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CO_2-ENRICHMENT AND NUTRIENT AVAILABILITY ALTER ECTOMYCORRHIZAL FUNGAL COMMUNITIES

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Abstract. Ectomycorrhizal fungi (EMF), a phylogenetically and physiologically diverse guild, form symbiotic associations with many trees and greatly enhance their uptake of nutrients and water. Elevated CO_2, which increases plant carbon supply and demand for mineral nutrients, may change the composition of the EMF community, possibly altering nutrient uptake and ultimately forest productivity. To assess CO_2 effects on EMF communities, we sampled mycorrhizae from the FACTS-I (Forest–Atmosphere Carbon Transfer and Storage) research site in Duke Forest, Orange County, North Carolina, USA, where *Pinus taeda* forest plots are maintained at either ambient or elevated CO_2 (200 ppm above ambient) concentrations. Mycorrhizae were identified by DNA sequence similarity of the internal transcribed spacer ribosomal RNA gene region. EMF richness was very high; 72 distinct phylotypes were detected from 411 mycorrhizal samples. Overall EMF richness and diversity were not affected by elevated CO_2, but increased CO_2 concentrations altered the relative abundances of particular EMF taxa colonizing fine roots, increased prevalence of unique EMF species, and led to greater EMF community dissimilarity among individual study plots. Natural variation among plots in mean potential net nitrogen (N) mineralization rates was a key determinant of EMF community structure; increasing net N mineralization rate was negatively correlated with EMF richness and had differential effects on the abundance of particular EMF taxa. Our results predict that, at CO_2 concentrations comparable to that predicted for the year 2050, EMF community composition and structure will change, but diversity will be maintained. In contrast, high soil N concentrations can negatively affect EMF diversity; this underscores the importance of considering CO_2 effects on forest ecosystems in the context of background soil chemical parameters and other environmental perturbations such as acid deposition or fertilizer runoff.

Key words: biodiversity; CO_2; DNA sequencing; ectomycorrhizal fungi; forest productivity; Free Air CO_2 Enrichment (FACE); global change; internal transcribed spacers (ITS); mutualism.

INTRODUCTION

Atmospheric CO_2 concentrations are predicted to continue rising over this century (Cubasch et al. 2001). The degree to which this increase may be mitigated by increased carbon storage in terrestrial ecosystems will depend in part upon whether enhanced plant productivity, which has been observed in short-term experiments (e.g., Hamilton et al. 2002), will continue. In turn, as many forest ecosystems in temperate climates are perched upon relatively infertile soils, the ability of plants to acquire adequate nutrients is likely to be a major determinant of whether, or for how long, CO_2-enhanced productivity can be sustained.

Many forest tree species rely heavily upon their ectomycorrhizal fungal symbionts to obtain sufficient amounts of nutrients, especially nitrogen and phosphorus. Ectomycorrhizal fungi (EMF) form symbiotic organs with the fine roots of many conifer and angiosperm tree species (Smith and Read 1997). EMF receive photosynthetically derived carbon from host plants; an estimated 20% of the total carbon fixed by host trees is allocated to their fungal symbionts (Finlay and Söderström 1992), though values above 80% have been reported (Allen 1991). In exchange, EMF enhance plant nutrient uptake in a number of ways, especially with respect to nitrogen and phosphorus acquisition.

Despite the importance of EMF in improving plant nutrient status and their potential role in maintaining forest productivity under increased atmospheric CO_2 conditions, few large-scale field studies have examined the effects of elevated CO_2 on the EMF community (but see Fransson et al. 2001). Consequently, it is difficult to predict how particular EMF community attributes, such as species richness, will respond to CO_2 enrichment. On one hand, C fertilization may allow plants to grow more fine roots, increasing the resource base for EMF and allowing more fungal species to coexist. However, richness responses to increased productivity are not always positive, and depend upon initial conditions and the degree of productivity enhancement (Mittelbach et al. 2001). Communities in highly productive systems are also predicted to be more invasible than those in less

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productive sites (see review by Levine and D'Antonio 1999). If this is also true for EMF communities, greater productivity accompanying C fertilization may destabilize EMF communities, leading to enhanced community dissimilarity between fertilized plots. The goal of this study is to investigate changes of EMF composition and diversity in response to elevated CO_2 so that we may arrive at specific hypotheses for future testing.

While CO_2 effects remain largely unexplored, soil nutrients are known to affect EMF community composition and diversity. In particular, increased soil nitrogen (N) concentrations are often correlated with changes in a number of EMF community attributes, such as decreased fruiting body production, lower community diversity, and shifts in the relative frequencies of EMF community members (see review by Lilleskov 2005), due in part to variation among EMF taxa in their physiological abilities to utilize different N-containing compounds. We expect any underlying variation in N availability will alter the relative frequencies of EMF taxa. Therefore, to understand the relative importance of CO_2 enrichment for EMF, we must test for its effects against the background of known predictors of EMF community characteristics, such as N availability.

