The rhizosphere and hyphosphere differ in their impacts on carbon and nitrogen cycling in forests exposed to elevated CO₂

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Introduction

Temperate forests influence the global climate system by exchanging large amounts of carbon dioxide (CO₂) with the atmosphere. While temperate forests currently represent net sinks for anthropogenic carbon (C; Canadell et al., 2007), the degree to which these forests will persist as C sinks in the wake of rising atmospheric CO₂ is uncertain. Both empirical research and theory suggest that the fate of this sink hinges on plant–microbe interactions (Högberg & Read, 2006; Reich et al., 2006; Thornton et al., 2007; Ostle et al., 2009). Carbon uptake by trees depends on the release of growth-limiting nutrients from soil organic matter (SOM) by microbes, while microbial metabolism and enzyme activities depend on the supply of plant-derived C substrates to soil (Allison & Vitousek, 2005). Hence, the long-term persistence of temperate forests as sinks for CO₂ depends on plant–microbial exchanges of C and nutrients and the balance between C stored in plant biomass and that stored in soils.

Plant–microbial dynamics are often intensified in forests exposed to elevated CO₂ (Norby & Zak, 2011). Multiple experiments have demonstrated that CO₂-enriched trees increase the amount of C allocated to roots. Elevated root-derived C provides an energy subsidy to microbes to fuel microbial conversion of nitrogen (N) from unavailable forms to plant-available forms (Carney et al., 2007; Langley et al., 2009; Schleppi et al., 2012) — a process that increases N availability to both plants and microbes via a ‘priming effect’. While there is much indirect evidence of priming effects in forests (Carney et al., 2007; Phillips et al., 2011; Drake et al., 2012; Zak et al., 2012; Cheng et al., 2013), there is little consensus on whether priming results from enhanced root activities or enhanced mycorrhizal fungal activities. For example, trees may induce priming effects by releasing root exudates to the rhizosphere. Exudates, which are rich in C but poor in N, stimulate free-living microbes to produce extracellular enzymes that degrade N-rich SOM (i.e. inducing a rhizosphere priming effect). Alternatively, trees may induce priming by provisioning C directly to symbiotic mycorrhizal fungi to mine N from SOM (i.e. inducing a ‘hyphosphere priming effect’; cf. Talbot & Treseder, 2010).

At the Duke Forest free-air carbon enrichment (FACE) site, several lines of evidence support the view that stimulated priming effects are responsible for the 14 yr of sustained forest productivity under elevated CO₂ (Phillips et al., 2011; Drake et al., 2012). First, C allocation belowground has increased in response to elevated CO₂ as a result of increases in fine-root production (Pritchard et al., 2008a; Franklin et al., 2009; Taylor et al., 2007). These increases in root-derived C stimulate microbes to release nutrients from soil organic matter, the importance of root-versus mycorrhizal-induced changes in soil processes are presently unknown.

While multiple experiments have demonstrated that trees exposed to elevated CO₂ can stimulate microbes to release nutrients from soil organic matter, the importance of root-versus mycorrhizal-induced changes in soil processes are presently unknown. We analyzed the contribution of roots and mycorrhizal activities to carbon (C) and nitrogen (N) turnover in a loblolly pine (Pinus taeda) forest exposed to elevated CO₂ by measuring extracellular enzyme activities at soil microsites accessed via root windows. Specifically, we quantified enzyme activity from soil adjacent to root tips (rhizosphere), soil adjacent to hyphal tips (hyphosphere), and bulk soil.

During the peak growing season, CO₂ enrichment induced a greater increase of N-releasing enzymes in the rhizosphere (215% increase) than in the hyphosphere (36% increase), but a greater increase of recalcitrant C-degrading enzymes in the hyphosphere (118%) than in the rhizosphere (19%). Nitrogen fertilization influenced the magnitude of CO₂ effects on enzyme activities in the hyphosphere only. At the ecosystem scale, the rhizosphere accounted for c. 50% and 40% of the total activity of N- and C-releasing enzymes, respectively.

