

Experimental evidence that mycorrhizal nitrogen strategies affect soil carbon

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Abstract. Most land plants acquire nitrogen (N) through associations with arbuscular (AM) and ectomycorrhizal (ECM) fungi, but these symbionts employ contrasting strategies for N acquisition, which may lead to different stocks of soil carbon (C). We experimentally test this hypothesis with a mesocosm system where AM and ECM tree seedling roots, or their hyphae only, could access mineral soils with ¹³C- and ¹⁵N-enriched organic matter. We quantified loss of soil C and N, plant uptake of N and new inputs of plant C to soil. We found that AM, but not ECM, seedlings reduced soil C relative to controls. Soil C loss was greater in the presence of roots relative to hyphae only for both AM and ECM seedlings, but was correlated with plant N uptake for AM seedlings only. While new plant C inputs stimulated soil C loss in both symbioses, we detected plant C inputs more frequently and measured higher rates of decomposer activity in soils colonized by AM relative to ECM seedlings. Our study experimentally demonstrates how mycorrhizal strategies for N can affect soil C and C:N, even at the scale of an individual plant. Such effects may contribute to broad patterns in soil C across terrestrial ecosystems.

Key words: *arbuscular mycorrhizal fungi; carbon; decomposition; ecosystem biogeochemistry; ectomycorrhizal fungi; nitrogen; soil organic matter.*

INTRODUCTION

Soils contain the largest reservoir of carbon (C) in the terrestrial biosphere (Schlesinger and Andrews 2000), the stability of which depends on the balance of inputs and losses to the atmosphere and hydrosphere. The two biological fluxes that govern this balance, CO₂ fixation by plants and CO₂ release by soil microbes, are widely constrained by nitrogen (N; Thomas et al. 2015). While soil organic matter represents the primary source of N for both land plants and decomposer microbes, most plants associate with arbuscular mycorrhizal (AM) or ectomycorrhizal (ECM) fungi, whose distinct strategies of N acquisition (Read and Perez-Moreno 2003) may subsequently affect decomposer activity and the loss of CO₂ from ecosystems. In spite of the ubiquity of AM and ECM plants across terrestrial ecosystems, experimental comparisons of their N strategies are rare, and thus how they differentially affect soil C dynamics remains unresolved.

Our consideration of mycorrhizal symbioses centers on their strategies for N acquisition and how they differentially affect patterns of soil C retention and loss in terrestrial ecosystems. Arbuscular mycorrhizal fungi generally “scavenge” mineral N and therefore depend on (Lambers et al. 2008) and even promote (Hodge et al. 2001, Cheng et al. 2012) microbial decomposition of organic matter. In contrast, some ECM fungi can “mine” N directly from organic matter (Abuzinadah et al. 1986, Bending and Read 1997, Lambers et al. 2008), which may deplete N supplies for decomposers and suppress their activity (Averill and Hawkes 2016). But whether mining acts to stabilize soil C across terrestrial ecosystems depends on whether scavenging and mining strategies differ in how they affect soil C balance as a function of plant N gain. The observation that AM-dominated biomes and ecosystems tend to contain lower soil C or soil C:N relative to their ECM counterparts (Read 1984, Averill et al. 2014) suggests that the mining strategy stabilizes soil C. However, we lack direct experimental evidence for how strategies of N acquisition by AM and ECM plants, including their C exudation (inputs) and effects on decomposers (losses), and can alter the soil C balance.

Here, we examine whether N acquisition strategies of AM and ECM symbioses can affect the soil C balance.

Manuscript received 30 November 2016; revised 2 February 2017; accepted 22 March 2017. Corresponding Editor: Christine V. Hawkes.

