Biomass and compositional responses of ectomycorrhizal fungal hyphae to elevated CO$_2$ and nitrogen fertilization

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**Summary**

- The extramatrical mycelia (EMM) of ectomycorrhizal fungi make up a large proportion of the microbial diversity and biomass in temperate forest soils. Thus, their response to elevated CO$_2$ can have large effects on plant nutrient acquisition and carbon movement through forests.

- Here, the effects of CO$_2$ and nitrogen (N) fertilization on EMM biomass and community structure in *Pinus taeda* forest plots were examined using sand-filled mesh bags buried in the field, the contents of which were analyzed by phospholipid fatty acid (PLFA) and DNA sequencing.

- A total of 2138 sequences comprising 295 taxa were recovered; most (83.5%) were from ectomycorrhizal fungal taxa. No biomass increase was detected in elevated CO$_2$ plots relative to control plots, but individual taxa responded to both CO$_2$ and N fertilization, four of the six most abundant taxa were less frequent in N-fertilized plots. Thelephoroid and athelioid taxa were both frequent and abundant as EMM, and thelephoroid richness was extremely high. *Russula* and Cortinariaceae taxa were less abundant and boletoid taxa were more abundant as EMM relative to ectomycorrhizas.

- The EMM community, sampled across seasons and years, was dynamic with a high degree of interspecific variation in response to CO$_2$ enrichment and N fertilization.

**Key words:** CO$_2$, ectomycorrhizal fungi, extramatrical mycelia, free-air CO$_2$ enrichment, hyphal biomass, internal transcribed spacer (ITS) sequencing, nitrogen fertilization, phospholipid fatty acid (PLFA).

**New Phytologist** (2007) **176**: 164–174

doi: 10.1111/j.1469-8137.2007.02155.x

**Introduction**

Increasing atmospheric CO$_2$ concentrations and nitrogen (N) deposition are two of the most important aspects of anthropogenic global environmental change (Cubasch *et al*., 2001; Galloway *et al*., 2003). These increases may have important consequences for forest ecosystems by altering carbon (C) and N availability for both plants and soil biota. It is particularly important to consider the consequences for ectomycorrhizal fungi (EMF) because they represent a large proportion of microbial diversity and biomass in temperate and boreal forest soils (Wallander *et al*., 2001; Högberg & Högberg, 2002; O’Brien *et al*., 2005). EMF are also directly involved in the movement of C and N among plants, soil and the atmosphere (Smith & Read, 1997). Nutrients needed for both fungal and plant growth are acquired from soil and translocated between fungi and plants by fungal extramatrical mycelia (EMM) that emanate from colonized fine roots into the soil. EMM show considerable variation in both the quantity and the architecture of EMM (Agerer, 1987–2002). It is likely that such variation among species in EMM density, length, and aggregation into
rhizomorphs represents variation in foraging strategies (or ‘exploration types’; err, Aegerit, 2001). Morphological diversity of EMF taxa in exploration type may translate into variation in the amount of C required to sustain them, and the amount of nutrients they can provide to their plant hosts. Knowledge of the abundance and architecture of EMM for a particular species may therefore improve our understanding of its functional role in the mycorrhizal symbiosis.

Because EMF are almost entirely reliant upon C derived from plants, changing plant C allocation patterns resulting from elevated CO₂ may have important consequences for EMM production. An increase in atmospheric CO₂ concentrations has the potential to enhance forest productivity provided that the increase in nutrient demands needed to sustain greater growth rates can be met. Fine-root measurements taken from trees exposed to elevated CO₂ sustain greater growth rates can be met. Fine-root measurements taken from trees exposed to elevated CO₂ have shown that below-ground C allocation increases in CO₂-enriched plots (Matamala & Schlesinger, 2000; Norby et al., 2005). Given that CO₂ enrichment can cause plants to increase both the amount of C they allocate below-ground and the amount of nutrients they require, it has been hypothesized that EMM biomass may increase with rising CO₂ concentrations and may also serve as a below-ground C sink (Treseder & Allen, 2000; Staddon, 2005). However, the few studies that have examined the response of arbuscular and ectomycorrhizal EMM biomass to CO₂-enriched atmospheres have yielded conflicting results, with some documenting increases (Klironomos et al., 1997; Alberton et al., 2005), and some finding little or no response (Rouhier & Read, 1998; Gorissen & Kuyper, 2000; Kasurinen et al., 2005; Godbold et al., 2006).