Collectively, our results suggest that root exudates may contribute more to accelerated N cycling under elevated CO₂ at this site, while mycorrhizal fungi may contribute more to soil C degradation.

Summary

- While multiple experiments have demonstrated that trees exposed to elevated CO₂ can stimulate microbes to release nutrients from soil organic matter, the importance of root-versus mycorrhizal-induced changes in soil processes are presently unknown.
- We analyzed the contribution of roots and mycorrhizal activities to carbon (C) and nitrogen (N) turnover in a loblolly pine (Pinus taeda) forest exposed to elevated CO₂ by measuring extracellular enzyme activities at soil microsites accessed via root windows. Specifically, we quantified enzyme activity from soil adjacent to root tips (rhizosphere), soil adjacent to hyphal tips (hyphosphere), and bulk soil.
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- Collectively, our results suggest that root exudates may contribute more to accelerated N cycling under elevated CO₂ at this site, while mycorrhizal fungi may contribute more to soil C degradation.
mycorrhizal production (Pritchard et al., 2008b, 2014) and mass-specific root exudation (Phillips et al., 2011). Despite the additional inputs of root- and mycorrhizal-derived C to soil, there has been no additional change in mineral soil C, indicating that a proportional amount of C has probably been lost through the decomposition of indigenous SOM (Drake et al., 2012). Secondly, N cycling has been accelerated under elevated CO2 as a result of increases in root exudation (Phillips et al., 2011) and the turnover of mycorrhizal hyphae (Phillips et al., 2012). Finally, in a recent model of soil C and N dynamics under elevated CO2, Cheng et al. (2013) reported that the inclusion of increased priming effects in the model structure was necessary to correctly predict the observed changes in soil C and N uptake by trees under elevated CO2 at the Duke FACE site. While there is ample evidence of elevated priming at the Duke FACE site, none of the above-mentioned studies were designed to partition increased priming effects between roots and fungi. Enzyme activities and resultant patterns of SOM decomposition exhibit high spatial variability in the soil environment (Schimel & Bennett, 2004; Lindahl et al., 2010), and active soil microbes may occupy only a small percentage of the total soil volume (Parkin, 1987; Briar et al., 2011). Both roots and hyphae are thought to exude easily decomposable C substrates that enhance the activity of soil microorganisms. As such, microbial activities are generally much greater in the rhizosphere (the zone of soil adjacent to roots) and hyphosphere (the zone of soil adjacent to rhizomorphs and their associated hyphae) than in bulk soil. Consequently, extracellular enzyme activity and decomposition rates of SOM can be expected to peak at soil microsites around root and hyphal tips. While the importance of soil microsites for biogeochemical processes and ecosystem productivity has often been presumed, the proportional contributions of roots and mycorrhizal fungi have seldom been considered.

Understanding the relative contributions of roots and fungi to increased priming has important consequences for predicting C and nutrient turnover under future environmental conditions. If roots are the primary agents of stimulated priming, predicting the extent of plant–microbial exchanges of C and N will require more information on tree species differences in root and hyphal growth and mortality as well as in the quantity and chemical quality of root exudates. If, in contrast, mycorrhizal fungi are the primary priming agents, differences in the enzymatic capabilities of different mycorrhizal taxa will be needed to predict the magnitude of increased priming effects. Moreover, roots and mycorrhizal fungi do not always respond similarly to changes in elevated CO2 and temperature (Rillig et al., 1999; McCormack et al., 2010). Determining the relative roles of roots and mycorrhizal fungi represents a critical first step in understanding how and why forests differ in their ability to remove atmospheric CO2 and slow climate change.