Two major barriers to characterizing EMF communities are their speciose nature and the difficulty in distinguishing species on roots using solely morphological methods. Instead, molecular approaches either alone or in conjunction with morphological typing (morphotyping) are necessary to better characterize EMF communities. An additional challenge is that EMF species often have patchy distributions (see Horton and Bruns 2001, Lilleskov et al. 2004), resulting from variation among species in genet size, mode of dispersal, and soil physical and chemical properties. Preexisting heterogeneity among experimental units may make it more difficult to detect CO_2-related changes in the spatial distributions of EMF species. Both direct and indirect effects of elevated CO_2 on forest ecosystems, such as increasing fine root production and shifting competitive dominance of EMF taxa, respectively, may increase or decrease spatial heterogeneity of EMF communities exposed to increased atmospheric CO_2 concentrations. Thus, it is important to consider EMF spatial heterogeneity in both ambient and elevated CO_2 treatments.

In this study we used the FACTS-I (Forest–Atmosphere Carbon Transfer and Storage) experiment to examine the effects of CO_2 on the EMF community associated with *Pinus taeda* (loblolly pine, Pinaceae), an obligately ectomycorrhizal host tree species. FACTS-I is equipped with free air CO_2 enrichment (FACE) technology (described in Hendrey et al. 1999), which allows intact portions of forest to be enriched in atmospheric CO_2. We sampled mycorrhizae from control and elevated CO_2 plots, and used a DNA sequence-based identification approach to examine how CO_2 enrichment changes whole-community attributes as well as individual EMF species abundance. To assess the degree of EMF spatial heterogeneity, we compared EMF community similarity of subplots within vs. between plots. Because these plots also vary in a number of environmental factors, including N availability (Finzi et al. 2002, Finzi and Schlesinger 2003), we evaluated the influence of elevated CO_2 within the context of these other environmental variables. This is the first study to examine EMF community response to CO_2 in an intact forest environment using an entirely sequence-based approach for identifying EMF taxa, quantifying EMF richness, and documenting differences in the relative abundance of individual taxa.

Materials and Methods

Field site

Samples for this study were collected from the FACTS-I research site in Duke Forest, Orange County, North Carolina, USA (see Plate 1). These plots are located in an even-aged *P. taeda* stand that was planted in 1983. The soil at this site is an Ultic Alfisol in the Enon Series, which is somewhat acidic, N- and P-limited, and rich in clay (Schlesinger and Lichter 2001). CO_2 enrichment began in August 1996. Since that time, three circular, 30 m diameter experimental plots have been fumigated to achieve CO_2 concentrations 200 ppm above ambient (i.e., 565 ppm, or 1.5× current levels). Three control plots have been maintained at ambient CO_2 concentrations. Each plot is further subdivided into eight sectors. Four sectors are used for belowground sampling, and four sectors are used exclusively for aboveground vegetation studies. There are notable differences among plots in soil C and N pools (Schlesinger and Lichter 2001), and in N availability and potential net N mineralization rates determined from data collected in 1997–2001 and reported by Finzi and colleagues (2002, 2003), where methodological details can be found. Due to the considerable differences among plots, the six plots have been grouped into low, medium, and high N pairs. Additional details regarding FACE technology and site characteristics can be found in Hendrey et al. (1999) and Schlesinger and Lichter (2001).

Field sampling

Two sampling methods, fruiting body collections and mycorrhiza sampling (see Plate 1), were employed to survey the EMF community. Fruiting body surveys were conducted as part of the Duke Forest Mycological Observatory project, the goals of which included generating a database of DNA sequences from fruiting bodies to facilitate subsequent identification of DNA isolated from mycorrhizae or other environmental samples. Five surveys were conducted between fall 2000 and fall 2001. Representative fruit bodies of each species were collected from all plots, identified, photographed, and vouchered in the Duke University

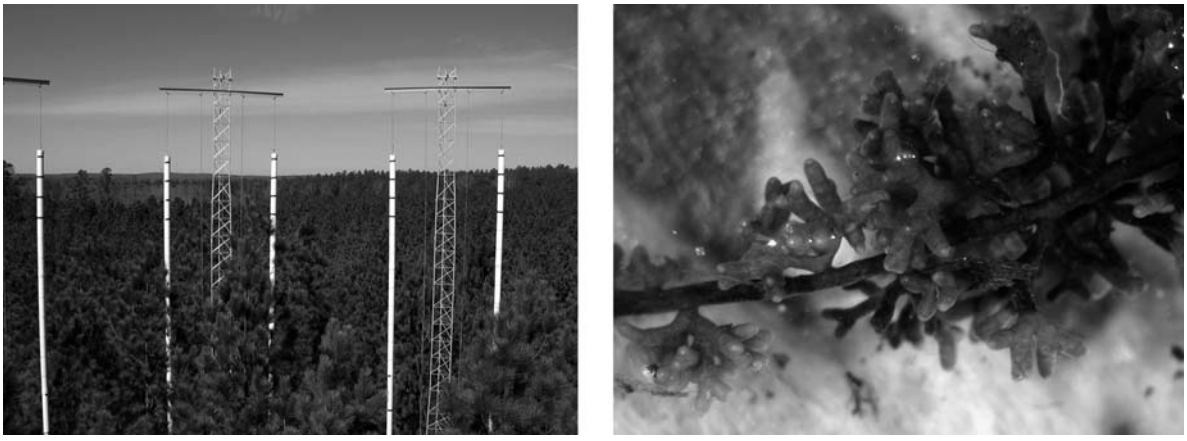

PLATE 1. (Left) Canopy view of the FACTS-I research site in Duke Forest, NC, taken from one of the plot's central towers. For several plots, the FACE system's circular array of suspended vent pipes responsible for delivering CO_2 to intact portions of forest can be seen. Photo credit: R. Vilgalys. (Right) Cluster of *Pinus taeda* mycorrhizae collected from the FACTS-I experimental research site. Photo credit: J. L. Parrent.