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We hypothesized that AM plants would promote greater soil C loss as a function of soil N uptake than ECM plants because they stimulate organic matter decomposition. We tested our hypothesis with plant-soil mesocosms that contained individual tree seedlings and a pouch of experimental soil with ^{13}C and ^{15}N -enriched organic matter. Experimental soil was contained in mesh that either allowed or prevented fine root ingrowth, but allowed the ingrowth of mycorrhizal hyphae. This approach allowed us to determine changes to soil C and N pool sizes as a function of plant size and plant N acquisition, quantify the amount of new plant C incorporated into soils, and isolate the effects of roots and mycorrhizal hyphae.

METHODS

We grew seedlings of five dominant tree species of mixed-deciduous forests of the southern Appalachians (Elliott and Swank 2008) that were AM (*Acer rubrum* L., *Liriodendron tulipifera* L.) or ECM (*Betula lenta* L., *Pinus strobus* L., and *Quercus rubra* L.). Seedlings grew in 650 cm³ pots (deepots, Stuewe and Sons, Tangent, Oregon USA) in a matrix of nutrient-poor peat and sand (1:1 by volume), with mesh pouches of experimental soil (~16 g dry equivalent) enriched in ^{13}C and ^{15}N . Soil pouches were constructed with two mesh sizes, one that facilitated hyphal ingrowth but prevented root ingrowth (small mesh, 50- μm polyester monofilament; Tetko Inc., Buffalo, New York, USA) and one that facilitated both hyphal and root ingrowth (large mesh, 2 mm fiberglass screen). This allowed us to concentrate the effects of N acquisition on soil C in a small volume, while manipulating the presence or absence of roots.

^{13}C - and ^{15}N -enriched soil organic matter

To generate experimental soils, we collected mineral soils (0–10 cm depth) under the canopies of our target tree species in mature forests at the Coweeta Hydrologic Lab in western North Carolina. We combined these soils and added ^{13}C - and ^{15}N -enriched *Q. rubra* leaf litter (from saplings that were pulsed with $^{13}\text{CO}_2$ and ^{15}N -enriched fertilizer), fragmented into 1-cm² pieces and applied at a rate equivalent to annual leaf-litter inputs. To accelerate decomposition, we added ~30 earthworms (*Lumbricus rubellus*) and kept the mixture moist at 20°C in a laboratory incubator. After 9 months, leaf fragments were no longer discernable, earthworms were removed, and the soil was fully homogenized. Each pouch received the moist equivalent of ~16 g soil from this homogenized mixture. Our analysis of seven subsamples of soil prior to the experiment demonstrated low variability in total C (6.4% \pm 0.18% [mean \pm SE]), total N (0.44% \pm 0.008%), $\delta^{15}\text{N}$ (541.2‰ \pm 12.0‰) and $\delta^{13}\text{C}$ (-3.97‰ \pm 0.72‰), and thus each pouch contained ~1.04 g C and 68.4 mg of N.

Experimental mesocosms

Our experimental design consisted of two mycorrhizal types (AM vs. ECM) represented by two and three tree species, respectively, crossed by mesh size (small vs. large) and each combination was replicated 10 times. A soil (no-plant) control was replicated five times for each mesh size, for a total of 110 experimental units. In our final analysis, we excluded mesocosms with dead plants or ruptured soil pouches, leaving 81 units and 9 controls. Plant N and $\delta^{15}\text{N}$ were not analytically detectable in nine of our plant samples, reducing our sample size to 72 for analyses of N uptake. We grew an equivalent number ($n = 93$) of seedlings in unenriched soil under the same conditions (i.e., with large and small mesh pouches that contained soil that had been incubated with unenriched *Q. rubra* leaf litter) to determine species-specific natural abundance $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$.

Seeds (Sheffield Seed Co., Locke, New York, USA) were germinated in a peat and sand mixture and grown 1–3 months before transplanting in experimental cones. During the transplant, seedling root systems were dusted with a mixture of pulverized, freshly collected roots and field soil to ensure adequate inoculation by mycorrhizal fungal and microorganisms. Seedlings of each species varied in age (i.e., 1–3 months old) to better resolve effects of size on soil N uptake and soil C loss. Mesocosms of each treatment, including unenriched controls, were distributed equally between two growth chambers with 12-h photoperiods and maintained at 25°C.

