Rapid Turnover of Hyphae of Mycorrhizal Fungi Determined by AMS Microanalysis of ^{14}C

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Processes in the soil remain among the least well-characterized components of the carbon cycle. Arbuscular mycorrhizal (AM) fungi are ubiquitous root symbionts in many terrestrial ecosystems and account for a large fraction of photosynthate in a wide range of ecosystems; they therefore play a key role in the terrestrial carbon cycle. A large part of the fungal mycelium is outside the root (the extraradical mycelium, ERM) and, because of the dispersed growth pattern and the small diameter of the hyphae (<5 micrometers), exceptionally difficult to study quantitatively. Critically, the longevity of these fine hyphae has never been measured, although it is assumed to be short. To quantify carbon turnover in these hyphae, we exposed mycorrhizal plants to fossil ("carbon-14–dead") carbon dioxide and collected samples of ERM hyphae (up to 116 micrograms) over the following 29 days. Analyses of their carbon-14 content by accelerator mass spectrometry (AMS) showed that most ERM hyphae of AM fungi live, on average, 5 to 6 days. This high turnover rate reveals a large and rapid mycorrhizal pathway of carbon in the soil carbon cycle.

Most terrestrial plants form symbiotic associations between their roots and mycorrhizal fungi, of which AM fungi are the most common (*1*). AM fungi obtain all their C from their host plant and supply the host with various benefits, such as improvements in nutrient acquisition from the soil (*2*). AM fungi can account for up to 20% of plant photosynthate (*3*) and therefore represent a substantial pathway for C flow to the soil and a key link in the terrestrial C cycle (*4*). However, the proportion of C entering the soil via AM fungi that is simply respired back to the atmosphere is not known. Despite the importance of mycorrhizae in community functioning (*5*, *6*), the biology of AM fungi, especially their external (extraradical) phase in soil, remains obscure. The rate at which the ERM turns over has never been measured, despite its central importance to understanding the role of mycorrhizal fungi in the soil C cycle. It has been hypothesized that AM fungal hyphae turn over in days rather than weeks (*7*), because observations have shown that over half of a population of fungal hyphae in soil, some of which may have been mycorrhizal, survived for less than one week (*8*).

The residence time of C in the ERM of AM fungi must be known if this important link in the C cycle is to be quantified (*4*). The turnover rate of AM fungal hyphae has not been quantified because of the difficulty of obtaining sufficient quantities of pure mycorrhizal hyphae other than by painfully slow and labor-intensive microscopic collection by hand. By including both hyphae and the unusually large spores of AM fungi, we previously used measurements of $\delta^{13}C$ to determine C transfer from root to fungus (*9*). Inclusion of spores gave samples that were sufficiently large to permit $\delta^{13}C$ determination of the combined tissue but that prevented measurement of hyphal turnover.

In this study, we grew seedlings of *Plantago lanceolata* in pots in a greenhouse with an inoculum containing several AM fungi, all in the genus *Glomus* (*10*, *11*). Experimental plants were exposed for 5 hours to an atmosphere in which all CO_2 was from a fossil source and hence contained no ^{14}C (*12*, *13*). Over the following 4 weeks, 21 plants (including 6 controls exposed to a normal atmosphere with ambient $^{14}CO_2$ concentrations) were harvested, and samples of live ERM hyphae were carefully collected from their roots (*14*). We analyzed these samples for their ^{14}C content by accelerator mass spectrometry (AMS) (*15*) using a gas-ion source (*16*). This required the development of a method for samples containing <100 μg C. The samples used in this study were substantially smaller than those commonly analyzed for radiocarbon content in ecological research.