In this study, we determined the extent to which the stimulated priming effects observed at the Duke Forest FACE site were driven by roots/rhizosphere microbes versus mycorrhizal fungi. We quantified the activities of four extracellular enzymes in soil microsites via large root windows, a design that enabled us to collect soil from areas adjacent to root tips (i.e. the rhizosphere), soil from areas adjacent to rhizomorphs and their associated mycorrhizal hyphal strands (i.e. the hyphosphere), and bulk soil. We then matched the enzyme activities with plot-specific measurements of root and fungal cord length to generate estimates of the spatial extent of the rhizosphere and hyphosphere, respectively. By relating enzyme activities measured in the rhizosphere, hyphosphere, and bulk soil to the estimated volumes of soil occupying these three microsite types, we were able to scale enzyme activity to the forest ecosystem. We hypothesized that stimulated priming effects would be greater in the rhizosphere, which is influenced by both root exudation and fungal enzyme production, than in the hyphosphere, particularly for enzymes involved in the decomposition and release of growth-limiting N, and that the magnitude of priming effects would be reduced in plots released from progressive N limitation (i.e. plots with elevated CO2 and N fertilization).

Materials and Methods

Study site and sample collection

This research was conducted at the Duke FACE experiment, North Carolina, USA. At this site, a loblolly pine (Pinus taeda L.) plantation was established from 3-yr-old seedlings in 1983 following clear-cutting and burning, and has subsequently undergone recruitment of hardwood tree species (i.e. Liquidambar styraciflua, Ulmus alata, Cornus florida, Acer rubrum, and Cercis canadensis) in the understory. From 2008 to 2010, the years this study was conducted, loblolly pine comprised c. 80% of the basal area of the stand. The site is located in the region of the Piedmont plateau on low-fertility, slightly acidic Hapludalfs (Enon series) derived from diabase (Oh & Richter, 2005). The climate is humid subtropical, characterized by warm, humid summers and relatively moderate winters. Mean annual precipitation is 1140 mm and mean annual temperature is 15.5°C.

The Duke FACE experiment consisted of eight randomly selected 30-m-diameter plots, which included one prototype control plot with no instrumentation, three fully instrumented control plots receiving ambient air, and four treatment plots receiving elevated atmospheric CO2 at a target concentration of ambient plus 200 ppm (c. 580 ppm average daytime concentration, as anticipated for the year 2050; IPCC, 2013). For technical details of the FACE technology, refer to Hendrey et al. (1999) and for more information about how the plots were chosen refer to Schlesinger et al. (2006). Fumigation started in August 1996 and was ongoing for 14 yr until October 2010. An N fertilizer (NH4NO3) treatment was added to half of each plot beginning in spring 2005, to achieve a total annual fertilization rate of 11.2 g N m⁻² yr⁻¹ (Oren et al., 2001). This rate was one order of magnitude higher than the rate of local N deposition (c. 0.6–0.7 g N m⁻² yr⁻¹; Finzi et al., 2002). The experimental design was a split-plot in a randomized complete block design with four replicates; CO2 treatment was the whole-plot factor and N treatment was the subplot factor.

In 2008, we excavated a 60 × 60 × 30 cm cuboid in each subplot and installed two clear acetate windows on two opposite...
The N mineralization potential was determined using 15N isotope pool dilution. Samples were initially labeled with 0.5 ml of a 92 mg l⁻¹ 99-atom% enriched (15NH₄)Cl solution (Hart et al., 1994). Inorganic N was extracted immediately from soil by shaking with 2 M KCl (1 : 8) and was analyzed by photometrical measurement with an autoanalyzer (flow injection analysis; QuikChem; Lachat Instruments, Loveland, CO, USA). Net N mineralization, that is, the net difference between mineralization and microbial immobilization, was calculated as the change between initial and final concentrations of NH₄⁺ and NO₃⁻ after incubation for 4 h at room temperature. For the determination of gross (or total) N mineralization, which excludes microbial immobilization calculative, samples were further prepared by diffusing the N in each sample onto an acidified filter disc. The atom% 15N excess of the initial and incubated sample was analyzed on a continuous-flow isotope ratio mass spectrophotometer at the Boston University Stable Isotope Laboratory (Boston, MA, USA). Soil moisture was determined by drying (105°C for 48 h) and weighing. Organic matter content was measured as loss on ignition (LOI) after 1 g of soil dry mass was oxidized at 550°C in a muffle furnace for 24 h. The difference in soil mass before and after heating represented the SOM content.