CO₂ enrichment may also induce species-specific responses in EMM abundance. Previous studies showed that the relative abundances of EMF species on fine roots were affected by both CO₂ concentration and background nutrient content (Fransson et al., 2001; Parrent et al., 2006). However, these studies did not determine the relative contribution of mycorrhizal taxa to EMM biomass, or whether EMM distribution and abundance are affected by CO₂ and nutrient content in a similar manner to fine roots. Mycorrhizal taxa that produce the greatest amount of EMM can explore and acquire nutrients from a larger portion of the soil system; these taxa may therefore represent the most beneficial plant partners. Alternatively, these taxa may be a larger cost to plants than taxa that produce less EMM if greater C and nutrients are required to sustain a larger EMM, and if these costs are not offset by greater nutrient transfer to plants. Given their greater exploration potential combined with increased plant nutrient demand in elevated CO₂ conditions, one plausible hypothesis is that CO₂ enrichment will favor taxa producing the greatest amounts of EMM (Rygiewicz et al., 2000).

One reason for the scarcity of knowledge about EMM is the methodological challenge of studying the EMF community in soil. The ectomycorrhizal nutritional mode is an evolutionarily labile trait (Hibbett et al., 2000); EMF taxa are therefore intercalated among closely related nonmycorrhizal fungi in the phylogeny, making it unfeasible to develop molecular markers that target EMF and exclude other fungi. As a result, directly assaying the soil substrate using available molecular methods will not only recover the desired EMF taxa, but will also amplify saprotrophic fungi foraging in the soil (O’Brien et al., 2005), as well as DNA from nonactive fungal structures such as dead hyphae and resting structures such as spores.

In the last several years, a number of studies (e.g. Wallander et al., 2001; Hagerberg & Wallander, 2002) have used an ingrowth bag approach to study mycorrhizal EMM biomass. Bags constructed of fine-gauge nylon mesh, to prevent roots and larger soil fauna from entering, are filled with a C-free substrate (acid-washed sand) to reduce ingrowth of saprotrophic fungi. Natural abundance isotope data and DNA sequences from bags incubated in forest soil confirm that the majority of the EMM community is comprised of mycorrhizal taxa (Wallander et al., 2001; Kjøller, 2006), thus allowing examination of the EMF contribution to EMM biomass.

In this study, the ingrowth bag method was employed to examine EMF communities in intact forest soils that have been exposed to elevated CO₂ and N fertilization conditions. Ingrowth bags were buried for 8 wk in temporal succession at the forest–atmosphere carbon transfer and storage (FACTS-I) site in Duke Forest, NC, USA, in the summer and autumn of 2004 and 2005. DNA sequencing of bag samples was carried out to identify the EMF taxa growing as hyphae. Phospholipid fatty acid (PLFA) analyses were also performed to estimate hyphal biomass. These data allowed determination of the major EMF contributors to EMM in soil, and quantification of seasonal, CO₂ and N fertilization effects on EMM composition and biomass. Using this approach, we tested the hypotheses that increased below-ground C allocation associated with elevated CO₂ increases EMM biomass, and that CO₂ and N fertilization alter EMM community composition. To our knowledge, this is the first study to examine the EMF community response of EMF taxa to CO₂ and N fertilization in a field setting.

Materials and Methods

Field site

The field site used in this study is the FACTS-I research site in Duke Forest, Orange Co., NC, USA. Six circular, 30-m-diameter plots have been erected in an even-aged Pinus taeda stand. Beginning in 1996, three experimental plots have been exposed to a CO₂-enriched atmosphere (200 ppm above ambient), and three reference plots have been maintained under ambient conditions. Each plot is subdivided into eight sectors; all four sectors designated for below-ground studies (soil sectors) were sampled in every plot. Soils at this site are clay-rich, low-fertility Ultic Alfsols in the Enon series (Schlesinger & Lichter, 2001). Beginning in 2005, a N
fertilization regime was implemented in half of every plot. In March and April 2005, NH₄NO₃ pellets were broadcast by hand to achieve a total annual fertilization rate of 11.2 g N m⁻² yr⁻¹. Additional information regarding site characteristics can be found in Schlesinger & Lichter (2001).

Ingrowth bags

Ingrowth bags were constructed following the protocol of Wallander et al. (2001). Fine-gauge (48-µm-diameter) nylon mesh was fashioned into bags approx. 4.5 × 4.5 × 1.5 cm in size. Quartz sand was sieved to remove fine particles (<0.5 mm), acid-washed, thoroughly rinsed and autoclaved. Approximately 20 g of sterile, acid-washed sand was then added to each bag and the bag was heat-sealed.