The ^{14}C content of extraradical mycorrhizal hyphae linked to the roots of plants fed with ^{14}C-dead CO_2 was almost always at or below the control values (Fig. 1), meaning that exposure to ^{14}C-dead CO_2 successfully reduced the ^{14}C content of the fungal tissue and that the mycorrhizal hyphae were growing at the expense of current photosynthate during the experimental period. The C pools in the mycorrhizal mycelium would have been differentially depleted in ^{14}C because of their different turnover rates. The two main pools in the ERM are those for growth and maintenance; the AM storage pool is principally located in the AM fungal component inside the roots (*1*). The maintenance pool is primarily composed of recently fixed C (24 hours or less) (*17*). In this experiment, therefore, we were following the C incorporated into growing hyphae.

The density of ERM in pots does not increase continuously (*18*), and we showed in a previous experiment conducted under similar conditions to the research reported here that the density of ERM was relatively constant over a comparable period, that is, 50 to

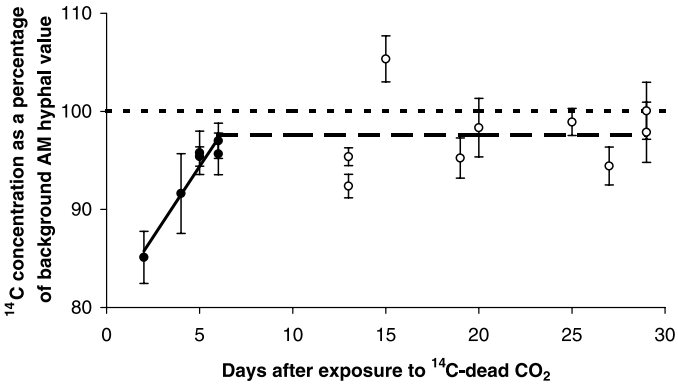

Fig. 1. The ^{14}C content of extraradical mycorrhizal fungal hyphae over a 1-month period after exposure of their host plants to ^{14}C-dead CO_2. The ^{14}C depletion is expressed relative to the background hyphal value (arbitrary baseline is 100). The data are best split in two and described by two fitted lines: the initial slope region (solid line and filled circles) and the plateau region (dashed line and open circles). Most of the experimental points remain below the average background value (100 ± 2.0 SE) for ^{14}C content (dotted line). The regression equation for the slope region is $y = 2.87x + 80.0$. The bars represent analytical uncertainty (*15*) and are a function of sample size. The mean value for the plateau region is 97.5 (± 1.4 SE), or, in other words, the depletion in ^{14}C as compared to the background hyphal value is 2.5% for the plateau region.

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71 days after planting (*19*). Even if the ERM was growing, the increase in ERM density over 5 days would only have had a small dilution effect on the total hyphal ^{14}C concentration. Also, if ERM biomass was increasing, the plateau ^{14}C depletion value would have continually converged to the background ^{14}C value. We are therefore confident that the measured changes in ^{14}C hyphal concentration were primarily because of hyphal turnover rather than any dilution effect as a result of increased ERM biomass.

The trends in the ^{14}C content values for the samples exposed to ^{14}C-dead CO_2 were best described by two fitted lines as shown by the F-ratio method of fitted lines comparison (*20*) ($P < 0.05$). These two lines can be termed the slope and plateau regions. The linear regression fitted to the slope region was highly significant ($P = 0.0014$) and explained most of the variation in ^{14}C content of the mycorrhizal hyphae ($r^2 = 0.94$). By 6 days after exposure, the ^{14}C content of the mycorrhizal hyphae had reached the plateau value (this can be seen directly on the graph but is also the predicted value from the regression equation). Therefore, almost all the C imported into the hyphae during exposure to ^{14}C-dead CO_2 was replaced in 6 days or less. However, ^{13}C studies have shown that host-plant photosynthate enters mycorrhizal hyphae within a few hours of fixation and that within 24 hours most of this C has been respired by the ERM (*21*). The maximum ^{14}C depletion content of the mycorrhizal hyphae was therefore most likely to be around 1 day after labeling (*21*), so the hyphal turnover time was probably closer to 5 days.