Harvesting the experiment

After 6 months of growth, we harvested mesocosms. Roots were removed from pouches (large-mesh only) and soils were homogenized. A subsample of pouch soil was used for measures of soil respiration, and the remaining soil and whole-plant biomass was dried, weighed, and ground to a fine powder to determine soil and whole-plant total C and N and $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ (combustion coupled to an isotope ratio mass spectrometer). Changes in soil C and N were calculated as the difference between end-of-experiment values and pre-experiment values, were mass-based and did not depend on isotope values. To quantify plant N uptake from the pouch soil, we calculated atom percentage of ^{15}N enrichment of seedlings relative to unenriched controls, and the isotopic enrichment of soil N relative to unenriched soils, to estimate the plant N derived from pouch soils.

To quantify rates of soil respiration, we transferred ~10 g dry equivalent of fresh soil from each pouch to 250-cm³ mesocosms in a laboratory incubator maintained at 25°C. After a two-week acclimation period to allow soils to stabilize from disturbance, mesocosms were flushed with N₂, capped with a gas-tight sampling lid, and analyzed for CO₂ concentrations four times over a 12-h incubation. At each sampling point, mesocosm

head space was mixed, sampled (3 cm³) and analyzed with an infrared-gas analyzer (LiCor 6252, LiCor, Lincoln, Nebraska, USA) to determine the rate of respiration ($\mu\text{mol CO}_2\text{g C}^{-1}\cdot\text{h}^{-1}$).

We quantified inputs of new plant C in the soil C pool using a mass balance approach parameterized by the mass and isotopic signatures of plant tissue, initial soil C, and lost soil C

$$I = \frac{M_f + a_1L - M_0}{a_p}$$

where I is the input mass (g) of new plant C to soil, M_f is the mass (g) of ¹³C at a given fraction of soil C remaining relative to the initial soil C, a_1 is the atom percentage of ¹³C of soil C losses, L is soil C loss (g), M_0 is the initial mass of ¹³C in soils, and a_p is the atom percentage of ¹³C of plants. We calculated a_1 as pre-experiment mass (g) of soil ¹³C – control (no plants) mass (g) of soil ¹³C/total soil C (g). We calculated L as the average initial soil C (g) – soil C (g) remaining at the end of the experiment. We then calculated a conservative estimate of new plant C by subtracting the mean $I + 1$ SD calculated from the control (no plant) soils.

We verified mycorrhizal colonization on root samples (~20 cm length) from 5–10 individuals of each species. Arbuscular mycorrhizal colonization was determined for *A. rubrum* and *L. tulipifera* (46% and 58% of root length, respectively) by clearing and staining roots (Wurzburger and Wright 2015). Ectomycorrhizal colonization for *B. lenta*, *P. strobus*, and *Quercus alba* (30%, 26%, and 20% of root length, respectively) was determined from the presence of a fungal mantle using a grid-line intercept method; Wurzburger and Bledsoe 2001). We did not determine if *Q. alba* seedlings also possessed AM colonization.

Statistical analyses

To determine differences between mycorrhizal types, we constructed linear mixed-effects (or generalized linear mixed-effects) models in R (version 3.3.1; R Development Core Team, Vienna, Austria), where tree species were treated as a random effect. For all linear mixed-effects models, we conducted F tests using Kenward-Roger approximated degrees of freedom using the lme4 (Bates et al. 2015) and pbktest packages (Halekoh and Højsgaard 2014), followed by post hoc mean separation tests (Tukey HSD). Fixed effects in our models included mycorrhizal type, mesh size, and their interaction. When mesh size or interactions with mesh size or mycorrhizal type were not significant, we removed them from the model. Some variables required a square-root transformation to achieve normality and normal error distributions. We calculated the marginal (R_m^2) and conditional R^2 (R_c^2) to determine the variance explained by fixed and random effects, respectively, using the piecewiseSEM package (Lefcheck 2016).