Cryptogamic Herbarium (collection and DNA sequence data *available online*).[2]

Mycorrhizal root tips of *P. taeda* were collected in July 2002. In order to sample extensively while minimizing disturbance to the site, sampling relied on excavation of individual fine roots of *P. taeda*. Twenty-five roots were collected in each soil sector at the intersection points of a grid with a cell size of 2×2.5 m, for a total of 100 fine roots sampled from every plot. Samples were placed on ice in individual bags and returned to the laboratory. Roots were gently rinsed with deionized water, and two root tips were chosen at random from each root (one for immediate analysis and one to be stored at −20°C for use if needed). Root tip turgidity, morphology, and color characteristics as well as the absence of fine root hairs were assessed using a stereomicroscope for each sample to confirm that they were alive and colonized. Each live root tip was then placed in a microcentrifuge tube containing 250μL 2X CTAB buffer (Zolan and Pukkila 1986) pending DNA extraction.

Molecular methods and EMF identification

DNA was extracted from each mycorrhizal sample according to the protocol of Zolan and Pukkila (1986). For each DNA sample, the ribosomal internal transcribed spacers (ITS) and 5.8S ribosomal RNA gene were amplified by PCR and sequenced. For PCR amplification, the fungal-specific forward primer ITS 1F (Gardes and Bruns 1993) was used with a basidiomycete-selective reverse primer that we have designed, ITS 4NA (5′-CTTTTCATCTTTCCCTCACGG-3′). Cycle sequencing of amplified fragments was conducted using Big Dye version 3.1 and visualized on an ABI3700 automated sequencer (Applied Biosystems, Foster City, California, USA). Sequences were assembled and manually edited using Sequencher version 4.2 (Gene Codes, Ann Arbor, Michigan, USA). We used basidiomycete-selective primers in this study because a large proportion of roots were infected with endophytic and/or pathogenic ascomycetous fungi, resulting in mixed PCR products when universal fungal primers were used, making DNA sequencing of these products impractical. Consequently, ascomycete EMF, which account for <5% of EMF species on *P. taeda* at this site (J. L. Parrent, *unpublished data*), were not included in this study.

Pairwise comparisons were made between all sequences, which were then clustered together based on percentage similarity. Several factors must be taken into consideration when assembling fungal ITS sequences into clusters based on sequence similarity: (1) inherent intraspecific genetic variation, which is common in large, sexually outcrossing populations (Kauserud and Schumacher 2003); (2) variation among ITS repeats within an rDNA array or between arrays within an individual (Ko and Jung 2002); and (3) variation caused by PCR error (Keohavong and Thilly 1989). Allowing for these sources of expected variation, 97% or greater sequence similarity was considered to define a phylotype, or operational taxonomic unit. To determine the genus-level or, where possible, the species-level identity of each phylotype, sequences were queried against the GenBank database using BLAST (Altschul et al. 1997) and the database of ITS sequences derived from fruiting bodies previously described. The same 97% sequence similarity criteria was used for assigning a species name to a phylotype, and a combination of similarity and phylogenetic analyses was used to classify to genus samples that we were unable to assign to a known, previously sequenced species. One representative sequence of each phylotype was deposited in Genbank (accession num-

[2] ⟨www.biology.duke.edu/fungi/mycolab/DFMO.html⟩

bers DQ377369–DQ377440). Appendix C provides additional details regarding phylotype determination from ITS sequence data.

Data analysis

Shannon-Weiner and Simpson diversity indices were calculated for each plot using EstimateS (Colwell 2005). Total species richness was inferred using the nonparametric abundance-based coverage estimator (ACE; Chao et al. 2000). Due to differences among plots in the number of samples successfully sequenced, diversity estimates were also computed by rarefying samples from each plot to the number in the plot with the smallest sample size, and calculating the mean and 95% confidence intervals of Shannon Wiener diversity from 1000 replicates using EcoSim (Gotelli and Entsminger 2001). Assumptions for normality were met (Shapiro-Wilk W, $P > 0.05$) and t tests were performed to compare mean richness and diversity between ambient and elevated CO_2 plots for actual and rarefied data. Linear regressions were also conducted using mean potential net N mineralization as the explanatory variable and either rarefied richness or diversity as the dependent variables.

We tested whether CO_2 enrichment might influence the degree of spatial heterogeneity in the EMF community by using similarity indices based on presence–absence data (Jaccard's, Sorenson's, and Chao Jaccard's; Chao et al. 2005), and abundance data (Bray-Curtis) as implemented in EstimateS (Colwell 2005). All incidence-based indices were consistent in sign as well as significance; therefore only Chao Jaccard's index is presented as a representative analysis. Pairs of sectors were compared within vs. between plots to assess local vs. larger scale similarity. A one-way analysis of variance followed by Student's t means comparison was performed with similarity either within or among plots as the response variable. When necessary, similarity indices were arcsine square-root transformed to satisfy conditions of normality prior to analysis. For the Bray-Curtis similarity index, normality was not achieved by either arcsine square-root or logit transformation, so significance was assessed with nonparametric Wilcoxon comparison among means and Tukey-Kramer hsd analyses.