If maximum dilution of ^{14}C in the mycorrhizal hyphae occurred 1 day after exposure to ^{14}C-dead CO_2, we can estimate that the maximum dilution was around 16%. This means that the 5 hours of exposure of the host plants to ^{14}C-dead CO_2 resulted in a replacement of circa (ca.) 16% of extraradical mycorrhizal hyphal carbon. Because the exposure occurred during the most photosynthetically productive part of the day, this value cannot be extrapolated to estimate the total amount of hyphal carbon replaced per day. Nonetheless, that value would also indicate that the turnover of most mycorrhizal hyphae was within a week (that is, 1/0.16 is ca. 6 days). The mycorrhizal hyphal network is known to contain a large proportion of finely branched hyphae or branched absorptive structures (*22*), which may be the principal sites of nutrient uptake from the soil. These structures were likely to be responsible for the observed rapid turnover of ERM.

The plateau ^{14}C content average was less than the background (control) value in seven of the nine measurements, suggesting that some of the C imported into the ERM during exposure to ^{14}C-dead CO_2 remained for up to 30 days. The AM fungal hyphal network contains a few larger and probably longer-lived hyphae, called runner or trunk hyphae, that are thought to act as the "backbone" of the mycorrhizal hyphal network (*7*), a phenomenon paralleled in root system architecture, where turnover studies revealed a few roots that live far longer than others (*23*). The residual ^{14}C depletion of the mycelium probably reflected C in these longer-lived structures.

In this experiment, the plant-mycorrhizal fungi systems were grown under semisterile conditions in a growth medium (*10*) free of soil animals such as nematodes or collembola, which could have grazed the AM mycelium (*24*). This means that our measure of AM fungal hyphal turnover was the intrinsic turnover of the external AM mycelium under the conditions of this experiment. Under field conditions, the AM hyphal turnover could therefore be higher because of grazing by soil animals or environmental impacts such as drought.

These findings on external mycorrhizal hyphal turnover confirm that mycorrhizae act as a substantial pathway of C flow to the soil. Potentially, therefore, they could sequester some of this C in the soil. However, our data may suggest that most of the C transferred to the AM fungal hyphal network is rapidly recycled back to the atmosphere. There has been considerable research effort in understanding how global environmental change will affect the terrestrial carbon cycle (*25*–*27*), yet predictive modeling exercises are still often limited by the lack of understanding of the soil carbon cycle and soil ecosystem functioning at the most basic level (*28*).

This research shows that a large proportion of extraradical mycorrhizal hyphae, with the possible exception of runner hyphae, turn over in 5 to 6 days. This finding was made feasible by an application of AMS that allowed the microanalysis of hyphal samples containing as little as 10 μg of C, with only a small maximal depletion in ^{14}C content as compared to current ambient. AMS has been used previously, where sample size was not a constraint, to measure ^{14}C natural abundance in fungal sporocarps to determine the mycorrhizal status of fungi (*29*) and in fine roots to determine the age of fine-root C (*30*). We suggest that the level of analytical precision achievable by AMS will prove extremely useful in answering many other ecological questions, especially where limited sample weight is a constraint. This, along with other stable isotope approaches such as stable-isotope probing (*31*), provides exciting opportunities in functional ecological research.