To examine the effect of mycorrhizal type and mesh size on various plant and soil response variables, treatment (e.g., pre-experiment, AM, ECM and no-plant control) or mycorrhizal type (AM vs. ECM) and mesh size as fixed effects. To examine how plant N uptake scaled with plant size, and whether this differed between mycorrhizal types, we constructed linear mixed-effect models where mycorrhizal type (AM vs. ECM) and plant C were treated as fixed effects. To determine the effects of mesh size and N uptake on the fraction of soil C remaining (from pre-experiment levels), we included plant N uptake as a fixed effect.

We determined how soil respiration varied by mycorrhizal type by constructing a generalized linear mixed-effect model with a gamma distribution and soil moisture as a covariate. Since the variance components of the random effects were equal to 0, we constructed a generalized linear model (GLM) with a similar structure followed by a post hoc mean separation test (Tukey HSD). We excluded samples with soil moisture <10% ($n = 13$), because CO₂ did not accumulate during the incubation.

To determine if the extent of soil C loss was affected by inputs of new plant C, we constructed a linear mixed-effects model where mycorrhizal type, mesh size, and new plant C were fixed effects. Since several samples produced values below our detection limit, we analyzed the data set with new plant C as a binary response to determine if the frequency depended on mycorrhizal type (generalized linear mixed-effect model with a binomial distribution). In this model the variance components of the random effects were equal to 0, so we constructed a GLM with a similar structure.

RESULTS

Overall, we found that mycorrhizal type explained more variance in our linear mixed models (LMMs) than did individual species (bootstrapped 95% confidence intervals of random effect estimates include 0, and the portion of variance explained by fixed effects were often greater than those of random effects; Appendix S1: Table S1), and thus we focus exclusively on mycorrhizal effects. Seedlings of AM and ECM plants did not differ significantly in their final biomass, total C or N nor were they differentially affected by root inclusion or exclusion (i.e., mesh size; Appendix S1: Tables S1.1, S1.2, and S2), eliminating any potential experimental bias between mycorrhizal types. However, random effects (species) accounted for a large portion of variance in our model of total plant N, likely due to greater amount of seed N for *Quercus alba* relative to other species (Appendix S1: Tables S1.3 and S2).

We evaluated how mycorrhizal types affected soil C and N pools at the end of the experiment. Both AM and ECM seedlings triggered a net loss of soil C and N after 6 months of growth relative to pre-experimental soils (LMM, $n = 97$, $F_{3,4.10} = 8.94$, $P = 0.03$;