### Enzyme activities in the soil

A subsample of soil was stored at −80°C before enzyme analysis. The activities of four extracellular enzymes involved in the decomposition of C-, N-, and phosphorus (P)-containing compounds were assayed (Finzi et al., 2006). The four extracellular enzymes can be grouped into three functional groups based on their ability to decompose recalcitrant C constituents (i.e. lignin; polyphenol oxidase (Pheox) and peroxidase (Perox)) or depolymerize organic N (β-1,4-N-acetyl-glucosaminidase (NAG)) or P (acid phosphatase (AP); Table 1). The activities of AP and NAG were measured with methylumbelliferone as the substrate using a microplate fluorometer with 365-nm excitation and 450-nm emission filters (Saiya-Cork et al., 2002). Phenox and Perox activities were measured spectrophotometrically as absorbance at 460 nm using 1,3,4-dihydroxyphenylalanine (L-DOPA) as the substrate (Saiya-Cork et al., 2002). After correction for controls and quenching, enzyme activities were expressed in units of nmol or µmol substrate cleaved g⁻¹ h⁻¹.

### Scaling exercise

To scale our NAG and Perox activities from May to October 2010 to the plot-scale, we used length and diameter measurements of fine roots and fungal cords collected from each ring. Fine-root morphology was measured in 20-cm³ soil monoliths collected in the first week of November 2010. The roots were removed from the soil, washed and then analyzed for their morphology using WINRHIZO (Rgent Instruments, Québec, Canada) (Taylor et al., 2014b). The length of cord-like fungal structures in May, June, July, and October 2010 was measured using mini-rhizotrons. As a consequence of the absence of rhizomorph diameter data in 2010, we used the average rhizomorph diameter across all rings from mini-rhizotron measurements made from 2005 to 2007 on over 1600 rhizomorphs (S. G. Pritchard, unpublished). To convert the length data for fungal structures to similar units as those used for root length (i.e. mm cm⁻³), we calculated the total soil area surveyed based on the dimensions of the viewing frame of the mini-rhizotron and converted to volume using an empirically derived 0.78 mm depth of field for mini-rhizotron images (Taylor et al., 2014a).

### Table 1 Catalytic function of the investigated extracellular enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Abbreviation</th>
<th>Function</th>
<th>Substrate</th>
<th>Products</th>
<th>Decomposition rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid phosphomono-esterase</td>
<td>AP</td>
<td>Hydrolase</td>
<td>Organic P</td>
<td>Phosphate</td>
<td>Medium to slow</td>
</tr>
<tr>
<td>β-1,4-N-acetyl-glucosaminidase</td>
<td>NAG</td>
<td>Hydrolase</td>
<td>Chitin, peptido-glycan</td>
<td>N (and C)</td>
<td>Medium</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>Perox</td>
<td>Oxidoreductase</td>
<td>Lignin, humics</td>
<td>C (N and P)</td>
<td>Slow</td>
</tr>
<tr>
<td>Phenol oxidase</td>
<td>Phenox</td>
<td>Oxidoreductase</td>
<td>Lignin, humics</td>
<td>C (N and P)</td>
<td>Slow</td>
</tr>
</tbody>
</table>

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We calculated the volume of soil occupied by roots and fungal cords using the measurements of length and diameter to 20 cm depth assuming both were cylindrical. Given that the zone of influence of both roots and hyphae is not limited to the surface, we assumed that an exudate molecule or enzyme could travel 1 mm from the root and hyphal surface (Herman et al., 2006; Schenck zu Schweinsberg-Mickan et al., 2010; Marschner et al., 2012). Using this influence zone, we estimated rhizosol and hyphosol volumes (subtracting the respective root and fungal cord volumes) again assuming both were cylindrical and all spheres were independent and additive, thus ignoring any overlap of roots or hyphae and overestimating the spheres by this factor. As we based our calculation of the hyphosphere volume on measurements of fungal cord dynamics (ignoring extrametrical hyphae), we may have underestimated the total hyphosol volume relative to rhizosphere volume. We classified the remaining soil as bulk soil and converted each fraction’s volume to mass using measurements of bulk density in July 2010. Finally, for each enzyme, the activity was scaled to the plot level by taking the product of the amount of each soil fraction and the mass-specific rates of enzyme activity.