Bags were incubated at the site in the summer (June–August) and autumn (August–October) seasons of 2004 and 2005. Bags were placed at the organic–mineral soil interface in four randomly chosen locations in each soil sector of all six plots, giving a total of 16 bags per plot × 6 plots = 96 bags per sampling period. When the summer set of bags was harvested, the autumn bags were immediately placed in the identical location. Bags were removed from the soil after 8 wk. Bag contents were homogenized by mixing, and two 5-g subsamples were taken from each bag; one to be used for constructing clone libraries and one for measuring PLFA concentrations. For each sampling period, samples taken from bags within a sector were combined so that there was one sample for each soil sector for each sampling period (4 sectors per plot × 6 plots × 4 sampling periods = 96 samples). Samples were stored at –80°C before analyses.

Clone library construction and DNA sequencing

DNA was extracted from each of the 96 pooled samples. Internal transcribed spacer (ITS), 5.8S and partial large subunit (LSU) ribosomal gene regions were amplified by polymerase chain reaction (PCR) as a single fragment using the primer pair ITS-1F (Gardes & Bruns, 1993) and LR5 (Vilgalys & Hester, 1990). PCR products were ligated into the PCR 2.1-Topo vector and transformed into chemically competent Escherichia coli strain TOP 10 cells (Invitrogen, Carlsbad, CA, USA). Twenty-four colonies were collected from each library and colony PCR was performed using the forward and reverse primers M13F and M13R. Initially only single reads of ITS sequences were generated from each cloned PCR product using the forward primer ITS-1F (Gardes & Bruns, 1993). After ITS sequences had been analyzed, one representative partial LSU sequence from each unique ITS sequence type was generated using LR0R (Vilgalys & Hester, 1990). Cycle sequencing of amplified fragments was conducted using Big Dye Chemistry version 3.1 and visualized on an ABI3730 automated sequencer (Applied Biosystems, Foster City, CA, USA).

Sequence identification

Sequences were assembled and manually edited using SEQUENCHER version 4.2 (Gene Codes, Ann Arbor, MI, USA). Pairwise comparisons were made between all ITS sequences, which were clustered into groups that shared 97% or greater sequence similarity; these groups were considered to be the operational taxonomic units (OTUs) for this study (Parrent et al., 2006). Precautionary steps were taken to identify and remove possible PCR recombinants (chimeras) from the data set. Sequences considered at risk of being the product of PCR recombination events were those that either occurred only once (singleton) or were recovered from a single clone library, and did not closely match any known fungal (or other eukaryote) sequence across the entire length of the ITS and 5.8S gene regions. For these sequences, ITS1 and ITS2 regions were parsed and analyzed separately by BLAST (Altschul et al., 1997) searching the GenBank database, and their BLAST matches were compared to identify any apparent conflict. LSU sequences were also compared with ITS sequences to look for inconsistencies in sequence affinity between the different fragments.

Because of the large number of thelephoroid sequence types, some of which showed no close affinity to previously sequenced thelephoroid taxa, we took additional measures to rule out the possibility of having chimeras derived from closely related thelephoroid taxa disguised as novel taxa hidden in the data set. One representative of each thelephoroid sequence type from the present study and additional Tomentella and Thelephora sequences from GenBank and UNITE (Kõljalg et al., 2005) databases were imported and manually aligned using MACCLADE version 4.05 (Maddison & Maddison, 2002). Independent phylogenies for ITS1 and ITS2 were inferred using maximum parsimony implemented in PAUP 4.0b10 (Swofford, 2002) and statistical significance was assessed by bootstrap analysis. These phylogenies were inspected for topological conflicts between ITS1 and ITS2, and those sequences with incongruent topologies (≥70% bootstrap support) between these regions were considered possible chimeras. All obvious or suspected chimeras were removed from the data set. All nonfungal eukaryote sequences were also pruned from the data set before analysis. One representative of each ITS sequence group was deposited in GenBank (EF619623-EF619915).

Nutritional mode (saprotroph, EMF or pathogen) was assigned when possible to each OTU. ITS sequences with exact matches to species of known nutritional mode were assigned that nutritional category (e.g., Cenococcum geophilum = EMF). When there was no exact match for a given sequence, the phylogenetic affinity of the sequence was also considered. For example, if a sequence could not be identified to species level but could clearly be assigned to a particular genus, and the nutritional mode of that genus is constant, then the sequence was assigned to that nutritional mode.
However, if the achieved taxonomic resolution could not conclusively predict the nutritional mode of a sequence, or if nutritional status was highly variable for a particular lineage, the nutritional status of the OTU was labeled ‘unknown’.