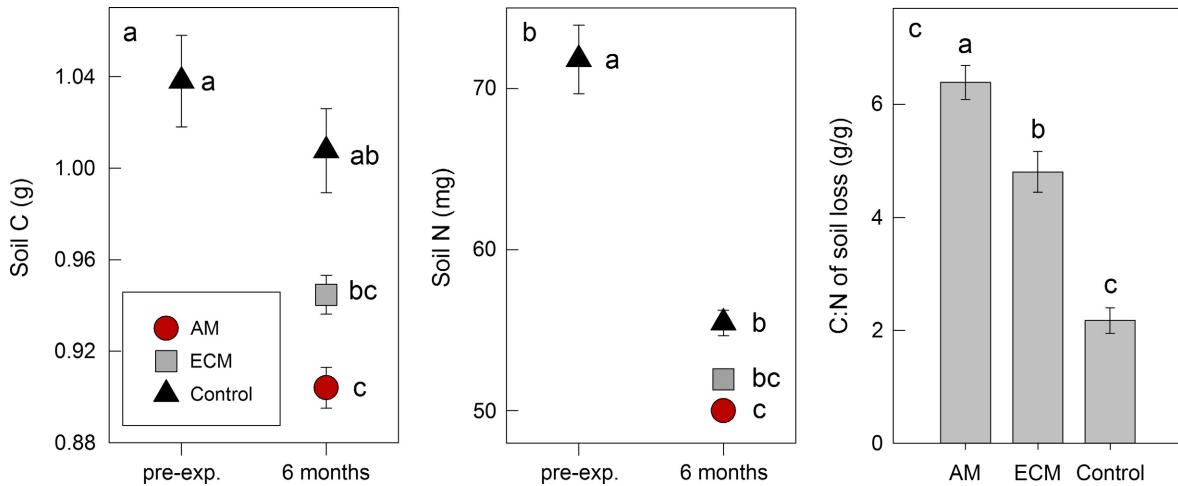


FIG. 1. Total soil C and N and C:N loss after growth of arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) tree seedlings in experimental mesocosms. (a) total soil C and (b) total N before (pre-exp. [experiment]) and after 6 months of growth relative to a no plant control. (c) The ratio of C loss to N loss after 6 months. Values are means \pm SE and significant differences ($P < 0.05$) among treatments denoted by different letters.

$F_{3,4.45} = 39.06$, $P = 0.001$, for soil C and N respectively), but only AM seedlings significantly reduced soil C and N at the end of the experiment relative to the no plant control (Tukey HSD, $P < 0.05$ for AM and $P > 0.05$ for ECM; Fig. 1a, b; Appendix S1: Table S1.4 and S1.5). Interestingly, we observed preferential loss of N over C between pre-experiment and no-plant controls, suggesting gaseous or hydrologic loss of N was more important than plant N uptake in determining the final soil N pool. The ratio of soil C loss to soil N loss differed by mycorrhizal type (LMM, $n = 92$, $F_{2,3.49} = 7.92$, $P = 0.049$), was greater in AM vs. ECM and control soils (Tukey HSD, $P < 0.05$; Fig. 1c;

Appendix S1: Table S1.6) and was also greater for large vs. small mesh ($F_{1,84.96} = 5.98$, $P = 0.02$).

We next examined the possibility that patterns in C loss were explained by differences in N uptake (determined by isotopic enrichment) between AM and ECM seedlings. Because N demand can vary with growth rates and biomass, we included plant biomass C at the end of the experiment as a covariate in the model. Nitrogen uptake (square-root transformed) increased more strongly with increasing plant C in ECM than in AM seedlings (LMM, $n = 72$, interaction of mycorrhizal type and plant C, $F_{1,54.60} = 12.10$, $P = 0.001$; Fig. 2a; Appendix S1: Table S1.7), and the exclusion of roots did