Statistical analyses

Statistical analyses were conducted with the package SAS, version 9.3 (SAS Institute Inc., Cary, NC, USA). Means and standard errors of soil and root parameters were calculated from each of four plots per treatment. The probability of a fit to a normal distribution was tested using a Shapiro–Wilk test ($P \leq 0.05$). Non-normally distributed data were log-transformed to meet normality criteria. Treatment means were compared by two-factorial analyses of variance (ANOVA) to analyze differences between treatments. Mixed variance-covariance models for fixed and random effects with the variables atmospheric CO2, N fertilization, soil microsites and sampling date were calculated to test for significant effects. Data likelihood was maximized to estimate the model parameters. FACE rings were included as random effects. We refer to a $P$ value of $0.05$ as statistically significant and indicate in some cases a $P$ value of $0.1$ as marginally significant.

Results

Enzyme activities at soil microsites

All extracellular enzyme activities were significantly influenced by the time of the year (Table 2). The potential activities of AP, NAG and Perox were also influenced by the location in the soil, while the activity of Phenox did not vary among different microsites. The activities of AP, NAG and Perox were positively correlated with each other (Table S2), while the activity of Phenox was independent of the other three. The activities of NAG and Perox had similarly high variation across the treatments and soil microsites (coefficient of variation 69% and 72%, respectively). AP varied only by about a third in its activity across the four treatments and the three microsites. According to the mixed effects models, AP and Perox activities were significantly influenced by N fertilization, while the activity of NAG was also influenced by both elevated atmospheric CO2 and N fertilization (Table 2).

The C and N mineralizing enzyme NAG had the highest activity in the elevated CO2 treatment in June (Fig. 1a–c); the activity increased more in the rhizosphere (significant increase by 91 nmol g$^{-1}$ h$^{-1}$) than in both the hyphosphere and bulk soil (Figs 1a–c, 2a). There was also a tendency for higher NAG activities in the rhizosphere under elevated CO2 in May and July, as well as for higher activities in the hyphosphere during the whole growing season (not significant; Fig. 1a–c). When trees were released from N limitation in N-fertilized plots, the increase in NAG activity in the rhizosphere was diminished under elevated CO2. The activity of the lignin-cleaving enzyme Perox was also increased in the elevated CO2 treatment in June, but, in contrast to NAG, its activity increased most in the hyphosphere (significant increase by 0.8 µmol g$^{-1}$ h$^{-1}$; Figs 1d–f, 2b). N fertilization decreased Perox activity in the rhizosphere (decrease by 0.4 µmol g$^{-1}$ h$^{-1}$ relative to the elevated CO2 treatment; Fig. 2b), but N availability did not change the elevated CO2 effect in the hyphosphere.

The amount of organic matter increased in unfertilized and fertilized plots exposed to elevated CO2 in all three spheres (Fig. 3a); this increase was significant for bulk soil and rhizosphere under elevated CO2. The concentration of N increased significantly in both the rhizosphere and hyphosphere of unfertilized plots exposed to elevated CO2, but not in bulk soil (Fig. 3b). Under elevated CO2 both net and gross N mineralization rates in the rhizosphere tended to increase (Fig. 3c,d), but had high variability. There was a significant positive effect of elevated CO2 on net mineralization rates in the hyphosphere of unfertilized plots. N fertilization increased net N mineralization rates in bulk soil and the rhizosphere but had no significant effect on gross N mineralization, indicating reduced microbial immobilization rates in bulk soil and the rhizosphere. By contrast, net and gross mineralization rates tended to decrease in the hyphosphere and the importance of microbial immobilization increased with N fertilization.