A generalized linear model (GLM) with a logit link function and Poisson error distribution was used to analyze the relative abundances of all EMF species to determine whether there was a significant effect of CO_2, block (which represents differences in mean potential net N mineralization rates), or a $CO_2 \times$ block interaction. Fisher's exact tests were conducted for the four most abundant taxa to compare their frequencies between ambient and elevated CO_2 plots. Significance values were adjusted using the Bonferroni correction to account for multiple comparisons. GLM analysis was performed using R version 2.0.1 (R Development Core Team 2005). All other analyses were carried out using JMP version 5.1 (SAS Institute 2003).

Results

EMF community composition

From mycorrhizal collections, we successfully amplified and sequenced 411 (52%) of the samples, yielding 72 basidiomycete EMF phylotypes (Appendix B). Eighty-three percent of unsuccessful samples either failed to amplify or resulted in mixed products; the remaining failed samples were failed sequences, and 11 samples were putative saprophytes and were excluded from the analyses. Given such high phylotype richness, accumulation curves did not reach saturation for ambient or elevated CO_2 treatments (Appendix A), but nonparametric richness estimators predict that approximately two-thirds of EMF taxa in these plots were sampled (observed richness, ambient = 43, elevated = 51; ACE-predicted richness, ambient = 65, elevated = 73). Consistent with other EMF community studies, a few common and many rare taxa were encountered, with 24 phylotypes (33.3%) detected only once. Only three of the 72 taxa encountered were unidentifiable below phylum. Of the remaining 69, 42 belonged to 14 distinct genera, and the remaining 27 were assigned to one of four distinct clades, the thelephoroid clade, athelioid clade, cantharelloid clade, or Sebacinaceae (Appendix B).

The three most abundant groups of basidiomycete EMF taxa were members of *Russula*, *Tylospora*, or the thelephoroid clade (Appendix B). Eleven ITS phylotypes were assigned to the genus *Russula*, which made up 37.7% of all samples. *Tylospora* was a much less speciose genus, with only two taxa, but they represented 11.2% of root tip sequences. The thelephoroid clade contained by far the greatest richness of all lineages in this study with 22 distinct phylotypes. However, most of these taxa were rare; only 13.4% of all samples belonged to the thelephoroid clade, but together they comprised 30.6% of total phylotype richness.

Magnitude of EMF community response to spatial effects and nutrients

Spatial factors.—If EMF community composition and structure show substantial spatial heterogeneity, similarity between pairs of sectors within a plot should be greater than between sectors from different plots. For the incidence-based similarity (Chao Jaccard's) we found overall similarity was higher between sectors within plots than between sectors in different plots (mean $\pm$ SE within plots = 0.75 $\pm$ 0.02; mean $\pm$ SE between plots = 0.68 $\pm$ 0.008; $t = 1.97$, $P < 0.01$). We found no statistical difference in similarity within plots vs. between plots when only ambient CO_2 plots were considered (Fig. 1). However, mean community similarity between elevated CO_2 plots was significantly lower than either ambient or elevated within-plot similarity (Fig. 1; $t = 1.98$, $P < 0.01$ for both comparisons). This result is consistent with the obser-

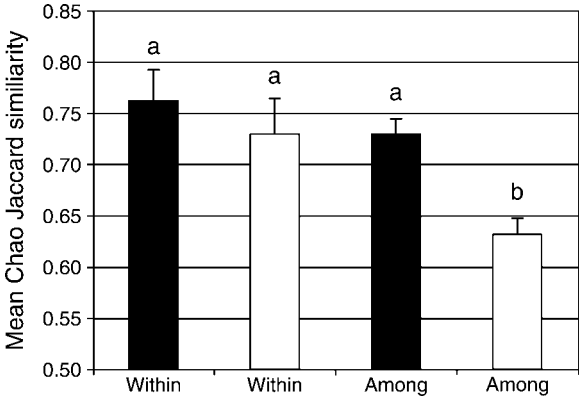

FIG. 1. Chao Jaccard's community similarity indices of sectors within vs. among plots (mean + SE; $n = 18$ for within-plot comparisons; $n = 48$ for between-plot comparisons). Filled bars are ambient CO_2 levels; open bars are elevated CO_2. Bars that do not share a common letter are significantly different (t tests, $\alpha = 0.05$).

vation that there is a greater proportion of unique species, species found in only one plot regardless of its frequency within that plot, in elevated CO_2 plots relative to control plots (Table 1). When abundance-based indices are considered (Bray-Curtis), between-plot similarity is lower than within-plot similarity for both ambient and elevated CO_2 treatments (data not shown). The different results for incidence-based vs. abundance-based indices suggest that under control but not elevated CO_2 conditions, EMF communities were relatively homogeneous with respect to community composition at the scales examined here, but differences in the relative abundances of EMF taxa drive declining similarity at larger spatial scales for both ambient and elevated CO_2 conditions.