PLFA analysis

Sand–mycelium samples were lyophilized and weighed (approx. 20 g was used in each sample), and 19 ml of the single-phase phosphate-chloroform-methanol buffer (1 : 6 : 4 : 2 volume/volume) was added to each sample. Samples were vortexed, sonicated for 2 min, and extracted according to the protocol of Bligh & Dyer (1954) as modified by White et al. (1979). Extracted lipids were then separated into neutral lipid, glycolipid, and polar lipid fractions with chloroform, acetone and methanol solvents, respectively, using silicic acid column chromatography (Guckert et al., 1985). Polar lipid fractions were esterified, and fatty acid methyl esters (FAMEs) were generated with mild alkaline methanolic transesterification (Guckert et al., 1985).

Identification and quantification of FAME compounds were performed with a gas chromatograph fitted with a flame ionization detector and a nonpolar column using methyl nonadecanoate as an internal standard (Hewlett-Packard 5890 Series 2 chromatograph). One sample from each set of extractions was also analyzed by gas chromatography/mass spectrometry to confirm peak identity (Hewlett-Packard gas 5890 Series 2 chromatograph with Hewlett-Packard 5971 mass selective detector). The concentration of the fungal-specific fatty acid 18:206 was used as a proxy for biomass.

Data analysis

For all EMF community analyses, all non-EMF taxa and taxa of unknown nutritional mode were pruned from the data set. Because half of all plots were fertilized in 2005 but data were collected from plots both before (2004) and after (2005) fertilization, sectors within a plot were aggregated to the half-plot level so that assessments of seasonal, interannual and CO2 effects could be analyzed without being confounded with possible fertilization effects. Relative frequency was calculated as mean per cent (± 1 standard error) of mycorrhizal sequences per half plot.

To examine how overall EMF community composition may be influenced by the abiotic factors of interest, nonmetric multidimensional scaling (NMS) multivariate analyses were conducted using PCORD software (McCune & Mefford, 1999). The input matrix contained the relative frequencies of taxa for each half plot in each sampling period; singletons were removed from the data set before analysis. A secondary matrix containing plot, CO2 concentration, fertilization, block, season, and year was included. To determine the optimum number of dimensions for the data set, a Monte Carlo test was conducted on an initial run of the data set with the following parameters: 200 iterations, 10 runs of real data and 20 runs of randomized data, a step-down in dimensionality from 6 to 1, and random seed starting value. The resulting graph file was then used as a starting seed for the final NMS run using the recommended three-dimensional solution determined from the results of the initial run. R2 values were determined for each of the three axes, and mean and 95% confidence intervals were calculated for CO2 and fertilized half plots and plotted along with the actual data on the graphs.

Logistic regression analysis was carried out for the six most abundant EMF taxa to assess the effects of CO2, fertilization, and season/year on their frequencies, with the binomial probability of a sample representing the taxon of interest used as the categorical response variable. Because three-way interactions are difficult to interpret, we used a stepwise approach to examine fertilization effects. First, models containing block, year, CO2, fertilization, CO2 × fertilization, and CO2 × fertilization × year parameters were analyzed. If the three-way interaction term was not statistically significant, it was removed from the model. With the simplified model, a significant fertilization × year interaction was interpreted as a fertilization effect as the fertilizer was applied between the first and second years of sampling.

Shannon–Weiner diversity indices were calculated using ESTIMATES (Colwell, 2005). Plots varied in the number of non-EMF taxa they possessed, which resulted in sample size differences among plots for the pruned EMF-only data set. In order to compare EMF richness and diversity among samples, mean rarefied richness and diversity estimates were calculated using ECO SIM (Gotelli & Entsminger, 2001). To assess the effects of CO2, fertilization, and their interaction on EMF community diversity or biomass, a split-plot analysis of variance (ANOVA) with plot nested within CO2 treatment was conducted with either rarefied richness or biomass as the response variable.

Results

Taxonomic affiliation and nutritional mode of EMM sequences

A total of 2138 fungal sequences comprising 295 taxa and representing all fungal phyla were recovered from the ingrowth bags. The majority of sequences (1840 sequences = 86.1%) belonged to Basidiomycete lineages, and comprised over half of all OTUs (162 = 54.9% of OTUs). Ninety-two OTUs (31.1%) were members of the phylum Ascomycota but they represented a much smaller fraction of the total number of sequences (236 sequences = 11%). Nine Zygomycota, nine Chytridiomycota and seven Glomeromycota OTUs were represented by 20, 11 and nine sequences, respectively. The remaining 16 OTUs, nearly all singletons (22 sequences), could not be identified to phylum level, but were unquestionably of fungal origin.
The majority of the fungal sequences (1787 = 83.5%) encompassing 134 taxa (45.4%) could be assigned to the EMF nutritional mode. Of the nonmycorrhizal taxa that could be assigned to other ecological strategies, there were 18 saprotrophs, seven arbuscular mycorrhizal fungi (AMF), six pathogens, and four taxa that shared close affinity with confirmed endophytes. Another 26 taxa belonged to lineages of known pathogens, but because they can also grow sapro-trophically they were given the more conservative designation of saprobe/pathogen. The remaining 99 taxa could not be assigned to any nutritional mode because their phylogenetic placement could not be determined, or the clade to which they belong contained multiple nutritional modes. A complete list of the identity and abundance of all sequences in this study can be found in Supplementary Material Table S1.