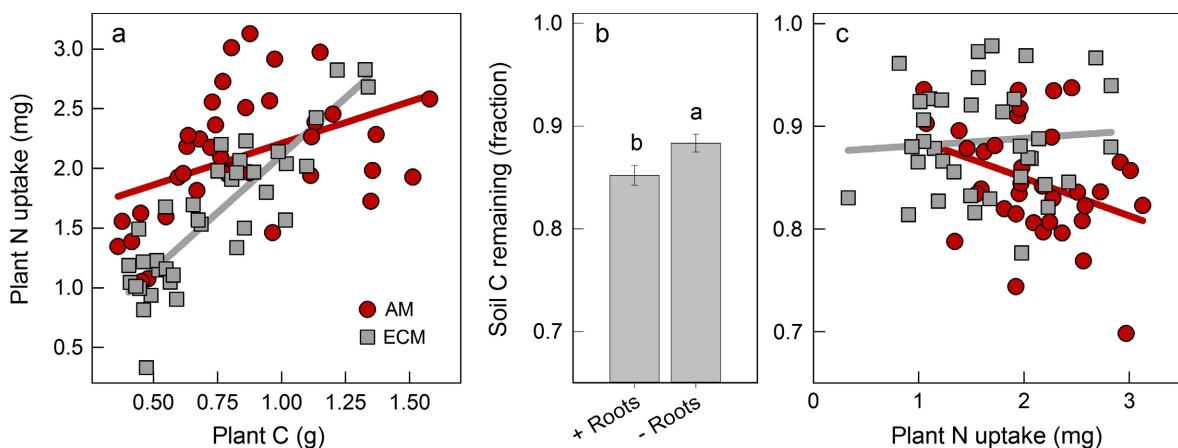


FIG. 2. Distinct relationships in N uptake and soil C between arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) tree seedlings. (a) plant uptake of soil N (square-root transformed) as a function of plant biomass C, (b) soil C remaining in the presence and absence of roots, and (c) relationship between soil C remaining and plant N uptake (square-root transformed) for AM and ECM seedlings. Mycorrhizal effects demonstrated by significant interactions with plant C (a; $P < 0.05$), a weak interaction with N uptake (c; $P = 0.08$) and a significant correlation of C remaining and N uptake for AM seedlings only (c; $r^2 = 0.11$, $P = 0.04$). Root effects on C remaining (means \pm SE) denoted by different letters ($P < 0.05$).

not change this relationship in either symbiosis (mesh size: $P > 0.05$), suggesting the size-dependency of plant N uptake was largely a function of hyphal uptake.

We next determined the relationship between plant N uptake and net soil C retention. Soil C declined in the presence of roots vs. hyphae only (LMM, $n = 72$, mesh size $F_{1,64.70} = 13.39$, $P = 0.0007$; Fig. 2b; Appendix S1: Table S1.8), but the mesh effect did not differ by mycorrhizal type nor the extent of N uptake. Soil C declined with increasing N acquisition (square-root-transformed) for AM seedlings ($r^2 = 0.11$, $P = 0.04$) but not ECM seedlings (interaction of mycorrhizal type and N uptake $F_{1,66.68} = 3.26$, $P = 0.08$; Fig. 2c; Appendix S1: Table S1.8). This pattern, although weak, is consistent with the idea that mycorrhizal strategies of N acquisition differentially affect soil C.

Our observations of N acquisition and C loss suggest that AM seedlings promoted the decomposition of organic matter relative to ECM seedlings. To evaluate this hypothesis, we quantified heterotrophic respiration in soils at the end of the experiment. Respiration rates were greater in AM and ECM soils vs. the no-plant control (GLM, $n = 81$, $F_{2,76} = 5.25$, $P < 0.01$, pseudo- $R^2 = 0.20$), were positively related to soil moisture ($F_{1,75} = 5.85$, $P = 0.01$), and were weakly higher in AM vs. ECM soils (Tukey HSD, $P < 0.059$; Fig. 3a). Although we isolated soils to perform these measurements, these findings support the idea that AM symbioses promote higher rates of microbial turnover of organic matter relative to ECM symbioses.

We evaluated whether the loss of ^{13}C from soil was affected by potential differences in lability of old enriched- vs. new enriched-organic matter in our experimental soils. Loss of ^{13}C was strongly related to soil C loss (LMM, $n = 81$, $F_{1,70.25} = 36.92$, $P < 0.0001$; Appendix S1:

Table S1.9), but did not interact with mycorrhizal type or mesh size, indicating that the decomposition of enriched organic matter did not vary by experimental treatments and bias our estimates of new inputs of plant C.

We then explored the possibility that patterns of soil C loss result from new inputs of plant C and its stimulatory effect on decomposition (i.e., priming). We quantified inputs of plant C in soils using simple isotope mass balance (see *Methods*). We found that soil C loss increased with increasing inputs of plant C (LMM, $n = 81$, $F_{1,26.75} = 41.82$, $P < 0.0001$; Fig. 3b; Appendix S1: Table S1.10) in both mycorrhizal types (i.e., no interaction with mycorrhizal type), and was greater in presence vs. absence of roots. Inputs of plant C varied considerably across all experimental units, but we observed C inputs more frequently in experimental soils colonized by AM vs. ECM seedlings (GLM, $n = 81$, AM vs. ECM $P = 0.0249$; Fig. 3c). These findings suggest that AM trees have a greater tendency than ECM trees to release C into soils and thereby stimulate decomposition. We cannot rule out the possibility that patterns in soil C loss are additionally explained by the quality of these plant C inputs.