Nitrogen availability.—For the community as a whole, there was a strong negative linear relationship between both community diversity and richness with mean potential net N mineralization rates (Fig. 2a, b). The GLM that best fit the EMF relative abundance data included a block × phylotype interaction term. Because block is strongly correlated with net N mineralization rates as well as other measures of N content in the plots (soil and foliar N content, microbial biomass N; Finzi et al. 2002), this suggests the abundances of EMF taxa vary in the direction and magnitude of their correlation with N availability depending upon phylotype identity. Of the 25 taxa that occur in two or more plots, 11 increased and 14 decreased in relative frequency in plots with increasing net N mineralization rates. When the four most abundant taxa (*Russula* G, *Tylospora* B, *Russula* B, and *Cortinarius mucosus*) are examined, differences in potential net N mineralization appear to be of as great or greater importance than CO_2 treatment in influencing their relative frequencies (Fig. 3a–d). *Russula* G, which showed no difference in mean relative frequency between control and elevated CO_2 plots, increased dramatically from 9% to 40% in plots with increased mean net N mineralization rates (Fig. 3a). Similarly, *Tylospora* B increased in relative frequency with increasing net N mineralization plot values regardless of CO_2 treatment, though relative frequency is consistently higher in the ambient CO_2 plots (Fig. 3b). In contrast, *Cortinarius mucosus* decreased in relative frequency as net N mineralization increased under both CO_2 treatments (Fig. 3d).

Other environmental variables.—In addition to spatial proximity and nutrient characteristics of the plots, we also considered the potential influence of other abiotic and biotic factors on EMF community dynamics. Although tree biomass in these plots is dominated by *P. taeda*, other woody species, some of which are EMF hosts, are present (J. S. Pippen and W. Cook, *unpublished data*). Although we only sampled *P. taeda* fine roots in this study, the presence and abundance of other EMF host species could indirectly influence EMF community richness and composition. However, EMF community richness was not significantly correlated with tree species richness, host abundance, or the proportion of EMF host trees above 2.5 cm diameter at breast height (tree richness, $P = 0.30$; EMF host tree abundance, $P = 0.89$; proportion EMF hosts, $P = 0.77$). We also found no statistically significant effect of mean soil moisture (average daily readings 1997–2002) on EMF community richness ($P = 0.14$). EMF diversity was similarly unaffected.

CO_2 effects

EMF richness and diversity.—Neither mean EMF richness nor Shannon-Weiner diversity differed significantly with regard to CO_2 treatment when assessed at the plot level (rarefied richness, $P = 0.46$; rarefied diversity, $P = 0.86$; Table 1). However, individual EMF taxa varied in their response to CO_2 concentrations. The most abundant phylotype, *Russula* G (overall frequency = 20.2%), was present and abundant in every plot, with nearly identical frequencies in ambient and elevated CO_2 plots ($P = 1.0$; Fig. 4). In contrast, other common taxa had a significantly lower (*Tylospora*, $P = 0.0004$) or higher (*Russula* B, $P = 0.0062$) mean relative frequency in the ambient CO_2 plots relative to plots with elevated CO_2 (Fig. 4).

DISCUSSION

We found that elevated atmospheric CO_2 concentrations did not affect EMF community richness and diversity. However, increased CO_2 levels did alter the relative abundances of EMF taxa colonizing fine roots, increasing the prevalence of unique EMF species and leading to greater EMF community dissimilarity between individual study plots. In addition to EMF community changes caused by elevated CO_2, natural variation among plots in mean potential net N mineralization rates was a key determinant of EMF community structure. Increasing rates of net N miner-

TABLE 1. EMF community response to elevated CO_2.

Parameter	Ambient CO_2 plots				Elevated CO_2 plots			
	1	5	6	Mean (SE)	2	3	4	Mean (SE)
No. samples	50	64	81	65 (9.0)	70	73	73	72 (1.7)
Phylotype richness	18	17	26	20.3 (2.8)	25	17	24	22 (2.5)
Mean rarefied richness		14.7 (12.2, 17.1)†	20.6 (17.5, 23.8)†	17.8 (1.7)	21.7 (19.0, 24.4)†	15.0 (12.6, 17.2)†	20.3 (17.5, 23.1)†	19.0 (2.0)
Unique species (%)	33.3	29.4	30.7	31.1 (1.1)	32	47.1	45.8	41.6 (4.8)
Rarefied Shannon diversity		2.16 (2.0, 2.3)†	2.68 (2.5, 2.9)†	2.48 (0.16)	2.85 (2.7, 2.9)†	2.09 (1.9, 2.3)†	2.66 (2.5, 2.8)†	2.53 (0.20)

Notes: Richness and diversity data were rarefied to a samples size consistent with plot 1, the plot with the smallest number of samples.

† Numbers in parentheses are 95% CL.