Fifty-nine sequences were determined to be the product of PCR recombination and were removed from the data set before any analyses. Out of the 35 thelephoroid OTUs known only from clone library sequences, six were determined to be PCR recombinants in this analysis and were removed from the data set before subsequent analysis. In every case these chimeras were represented by a single sequence that resulted from a recombination event between two abundant phylotypes that were found in multiple libraries and/or across multiple sampling dates. Therefore, while failure to detect chimeric sequences could cause overall estimates of richness to be inflated, these problematic sequences did not represent a large proportion (2.7%) of the total number of sequences in the data set.

**EMM community composition**

OTU richness was remarkably high and the shape of the rank abundance curves conformed to that of most microbial communities, with a few dominant and many rare OTUs. Overall, the eight most abundant taxa represented approx. 50% of all EMF sequences. Two *Tylospora* taxa, *Tylospora D* and *Tylospora B*, were the most commonly recovered taxa, with overall relative frequencies of 16.6 and 15.3%, respectively. The next 12 most frequently encountered taxa accounted for 30% of EMF sequences. Thirty-five taxa were recovered only once. The most abundant taxa were members of the athelioid clade, the thelephoroid clade, and the genus *Russula*.

**CO2 and N fertilization effects on EMM community structure and biomass**

The final three-dimensional solution for the NMS ordination yielded a cumulative $r^2$ of 0.743, with roughly equal amounts of variance explained by each of the three axes (incremental $r^2$: axis 1 = 0.211, axis 2 = 0.242, and axis 3 = 0.291). NMS analyses revealed overall differences in the community of EMM between ambient and elevated CO2 treatments when combined across all seasons and years, shown as separation of half plots along axis 3 (Fig. 1a). Differences were also detected between fertilized and unfertilized half plots, which also diverged along axis 3 and are most easily observed when graphed against axis 1 (Fig. 1b). This graph also highlights the interannual variability detected among half plots, indicated by separation of half plots through time along axis 1 (Fig. 1b).
Logistic regression results supported the NMS results and showed that both CO₂ and N fertilization affected the distribution of several EMF taxa as EMM. For the three taxa that could be analyzed with the full model, none showed a statistically significant effect of CO₂ × fertilization × year as assessed by likelihood ratio (LR) test; therefore, the simpler model without the three-way interaction term was used. Both *Tylospora* B and *Russula* B regression results showed a statistically significant increase in frequency in elevated CO₂ plots (*Tylospora* B, LR = 11.76, P < 0.0001; *Russula* B, LR = 6.493, P < 0.01) (Fig. 2a,c). *Tylospora* D, *Russula* G, and *Thelephoroid 1* all showed statistically significant declines in N fertilization half plots (LR = 17.56, 14.69 and 6.56, respectively; all d.f. = 1 and P < 0.01) (Fig. 2b–d).

EMM biomass, measured as the concentration of the fungal-specific fatty acid 18:2ω6, did not differ in elevated CO₂ plots relative to ambient plots (Fig. 3a). Similarly, no statistically significant biomass differences were found between fertilization treatments. In contrast to data from root tips for this site, EMF extramatrical mycelium richness was slightly greater in ambient than in elevated CO₂ plots. As with the biomass data, these results were not significantly different between treatments. Seasonal and annual variability in richness and biomass (although not statistically significant) was also detected (Fig. 3b).

**Environmental and temporal variability**

In 2005, the region in which the FACTS-I research station is located experienced severe drought conditions in the late summer and autumn seasons, reflected in reduced precipitation and soil moisture levels measured during this time (A. C. Oishi & J. S. Pippen, pers. comm.). Fungal richness and biomass from bags collected during this time were lower than in the samples of the previous year (Fig. 3a,b), although among-plot variability in these values was also large, and these differences are not statistically significant. Both *Tylospora* B and D declined in overall relative frequency in 2005 relative to 2004, but the remaining four most abundant taxa increased in frequency. However, as drought conditions progressed in
the autumn 2005 sampling periods, reductions in the abundance of these taxa were also observed.