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10. Twenty-day-old *P. lanceolata* seedlings were planted in pots (11.5 cm deep and 12.5 cm in diameter) with mycorrhizal fungal inoculum on 1 March 2001. The mycorrhizal inoculum included three unidentified *Glomus* species isolated from a hill grassland site (Sourhope Research Station, UK) and *G. mosseae* (UY 316). Plants were grown in a heated greenhouse (15-hour day length, 20°C day, and 15°C night), under natural light supplemented with light from a 400-W halogen bulb (giving a minimum photosynthetically active radiation of 100 $\mu mol\ m^{-2}\ s^{-1}$), in sand and Terragreen (an attapulgite clay soil conditioner; OilDri UK, Wisbech, UK) medium 1:1 by volume, with bonemeal at 1 $g\ l^{-1}$ as a slow-release phosphorus source, and fed with phosphorus-free nutrient solution. This growth medium, commonly used in mycorrhizal research, was chosen to enable the extraction of clean mycorrhizal hyphae.
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12. With the exception of the controls, plants were exposed on 19 April 2001 (48 days after planting) for 5 hours to ^{14}C-dead CO_2 (inexpensive CO_2 from a fossil-fuel source that is heavily depleted in ^{14}C compared to atmospheric CO_2). Exposure to ^{14}C-dead CO_2 occurred in purpose-built acrylic chambers at ambient atmospheric CO_2 concentration (370 $\mu l\ l^{-1}$) on a bright day.
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14. Extraradical mycorrhizal hyphae were carefully collected by pulling them from the surfaces of roots with fine forceps. Because hyphae were always collected from near the roots, the proportions of hyphal types (fine versus coarse or from different fungal species) were likely to have been constant; however, the thicker hyphae with greater growth potential may have been underestimated. Extraction of the roots and subsequent pulling of the hyphae from the root surfaces would have strongly discriminated against dead hyphae, which are easily broken when pulled; consequently, we followed ^{14}C concentration in the active mycorrhizal hyphal component. Care was taken to only include mycorrhizal hyphae, which can be visually distinguished from saprotrophs (uncommon in this system) by absence of septa, dichotomous branching, angular projections, and attachment to roots. The few spores present were removed from the hyphal samples, which were oven dried at 70°C overnight and weighed by microbalance. At harvest, shoots were also collected for dry weight. Shoot growth was constant over the experimental period (relative growth rate = 0.029 ± 0.003 SE, $P < 0.001$). On day 32 after exposure, the remaining pots were destructively harvested for the following parameters: shoot dry weight (4.48 ± 0.18 g), root dry weight (3.70 ± 0.44 g), percent root length colonized by mycorrhizal fungi (30% ± 6), and average extraradical mycorrhizal hyphal density in the pots (1.9 ± 0.22 $m\ g^{-1}$). Values in parentheses are means with standard errors.
15. Twenty-one mycorrhizal hyphal samples were obtained for AMS analysis: six controls (three immediately before the exposure date and three at the end of the sampling period) and 15 samples exposed to ^{14}C-dead CO_2 taken over a 4-week period from 2 to 29 days after exposure. The samples ranged from 20 to 116 μg (dry weight), equivalent to between ca. 10 and 60 μg C (9), and their ^{14}C signatures ranged from ambient to slightly depleted as compared with current ambient. The exact C content of each sample was not known, so it was not possible to bulk the samples to 1 or 2 mg in a carrier for analysis as required for conventional

mass spectrometry. Such small samples can only be analyzed by AMS. Sample combustion was performed with the use of a Carlo Erba EA1108 Elemental Analyzer (Carlo Erba, Milan, Italy) with tin combustion capsules cleaned in cyclohexane and distol acetone to reduce combustion blanks. After combustion, the CO_2 samples were cryogenically concentrated in a helium carrier gas with the use of a specially built two-stage capillary concentrator (capillary diameters were 0.75 mm and 0.25 mm), with the resulting CO_2-helium mixture directly injected into the AMS ion source. The combustion and gas-handling blank for "modern" (ambient radiocarbon concentration) was conservatively estimated to be 2.5 ± 0.5 μg C (typically 1.7 μg C of this being from the combustion itself); this blank makes very little difference to these results because they are very close to modern levels. With the use of a linear regression with sample size, we estimated the radiocarbon-free combustion blank to be 0.18 ± 0.10 μg C. The uncertainties in these contributions to the sample have been included in the error of the AMS analysis of ^{14}C content, which was 5% at 10 μg C and decreased to 1.5% at 100 μg C.