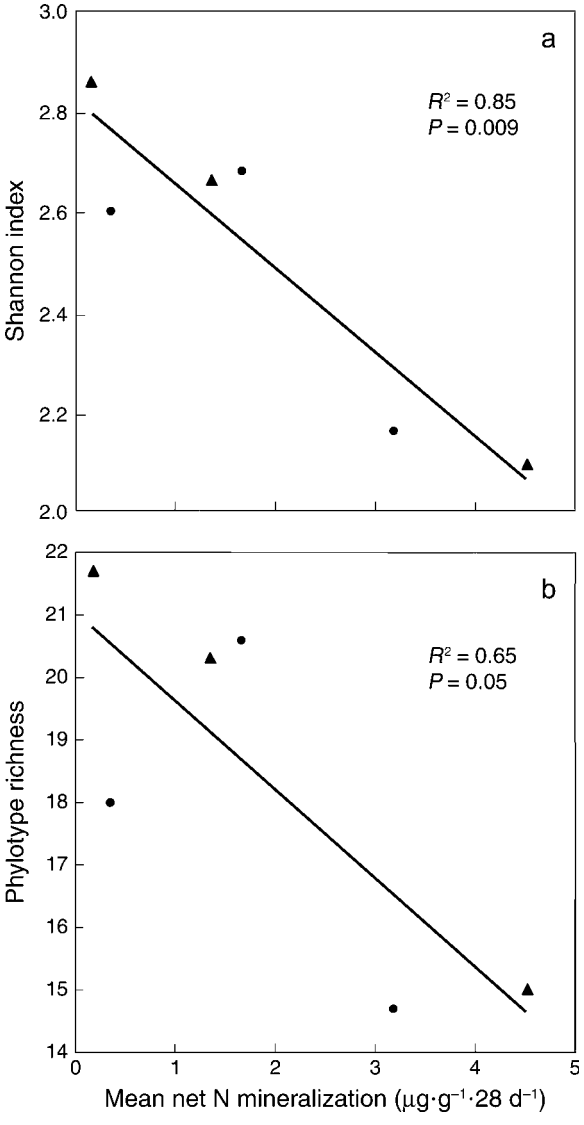

FIG. 2. Linear regression of (a) rarefied Shannon index and (b) rarefied phylotype regressed against mean potential net nitrogen mineralization rates for years 1997–2001 (Finzi et al. 2003). Each point on the graph represents the plot value. Triangles are elevated CO_2 plots; circles are ambient CO_2 plots. Data were rarefied to a sample size consistent with plot 1, the plot with the smallest number of samples.

alization were negatively correlated with EMF richness and diversity, and net N mineralization rates had variable effects on the abundance of different EMF taxa. We discuss these results in the context of relevant EMF community studies, and the implications of a CO_2-enriched atmosphere for plant productivity and the mycorrhizal mutualism.

Effects of CO_2 on EMF community diversity and composition

Due to increases in fine root biomass and productivity (Allen et al. 2000, Matamala and Schlesinger 2000), and carbon availability, more colonizable sites and resources, respectively, are available to the EMF community in elevated CO_2 plots. Because there is often a positive relationship between productivity and diversity, especially at low to intermediate productivity levels, we posited that C fertilization could lead to greater EMF community diversity. Although there was a trend toward greater diversity in elevated CO_2 plots, the differences were not statistically significant. There are a number of plausible explanations as to why diversity did not increase. First, the relationship between productivity and diversity may not be strictly positive, but rather unimodal or "hump-shaped," as has been shown in other systems (Mittelbach et al. 2001). Second, increased fine root production or carbon availability may not be sufficiently large to have a significant impact on EMF community diversity. Finally, biotic factors, such as host plant preference, or variation in competitive and/or dispersal abilities among EMF taxa may prevent all EMF species from benefiting equally from greater productivity.

Resource limitation via low productivity or complete resource utilization is often suggested as an important mechanism by which communities gain greater resistance to invasion (reviewed in Levine and D'Antonio 1999). In this system, C fertilization resulted in an increase in fine root productivity, suggesting a potential for C enrichment to increase the ability of novel EMF taxa to invade (Davis et al. 2000). We tested this prediction by comparing EMF community similarity and percentage of unique EMF species between CO_2 enriched plots. In