As a result of the drought conditions in 2005, it is not possible to discern consistent seasonal patterns in both 2004 and 2005. Logistic regression results for all taxa analyzed showed significant season × year interactions, suggesting that several taxa were more abundant in autumn relative to summer in 2004, but these trends were reversed in 2005. However, if we consider only data from 2004, which did not experience severe drought conditions, there is a trend towards greater biomass and richness in autumn than in summer. The increase in autumn biomass was greatest in the elevated CO₂ plots (Fig. 3a). This pattern is driven by particularly large increases in fungal biomass in plot 4. It is interesting to note that these results are mirrored in soil respiration measurements, which also show plot 4 exhibiting the greatest soil respiration values during this period (J. S. Pippen, pers. comm.).

Discussion

The potential for CO₂ enrichment to increase mycorrhizal biomass, and thereby enhance plant nutrient acquisition, has been suggested by many researchers and has been the subject of several reviews (Tingey et al., 2000; Treseder, 2004; Alberton et al., 2005). In the present study, we measured EMM biomass with fungal-specific PLFAs in the field using the ingrowth bag method refined by Wallander et al. (2001) and found no evidence for enhanced EMM biomass in elevated CO₂ plots. Overall species richness was also not affected by CO₂ enrichment but individual taxa did show large differences in their relative frequencies between CO₂ and N fertilization treatments. In the following paragraphs we discuss the potential mechanisms responsible for these results, and the insights gained by comparing mycorrhizal community data obtained from ingrowth mesh bags to those obtained from roots.

EMM biomass and elevated CO₂

In this study we did not find a significant increase in net EMM biomass resulting from elevated CO₂. These results are consistent with the findings of Kasurinen et al. (2005), who also found no response of EMM production to elevated CO₂ conditions. However, there are a number of additional mechanisms by which mycorrhizal fungi may respond to elevated CO₂ not measured in this study that are important to consider. First, it is possible that metabolic activity may be stimulated by greater CO₂ concentrations. Data from another free-air CO₂ enrichment (FACE) experiment in Rhinelander, Wisconsin, planted with Populus tremuloides, Betula papyrifera, and Acer saccharum, found evidence for increased fungal metabolic activity in elevated CO₂ plots (Chung et al., 2006). Laboratory experiments using Pinus sylvestris seedlings colonized by Laccaria bicolor also found increased soil respiration in elevated CO₂ treatments but no change in EMM biomass (Gorissen & Kuyper, 2000). Second, CO₂ enrichment may lead to proportionally greater C allocation to structural polymers such as chitin, or to intracellular fungal C storage compounds such as mannitol and trehalose. If C partitioning is altered in such a manner, the contribution of chitin to soil C storage may be significant (Staddon, 2005).

Gross EMM production may be increased under elevated CO₂ conditions although net production remains unchanged. This could result from greater mortality or turnover rates, as was found in P. sylvestris forest soils subjected to twice ambient CO₂ conditions using open-top chambers (Kasurinen et al., 1999). Alternatively, increased grazing by mycophageous organisms such as collembola or fungivorous mites in elevated CO₂ plots, although not inside the bags themselves, could also equalize any increases in EMM biomass, shifting CO₂-induced biomass increases from EMM to higher trophic levels.

One prerequisite for increased C availability to translate to greater EMM biomass is that sufficient quantities of other key nutrients must also be available. In several studies examining fine-root and mycorrhizal responses to elevated CO₂, biomass increases were only seen when soil N availability was increased (Markkola et al., 1996; Wiemken et al., 2001), and it was concluded that nutrient limitation prevented CO₂-induced biomass enhancement. The soils in the present study are classified as low-fertility Ultic Alfisols (Schlesinger & Lichter, 2001). Declines in forest-floor N content, a lack of response of decomposition rates to elevated CO₂ despite greater C inputs (Lichter et al., 2005), and microbial extracellular enzyme production data (Finzi et al., 2006) at this site all suggest a possible increase in nutrient limitation. It is therefore possible that the fungi in this system are experiencing nutrient limitation sufficient to attenuate EMM production even though CO₂ is increased. Given that research indicates N to be more limiting relative to phosphorus (P) at this site (Finzi et al., 2004), it follows that N fertilization could initially stimulate EMM biomass by alleviating N limitation; we did not find any biomass differences in the N-fertilized half plots, but samples were taken only 1 yr after fertilization began.