DISCUSSION

Our study provides evidence that mycorrhizal strategies for N acquisition affect soil C. While differences in AM and ECM symbiont identity, physiology and effects on decomposers have been noted for decades (Read 1984, Read and Perez-Moreno 2003, Lindahl and Tunlid 2015), we lack direct comparisons of how their N acquisition strategies may affect soil C pools. Such relationships between C and N are fundamental to understanding and predicting C stocks in the terrestrial biosphere (Finzi et al. 2015).

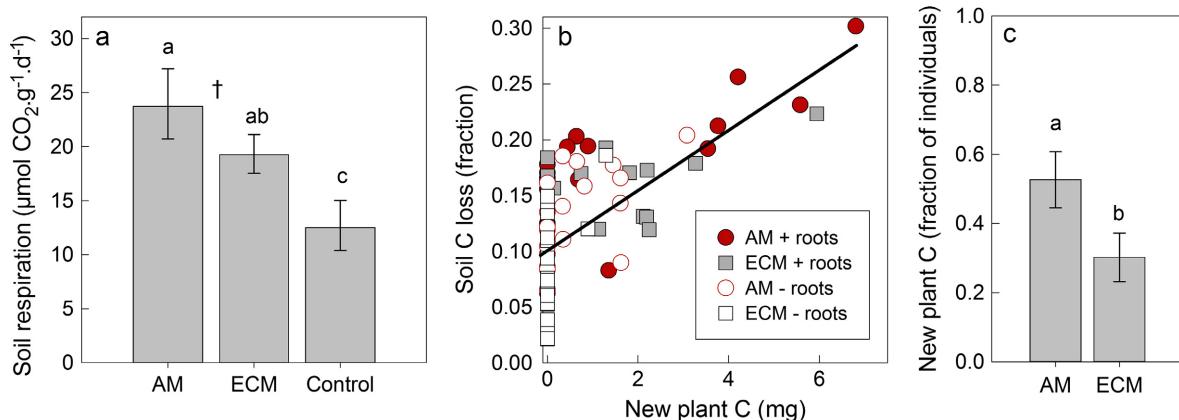


FIG. 3. Soil respiration and inputs of new plant C differ by mycorrhizal type. (a) Respiration rates among arbuscular mycorrhizal (AM), ectomycorrhizal (ECM), and control (no plant) soils. Values are parameter coefficients \pm SE, and differences ($P < 0.05$, $P < 0.10$) denoted by different letters or †, respectively. (b) Inputs of new plant C to soils, calculated from isotopic mass balance, were correlated with soil C loss for both arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) seedlings, but were greater in the presence vs. absence of roots. (c) Inputs of new plant C were detected more frequently among individuals of AM than ECM seedlings. Values are parameter coefficients \pm SE, and significant differences ($P < 0.05$) between treatments denoted by different letters.

Most remarkably, we found that only AM seedlings reduced soil C pools relative to the no-plant controls (Fig. 1a) and AM soils lost more C per unit N than ECM soils (Fig. 1c). Consistent with this finding, we detected new plant C inputs more frequently in AM vs. ECM soils (Fig. 3c) and greater heterotrophic respiration in AM soils (Fig. 3a). Further, we observed a negative, albeit weak, relationship between net soil C retention and N acquisition for AM but not ECM seedlings (Fig. 2c), which suggests that AM plants rely on decomposers to improve access to bioavailable N (Hodge et al. 2001, Cheng et al. 2012), and thus promote more C loss per unit N uptake. In contrast, ECM seedlings depended on plant C more than AM seedlings to acquire N (Fig. 2a), consistent with the idea that ECM plants rely on roots and hyphal networks, which excrete extracellular enzymes to mobilize organic N (Lambers et al. 2008). Our short-term experimental results thus provide a mechanism for observational findings of higher soil C and soil C:N in ECM than AM ecosystems (Read 1984, Averill et al. 2014) and the contention that mining stabilizes soil C (Orwin et al. 2011).

