 2.6.

transformed carrot roots (5) but also with *Medicago truncatula* (9).

Quantification of transferred carbon. The correspondence between ^{13}C enrichment in NLFA 16:1ω5 and ^{13}C enrichment in total mycelium (Fig. 2B) suggests that the flow of C from host plants to fungal mycelia can be calculated from ^{13}C enrichment in NLFA 16:1ω5. We used the data from the monoxenic P experiment to calculate a conversion factor to be used in the experiment with multiple plant hosts.

The total C flow to NLFA 16:1ω5 was calculated according to the following equation: NLFA 16:1ω5-C (μg) × ^{13}C enrichment of NLFA 16:1ω5 (%/100) = C flow to NLFA 16:1ω5 (equation 1).

The molecular weight of NLFA 16:1ω5 is 253 g mol^{-1} and contains 192 g of C mol^{-1}. There was a mean of 318 nmol NLFA 16:1ω5 of the mycelium collected from one culture, corresponding to 61 μg of C and the mean ^{13}C enrichment of NLFA 16:1ω5 was 4.0%, which gives a C flow to NLFA 16:1ω5 of 2.5 μg.

The total amount of the AM mycelium was determined from the weight of freeze-dried mycelium from the monoxenic P experiment (all three P treatments). The mean amount of mycelium was 0.44 mg. The biomass-C of the mycelium was assumed to represent 50% of its dry weight corresponding to 220 μg of C. The mean ^{13}C enrichment of total mycelium C was 3.0%, which gives a total C flow of 6.6 μg calculated according to the following equation: mycelium biomass C (μg) × total ^{13}C enrichment (%/100) = C flow to mycelium (equation 2).

By using the results from equations 1 and 2 we can calculate the ratio of C flow to NLFA 16:1ω5 to total C flow to AM fungal mycelium as follows: C flow to mycelium/C flow to NLFA 16:1ω5 = conversion factor (equation 3).

This ratio (6.6/2.5 = 2.6) can be used as a conversion factor to estimate the flow of plant C to the AM fungal mycelia based on measurements of the signature NLFA 16:1ω5. Thus, for every C atom incorporated in NLFA 16:1ω5, 2.6 C atoms were incorporated into the fungal mycelia.

In the experiment with multiple plant hosts, the largest C flow to fungal mycelium per g of root (and the largest NLFA 16:1ω5 concentration) was found in *Trifolium*, and the lowest was found in *Plantago*, which also had the lowest ^{13}C enrichment in NLFA 16:1ω5 (Table 3). Around 6,600 μg of ^{13}C was assimilated per pot, which can be compared to the 100 to 230 μg of enriched ^{13}C in intraradical AM fungal mycelium per g of root.

Sensitivity and specificity of the method. The natural abundance of ^{13}C ($\delta^{13}C$) is generally around δ −30 (corresponds to 1.15% ^{13}C) in C3 plants, and the ^{13}C abundance in AM fungi is similar to that of their host plant (30, 31, 46). Abraham et al. (1) provided various substrates to different microorganisms and observed that the ^{13}C abundance in the fatty acid 16:0 extracted from the microorganisms resembled that of the substrate. With a ^{13}C-labeled substrate, we reach a much higher sensitivity when the C metabolism was traced than can be obtained by using just the naturally occurring differences in ^{13}C abundance between various substrates.

The dominance of the fatty acid 16:1ω5 in AM fungi and its rareness in other fungi (28, 47) makes it a useful biomarker probably for all *Glomus* species (19, 34) and also for *Scutellospora* species (19, 49) but not for *Gigaspora* species (19). Neutral lipids are important for energy storage, and 16:1ω5 is particularly common in this fraction of AM fungal lipids (35). Triacylglycerols are the main type of neutral lipids found in AM fungal spores and vesicles (6, 10, 29), although other fractions, e.g., diacylglycerols and free fatty acids, also are important in *Glomus*. All of them are, however, dominated by 16:1ω5.

16:1ω5 is suitable for labeling because the amount of this fatty acid is correlated with total ^{13}C enrichment in hyphae and because this fatty acid represents a significant portion of the AM fungal biomass, which is consistent with the hypothesis of Bago et al. (4) that up to 50% of the hyphal volume may be lipid bodies. That a high proportion of AM fungal biomass is lipids is probably the main reason for the strong correlation between ^{13}C enrichment in hyphae and in NLFA 16:1ω5 extracted from the same hyphae (Fig. 2B). Higher ^{13}C enrichment of NLFA 16:1ω5 than of total C in mycelium indicates that lipids are the main C compounds translocated in AM fungal mycelia (39). Although the total ^{13}C enrichment varied between experiments and was reduced by the high-P treatment in the monoxenic P experiment, the relative allocation to NLFA 16:1ω5 was the same (Fig. 2B). High P availability reduces the allocation to AM in plants (33), but our results indicate that the relative allocation to neutral lipids within the mycelium is the same.

Timing of measurements. Timing is extremely important in all labeling experiments, particularly for studies of C allocation, because C is continually respired. However, a large proportion of AM fungal C is contained in lipids in vesicles inside plant roots and in spores on the external mycelium where they accumulate and may be stored for long periods of time. As long as the mycelium is expanding and spores are formed, there should be a continuous allocation of carbon to long-term storage. In field-labeled AM fungal mycelium, labeled respiration ceased within 7 days after pulse-labeling (23), indicating that only labeled stored material remained at that time. We observed a similar level of enrichment in the extraradical and intraradical mycelia after 7 days (Fig. 1), indicating that there was enough time for C translocation between different parts of the mycelium. Higher ^{13}C enrichment in lipids of the intraradical mycelia than in plant root lipids shows that lipid metabolism is more active in the intraradical mycelia than in the plant. This difference was particularly evident in the monoxenic ex-

periments with [^{13}C]glucose (Fig. 2A), which suggests that this substrate can be directly transferred to AM fungi without being metabolized first.

The method described here provides an objective method to compare C allocation to intraradical and extraradical AM hyphae and to track C allocation in plant roots to fungal symbionts. Plant regulation of C allocation can be followed by this method after the symbiosis has been established. The regulation of colonization establishment in AM has been well studied, whereas the regulation of the C transfer in established symbiosis has been little studied due to methodological difficulties. Our method may be used to test hypotheses regarding the relative C allocation to AM fungi under different environmental conditions and to estimate how much C different plant species allocate to the AM symbiosis. This method also might be used to calculate carbon fixation in AM fungi in field ecosystems, but this application needs further evaluation since the turnover rates in the field probably are lower due to lower mean temperatures and since the fungal community composition is much more complex than in the laboratory experimental systems.

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