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7 March 2003; accepted 11 April 2003

Mitochondrial Dysfunction in the Elderly: Possible Role in Insulin Resistance

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Insulin resistance is a major factor in the pathogenesis of type 2 diabetes in the elderly. To investigate how insulin resistance arises, we studied healthy, lean, elderly and young participants matched for lean body mass and fat mass. Elderly study participants were markedly insulin-resistant as compared with young controls, and this resistance was attributable to reduced insulin-stimulated muscle glucose metabolism. These changes were associated with increased fat accumulation in muscle and liver tissue assessed by ^1H nuclear magnetic resonance (NMR) spectroscopy, and with a ~40% reduction in mitochondrial oxidative and phosphorylation activity, as assessed by in vivo $^{13}C/^{31}P$ NMR spectroscopy. These data support the hypothesis that an age-associated decline in mitochondrial function contributes to insulin resistance in the elderly.

Type 2 diabetes is the most common chronic metabolic disease in the elderly, affecting ~30 million individuals 65 years of age or older in developed countries (*1*). The estimated economic burden of diabetes in the United States is ~$100 billion per year, of which a substantial proportion can be attributed to persons with type 2 diabetes in the elderly age group (*2*). Epidemiological studies have shown that the transition from the normal state to overt type 2 diabetes in aging is typically characterized by a deterioration in glucose tolerance (*3*, *4*) that results from impaired insulin-stimulated glucose metabolism in skeletal muscle (*5*, *6*). Measurements of muscle triglyceride content by biopsy (*7*) or intramyocellular lipid content (IMCL) by ^1H nuclear magnetic resonance (NMR) spectroscopy (*8–10*) have shown a strong relationship between increased intramuscular fat content and insulin resistance in muscle. Similar correlations have been established for hepatic insulin resistance and hepatic steatosis (*11–13*). Increases in the intracellular concentration of fatty acid metabolites have been postulated to activate a serine kinase cascade leading to defects in insulin signaling in muscle (*14–17*) and the liver (*18*), which results in reduced insulin-stimulated muscle glucose transport activity (*14*), reduced glycogen synthesis in muscle (*19*, *20*), and impaired suppression of glucose production by insulin in the liver (*11–13*).

To examine whether insulin resistance in the elderly is associated with similar increases in intramyocellular and/or liver triglyceride content, we studied healthy elderly and young people that we matched for lean body mass (LBM) and fat mass. All study participants were nonsmoking, sedentary, lean [body mass index (BMI) < 25 m^2/kg], and taking no medications. Sixteen elderly volunteers (ages 61 to 84 years, 8 male and 8 female) were screened with a 3-hour oral glucose (75 g) tolerance test and underwent dual-energy x-ray absorptiometry to assess LBM and fat mass (*21*). One elderly man was excluded from the study because of an abnormal glucose profile. Thirteen young volunteers (ages 18 to 39 years, 6 male and 7 female), who had no family history of diabetes or hypertension, were matched to the older participants for BMI and habitual physical activity, which was assessed by means of an activity index questionnaire (*22*). All participants underwent a complete medical history and physical examination, as well as blood tests to confirm that they were in excellent health (*23*).

Young and elderly participants had similar fat mass, percent fat mass, and LBM (Table 1) (*24*). The elderly participants had slightly higher plasma glucose concentrations (Fig. 1A) and significantly higher plasma insulin concentrations (Fig. 1B) during the oral glucose tolerance test, suggesting that they were relatively insulin-resistant as compared with the young controls. Basal plasma fatty acid concentrations (Fig. 1C) also tended to be higher in the elderly participants but were suppressed normally after glucose ingestion.

To determine what tissues were responsible for the insulin resistance, we performed hyperinsulinemic-euglycemic clamp studies, in combination with [6,6-$^2H_2$] glucose and [$^2H_5$] glycerol tracer infusions (*24*). Basal rates of glucose production were similar in the young and elderly participants (Table 2) and were suppressed completely in both groups during the hyperinsulinemic-euglycemic clamp. In contrast, the rates of glucose infusion required to maintain euglycemia during the clamp and insulin-stimulated rates of peripheral glucose uptake were ~40% lower in the elderly participants (Table 2). Basal energy expenditure and respiratory quotient both tended to be lower in the elderly participants (*24*).

To ascertain whether lipid accumulation in muscle might be responsible for the insulin resistance in the elderly participants, we used ^1H

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