In natural ecosystems and over longer time scales, factors regulating productivity and turnover among AM and ECM plants will determine whether such mycorrhizal differences in soil C can be sustained (Orwin et al. 2011). The observation that AM fungi stimulate decomposition (Cheng et al. 2012) suggests that this symbiosis promotes faster organic matter turnover in the plant-soil system relative to its ECM counterpart. Differences in decomposition may be further maintained by the mining actions of ECM fungi, which have direct suppressive effects on decomposers (Averill and Hawkes 2016). In our study, decomposition activity was associated with higher inputs of plant C, suggestive that priming improves plant access to N, and this may be particularly important for the AM symbiosis (Cheng et al. 2014). Such an effect may be reinforced by litter quality of the host plant, as AM plant litter tends to have a lower C:N and decompose more quickly than the litter of ECM plants (Midgley et al. 2015, Taylor et al. 2016). Over time, differences in litter quality and mycorrhizal strategies may give rise to nutrient feedbacks in the plant-soil system (Wurzburger and Hendrick 2009, Phillips et al. 2013) that involve distinct decomposer assemblages (McGuire et al. 2010). Interestingly, we observed mycorrhizal effects on soil C and C:N loss despite the fact that our experimental soils had a low C:N ratio (~14.5 on a mass basis), which may have promoted higher N availability, potentially favoring AM plants and dampening differences in C loss between AM and ECM plants.

While our study demonstrates that changes in soil C and N dynamics are detectable at the scale of an individual plant, we acknowledge potential limitations of our study design. First, we did not identify the precise form of N (i.e., organic vs. mineralized N) acquired by mycorrhizal fungi. However, because our experiment allowed

for dynamics among symbionts, decomposers, and organic matter to develop over the course of a growing season, it offers unique insights into the fate of organic matter C beyond those possible with short-term (i.e., hours) tracer experiments of N uptake. Second, we did not account for gaseous or hydrologic N loss from our experimental soils, and thus it remains a possibility that differences in loss pathways can also contribute to patterns in soil N (or C:N) stocks between mycorrhizal types. Third, since plant species within AM and ECM guilds demonstrate remarkable variability in nutrients foraging (Chen et al. 2016), their effects on soil C may also vary similarly. In our study, however, mycorrhizal effects on soil C retention and respiration were stronger than those of individual species, and thus, point to mycorrhizal differences overwhelming those of species on C loss dynamics.

Our findings suggest that differences in mycorrhizal N strategies alone may be sufficient to affect soil C or soil C:N. However, across terrestrial biomes and ecosystems, other factors (e.g., climate, evolutionary tradeoffs, disturbance) regulating the abundance, productivity and species composition of AM vs. ECM plants likely influence the extent and persistence of mycorrhizal-driven patterns in soil organic matter development. While broad geographic differences in soil C and mycorrhizal types lend support for such persistence (Read 1984, Averill et al. 2014), how species of these contrasting mycorrhizal types respond to global change factors will likely affect the stability of soil C pools in the future.

ACKNOWLEDGMENTS

We are grateful to S. Wilson, A. Martin, P. Hendrix, J. Minucci, C. Phillips, D. Abdulhadi, J. Tierney, M. Ament, F. Ballantyne, E. Kruger, R. Lankau, J. P. Schmidt, and C. Song. This research was supported by the University of Georgia Research Foundation, the U.S. Department of Agriculture Forest Service, Southern Research Station, and the National Science Foundation (NSF) Long-Term Ecological Research Program. Any opinions, findings, conclusions or recommendations expressed in the material are those of the authors and do not necessarily reflect the views of the USDA or the NSF. We thank two reviewers for their constructive criticism of our manuscript.

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