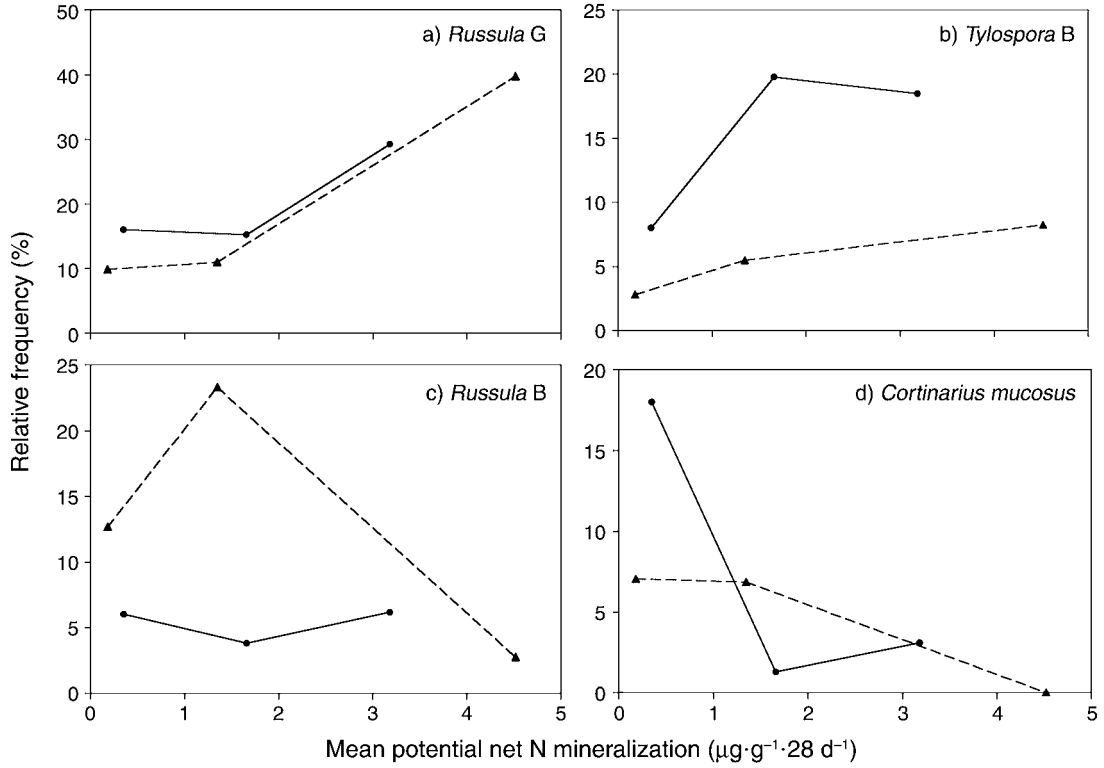

FIG. 3. Relative frequency (%) vs. mean potential net N mineralization rate in each plot for the four most abundant EMF phylotypes: (a) *Russula* G; (b) *Russula* B; (c) *Tylospora* B; and (d) *Cortinarius mucosus*. Filled circles connected by solid lines are the three ambient CO_2 plots; filled triangles connected by dashed lines are elevated CO_2 plots.

control plots, we detected no significant differences between Chao Jaccard's similarity indices measured within plots and between plots, suggesting there was no significant preexisting spatial heterogeneity in composition. In contrast, sectors from different elevated CO_2 plots were significantly less similar than sectors of different control plots. This increase in community dissimilarity was due to elevated CO_2 plots containing substantially greater proportions of unique phylotypes than in ambient plots. Also, one of the most abundant species, *Tylospora* B, showed a statistically significant decline in relative frequency in elevated CO_2 plots. These results support the prediction that disturbances leading to increased productivity and declines in the abundance of competitively dominant species may enhance EMF community invasibility, though the identity of invading species may vary from plot to plot.

The importance of nitrogen availability on EMF communities

Free air CO_2 enrichment technology provided a unique opportunity to study EMF community change resulting from elevated CO_2 in a realistic environment, which includes variation in N availability, potentially an important determinant of EMF species distributions and abundances. Mean net N mineralization rate was negatively correlated with EMF community richness. The relative frequency of both *Russula* G and *Tylospora* B increase, and both *Russula* B and *C. mucosus* decrease in plots where mean net N mineralization rates are increased. Other studies have found certain *Cortinarius* and *Russula* species decline where N increases (Peter et al. 2001, Lilleskov et al. 2002*a*), and *Tylospora* species abundance have been shown to either increase (*T. asterophora*; Peter et al. 2001) or not differ (*T. fibrillosa*; Lilleskov et al. 2002*a*) between plots with greater N concentrations relative to low N sites. These results also suggest that certain lineages may show a great degree of variation among closely related species in their physiological tolerance to various soil chemical properties, limiting our ability to predict the response of some species to environmental variation given the response of congeners to the same environmental factors.

Given the large increase in relative frequency of *Russula* G to >40% of all samples and complete absence of *Cortinarius mucosus* in the "high N" plot, it remains uncertain whether the changes in relative frequency of EMF phylotypes with variation in N are a direct effect of N itself; whether they stem from other more complicated indirect effects, such as shifts in the competitive abilities of particular EMF taxa or host plant response to increased N levels; or whether both EMF communities and N availability are influenced by additional unmeasured factors. It is also plausible that

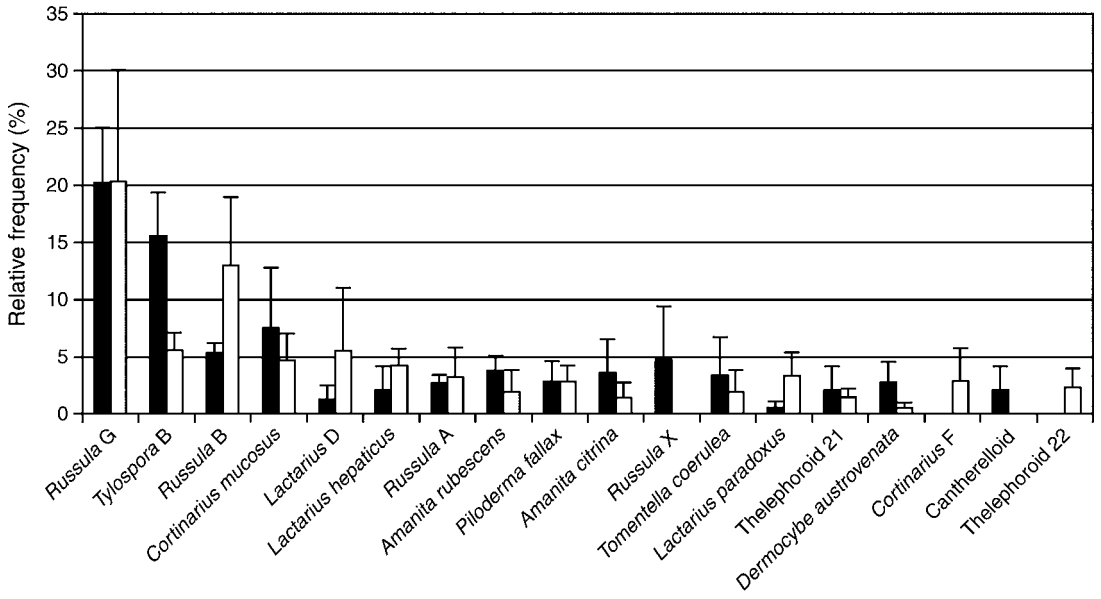

FIG. 4. Relative frequency (%; mean + SE) of EMF phylotypes comprising 1% or more of the total number of EMF samples. Filled bars are ambient CO_2 plots; open bars are elevated CO_2 plots.