EMM community response to CO₂ and N fertilization

Although no changes in EMM richness or diversity were detected, NMS ordination demonstrated EMM community-level differences between ambient and elevated CO₂ plots. Singletons were excluded from these analyses, and therefore differences observed between treatments are driven by changes in the relative frequencies of the more abundant EMM taxa. Of the six most abundant taxa, three increased in relative frequencies in elevated CO₂ plots (Tylospora B, Tylospora D and Russula B), two did not differ (Russula G and Athelioid 3), and one taxon declined (Thelephoroid 1). The few studies that have also examined CO₂ effects on EMF communities colonizing roots have found shifts in the relative abundances
of particular taxa at increased CO₂ concentrations (Godbold & Bernston, 1997; Kasurinen et al., 1999; Rygiewicz et al., 2000; Fransson et al., 2001; Parrent et al., 2006), or in conjunction with season (Rygiewicz et al., 2000). However, a large proportion of the taxa in these studies could not be identified to clade or species level, which limits our comparisons with the present study. Godbold et al. (1997) found that elevated CO₂ increased the abundance of a morphotype that produced greater amounts of EMM than the other morphotypes. Kasurinen et al. (1999) found that the dominant morphotype in their study (identity unknown) declined with increased CO₂ concentrations, and Rygiewicz et al. (2000) reported a Rhizopogon-like morphotype to be more abundant in the summer at elevated CO₂ while Cenococcum geophilum was more abundant in CO₂-enriched chambers in the winter. We also found that C. geophilum, although present at low overall frequencies, showed a positive response to elevated CO₂ (3.7% in elevated CO₂ plots vs 1.1% in ambient CO₂ plots).

The EMF community response to CO₂ at this site is not entirely consistent between hyphae and roots. For example, Tylospora B, which showed a statistically significant decline in relative frequency in elevated CO₂ plots as determined from root tips (Parrent et al., 2006), showed a small but significant increase in hyphae with elevated CO₂ (Fig. 4). This pattern may represent resource redistribution within these different compartments of the mycorrhizal fungus (fine root vs EMM), perhaps as a result of increased host nutrient demand. Alternatively, greater fine-root turnover of particular taxa could also result in lower abundances on roots in elevated CO₂ relative to EMM abundance (Hoeksema & Kummel, 2003).

Although other studies have shown that N fertilization can cause changes in EMF root tip communities in as little as 3 yr (Peter et al., 2001), and EMM biomass reductions after 10 yr of N fertilization (Nilsson & Wallander, 2003), it was striking that the effects of N fertilization on EMM composition were apparent in the first year of fertilization. After accounting for pre-existing spatial and interannual differences, three of the six most abundant taxa showed a statistically significant decline in their abundance after fertilization. Given the dominance of these taxa as foragers and as producers of hyphae in the soil, their decline – if not offset by a concomitant increase in other taxa – could ultimately result in large losses in EMM production in this system.

Contrasting views of EMF communities from hyphae and roots

Just as comparisons between fruit bodies and root tips have revealed marked differences in EMF abundances with the different sampling methods (e.g. Gardes & Bruns, 1996), notable differences are also observed between EMF communities sampled as hyphae or root tips. In our study, the athelioid, thelephoroid and russuloid clades were dominant both as root colonists and as hyphal foragers; however, the relative importance of particular taxa within these clades differs substantially between the root tip and hyphal compartments (Fig. 4, inset). χ² contingency tests (excluding samples subjected to N fertilization) showed statistically significant differences between the relative frequencies of species in EMM and EMF root tip communities (χ² statistic = 571.56, d.f. = 10, P < 0.01); this result was also found when only EMF root tip samples and EMM from the same CO₂ treatments were compared (χ² statistic: ambient CO₂ = 268.98, elevated CO₂ = 339.74; d.f. = 10 and P < 0.01 for both). Tylospora D, the most abundant forager, representing on average 20.15% of all non-N-fertilized mycorrhizal sequences, was only recovered from two root tips (Parrent et al., 2006). Furthermore, a number of taxa, such as Suillus hirtellus and Amanita rubescens, have never been recovered from roots at this site, although they are found as EMM and fruiting bodies. This may mean that these taxa colonize a small proportion of root tips, that they preferentially colonize...
root tips at greater depths in the soil but forage in the surface soil layers, or that they are simply caught as they journey to the soil surface where their mushrooms are produced.

The disparity between the distribution of taxa on fine roots and as EMM is interesting for the insight it may provide regarding the physiology of these taxa, the consequences for plant nutrient acquisition, and the nature of the mycorrhizal mutualism. An increasing number of studies are also using molecular methods to examine the mycorrhizal community from the hyphal perspective, either by directly assaying mycelium in soil (e.g. Landeweert et al., 2003a; Koide et al., 2005) or using the ingrowth bag method that we have used in this study (Wallander et al., 2003; Kjoller, 2006; Korkama et al., 2006), and some general trends for particular groups are beginning to emerge. Thel ephemroid and athelioid taxa are both frequent and abundant as EMM, although thelephoroid richness appears to be much greater than that of the athelioid group (Wallander et al., 2003; Kjoller, 2006; Korkama et al., 2006). Consistent with results obtained in the present study (Fig. 4), Kjoller (2006) found boletoid taxa to be more abundant, and Russula and Cortinariaceae taxa less abundant as EMM relative to roots. These results complement observations regarding exploration types of different EMF taxa made by Agerer (2001).