EMF themselves influence (rather than respond to) differences in net N mineralization rates in the field. However, estimates of potential net N mineralization ratios used in this study's analyses were generated from laboratory incubations conducted with sieved, root-free soil and are therefore unlikely to reflect the EMF contribution to these rates. Additional studies examining the effects of N concentrations on individual taxa in the absence of other EMF species would help to disentangle the potential indirect effects of competition with other EMF taxa from the direct effects of N concentration.

Implications for forest productivity and the mycorrhizal mutualism

Plots subjected to increased atmospheric CO_2 concentrations have sustained increases in NPP at this site without significant increases in plant C:N ratios or other signs of nutrient limitation (Finzi and Schlesinger 2003, Zak et al. 2003), at least through the time that our study was conducted (but see Oren et al. 2001). The EMF community may be playing an important role in the acquisition and translocation of sufficient amounts of nutrients needed to sustain increased productivity, perhaps delaying the predicted decline in forest productivity resulting from nutrient limitation. Previous studies have found that EMF species can vary dramatically in their ability to access and translocate different forms and amounts of inorganic and organic nutrients to their plant hosts (Abuzinadah and Read 1986, 1988, Lilleskov et al. 2002*b*). Given the heterogeneity in nutrient acquisition abilities of different EMF taxa, maintaining associations with many EMF taxa in environments with lower soil inorganic N content may allow plants to access a wider range and greater amount of N-containing compounds. Thus, maintenance of EMF richness and diversity at this site may facilitate greater productivity, or at least mitigate the onset of nutrient limitation under increased CO_2 concentrations.

In this study we observed changes in the relative frequency of a number of EMF taxa with elevated CO_2. The question remains as to whether these shifts in community structure serve to maintain, or perhaps enhance, the benefit conferred to these trees by their mycorrhizal symbionts. It is currently unknown to what degree an individual plant host may selectively associate with specific EMF taxa to maximize the benefit : cost ratio of the symbiosis. In other mutualisms, mechanisms have been discovered that allow hosts to favor beneficial partners and penalize less beneficial or "cheater" symbionts, such as selective fruit abortion in the yucca–yucca moth mutualism (Pellmyr and Huth 1994) and sanctions in the legume–*Rhizobium* mutualism (Denison 2000). Marx et al. (1977) experimentally demonstrated that individual *P. taeda* roots colonized by EMF strains demanding greater amounts of carbon had decreased longevity relative to strains of the same species that were less costly to their plant host. The proportion of colonized root tips and abundance of EMF and arbuscular mycorrhizal fungi have also been shown to decline when plants are fertilized with nitrogen or phosophorus (see review by Treseder 2004). These examples provide evidence that plants may discriminate among the suite of compatible fungi with whom they may associate, or increase turnover rates of roots colonized by less beneficial EMF (Hoeksema and Kummel 2003). Studies examining the composition and abundance of EMF hyphae in the bulk soil in these

plots are currently underway to determine the relative importance of different EMF species in nutrient acquisition. These studies, in concert with greenhouse experiments assessing plant benefit of relevant EMF taxa, may help us to determine whether the EMF community shifts we detected reflect choice on the part of host trees or an increase in carbon parasitism by EMF taxa taking advantage of increased carbon availability in a CO_2-enriched atmosphere.

Using sequence data for identifying and quantifying EMF taxa

The number of taxa we recovered in this study is much greater than similar studies that have relied on morphology-based identification methods. In a study of *Picea abies* EMF communities subjected to elevated CO_2 and nitrogen fertilization regimes, Fransson et al. (2001) sampled 5765 mycorrhizae across two years and identified 58 and 42 EMF morphotypes, of which 76% and 64%, respectively, remained unidentified. Rygiewicz et al. (2000) surveyed 156 000 root tips over four years from *Pseudotsuga menziesii* seedlings grown in forest soil from Oregon and exposed to elevated CO_2 and temperature regimes, and delimited 40 morphotypes, ascribing genus-level identity to just two types. It is likely that inherent differences among geographic regions, host species, and host ages are responsible in part for the differences in EMF richness between these studies and our own. However, by using sequence data we found greater community richness and could determine the taxonomic affiliation of a much greater proportion of mycorrhizal types, underscoring the utility of this approach to describe and analyze microbial communities. Increased publicly available fungal sequences and our development of a sequence database derived from fruiting bodies in the same site further allowed us to confidently assess relative abundances of individual taxa, and to minimize artificially lumping unique taxa into larger groups, which can mask differences in the responses of morphologically or taxonomically similar species to environmental perturbations.

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APPENDIX A

Phylotype accumulation curves (*Ecological Archives* E087-138-A1).

APPENDIX B

Phylotype distribution and abundance among ectomycorrhizal fungal lineages (*Ecological Archives* E087-138-A2).

APPENDIX C

Additional methods and results pertaining to the identification of EMF taxa from ITS sequence data (*Ecological Archives* E087-138-A3).