Methodological considerations

Clone library construction followed by subsequent sequencing is an efficient and powerful technique for examining soil microbial communities. However, although measures can be taken to minimize PCR recombination, a great deal of caution must be exercised when annotating and enumerating sequences using this approach. This is particularly true for taxonomic groups that are extremely diverse, for which taxonomic coverage is poorly represented in sequence databases, or that have received little attention from taxonomic experts. In these instances, comparison of the topological congruence of phylogenies derived from sequence fragments that flank known recombination hotspots (such as 5.8S) can be a useful approach for detecting chimeras.

Additional concerns with clone library/sequencing approaches warrant caution when using such data sets in a quantitative manner. There is uncertainty in the degree to which sequence abundance and species abundance are correlated, and a lack of true independence for sequences generated from a single clone library. These factors are influenced both by biological factors, such as species variation in nuclear abundance per unit biomass and the length of the amplified fragment, and by methodological factors, such as the choice of the locus to target and PCR conditions. Despite these concerns, there are several justifications for using clone libraries in the present study to estimate species frequencies rather than discarding these data and taking a more conservative presence/absence only approach. First, overall frequencies of the species for the entire site correlated very well with their presence/absence from individual subsamples. Secondly, there were high degrees of diversity detected within individual samples, suggesting that PCR biases did not dominate the outcome. Thirdly, results from a study using methods similar to those used in this work found that ITS clone libraries generated from fungal communities performed reasonably well in approximating species frequencies and did not exhibit phylogenetic bias (Taylor et al., 2007). Overall, we feel that this is a useful way to survey an extremely diverse community and to quantitatively assess the relative importance of different taxa, particularly if sites are sampled multiple times and if many, independently derived clone libraries are constructed and sequenced for a given site. However, changes in species frequencies over space or time observed in studies of microbial community structure should be viewed as hypotheses that warrant further and more direct testing, such as by quantitative PCR (Landeweert et al., 2003b).

The ingrowth bag method refined by Wallander et al. (2001) has greatly improved the feasibility of studying EMM in the field, but like any method it is not without its shortcomings. While the majority of fungal sequences (83.5%) generated from ingrowth bags in this study were ectomycorrhizal, nonmycorrhizal fungi were not entirely eliminated. Although non-EMF taxa represented a minority of sequences, approximately half of all OTUs recovered from bags were either non-EMF or were taxa that could not be assigned to a nutritional mode. This was particularly true with the ascomycete taxa belonging to the orders Pezizales and Helotiales, highlighting the need for greater attention to be paid to these groups, both taxonomically and ecologically (Chao et al., 2005; Tedersoo et al., 2006). Sand may reduce nonmycorrhizal colonization of ingrowth bags, but some EMF taxa may also avoid colonizing sand, which could result in lower EMM colonization levels than if a different substrate was used. Hendricks et al. (2006) found that bags filled with sand contained lower hyphal biomass than those buried in the same site that were filled with sterile, native soil, although molecular data are needed to assess the degree to which non-mycorrhizal colonization was also enhanced. Even if sand-filled bags reduce absolute EMM colonization, they are still useful for comparing EMM biomass across sites and treatments. On the whole, this method shows great promise for enabling researchers to move beyond roots and begin to examine the distribution, abundance, and function of mycorrhizal fungal hyphae.

Acknowledgements

Funding for this project was provided by National Science Foundation Doctoral Dissertation Improvement Grants DEB-0408079 to JLP and W. F. Morris, MCB-00-84207 to RV, and DEB-0235425 to R. B. Jackson. The Department of Energy and Electric Power Research Institute and Brookhaven...
National Laboratory provide site support and maintenance. We gratefully acknowledge T. A. Mao for field and laboratory assistance and D. C. White and A. D. Peacock for laboratory and technical assistance with PLFA analyses. We thank A. C. Oishi and J. S. Pippen for providing soil moisture and soil respiration data. Many thanks to W. F. Morris and A. F. S. Taylor along with three anonymous reviewers for their extremely helpful comments on this manuscript.

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**Supplementary Material**

The following supplementary material is available for this article online:

**Table S1** List of all unique operational taxonomic units (OTUs) generated from ingrowth mesh bags in this study. Taxonomic identity and classification, nutritional mode, number and location of occurrences are indicated for each OTU

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1469-8137.2007.02155.x

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