Table 2 Significance of the effects of elevated CO2, nitrogen (N) fertilization, soil microsite, and time of the year on the variance of measured enzyme activities (mol g$^{-1}$ h$^{-1}$) in a loblolly pine forest according to mixed variance-covariance models for fixed and random effects

<table>
<thead>
<tr>
<th>Enzyme activity</th>
<th>CO2 × N</th>
<th>Site</th>
<th>CO2 × N × site</th>
<th>Season</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP activity</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>NAG activity</td>
<td>*</td>
<td>(*)</td>
<td>(*)</td>
<td>***</td>
</tr>
<tr>
<td>Perox activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenox activity</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Effects: CO2, elevated CO2; N, N fertilization; site, soil microsite; season, time of the year. Enzymes: AP, acid phosphatase; NAG, β-1,4-N-acetyl-glucosaminidase; Perox, peroxidase; Phenox, phenol oxidase. numDF (numerator degrees of freedom) = 1, denDF (denominator degrees of freedom) = 176. (*), $P \leq 0.1$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. 


Enzyme activities at the ecosystem scale

Root and fungal cord lengths in the upper soil layer increased by 95% and 60%, respectively, in unfertilized plots under elevated CO2; for root length this effect was significant (Fig. 4; Taylor et al., 2014b). Subsequently, rhizosphere volume increased from about 23% to 26% in ambient and fertilized plots to 42% in plots exposed to elevated CO2 (Table 3). As a consequence of the significant increase in root length or rhizosphere volume and in specific activity in the rhizosphere, 47% and 46% of the total enzyme activity of NAG and AP, respectively, took place in the rhizosphere, while bulk soil and the hyphosphere contributed only c. 23–31% each (Fig. 5a; Table 3). This is in contrast to the ambient and fertilized treatments, where the greatest contribution to total NAG and AP activity, respectively, could be found in the bulk soil (52–57% of total NAG and AP activity; Table 3). Perox activity was also increased in both the rhizosphere and the hyphosphere of elevated CO2 plots in comparison to ambient CO2 plots (Fig. 5b). The relative contribution of the rhizosphere to total Perox or Phenox activity was similar to the relative contribution of bulk soil (35–39%; Table 3).

Discussion

The lack of understanding of the contribution of roots and mycorrhizal fungi to biogeochemical processes represents a key barrier to incorporating belowground dynamics into ecosystem models and to investigating biospheric feedbacks to climate change (Heimann & Reichstein, 2008; Ostle et al., 2009). In this study, we found that both rhizosphere microbes and cord-forming fungi increased extracellular enzyme activity in a forest...
exposed to elevated CO₂, and that the magnitude of this effect can be quantitatively important at the ecosystem scale (Fig. 5). Notably, we found that, under CO₂ enrichment, enzymes that primarily mediate N acquisition were stimulated in the rhizosphere whereas enzymes that promote soil C degradation were stimulated to a greater extent in the hyphosphere (Figs 2, 5). These findings indicate that increased priming effects may be caused by root and fungal activities, and that the consequences of increased priming for C and N cycling may depend, in part, on the relative amount of root versus mycorrhizal production, activity and turnover.

Nutrient limitation in forests exposed to CO₂ enrichment

Despite some evidence of nutrient constraints on ecosystem responses to CO₂ enrichment (Norby et al., 2010), many forests have shown a capacity to sustain enhanced levels of productivity by accelerating rates of N cycling through stimulated priming effects (Gielen et al., 2005; Langley et al., 2009; Drake et al., 2011; Zak et al., 2012). Priming effects occur when inputs of plant-derived C stimulate soil microbes to break down SOM (presumably as a means to release nutrients). While there is still much uncertainty about the mechanisms that control priming (Kuzyakov, 2010; Cheng et al., 2013), there has been increasing recognition that roots and root-derived products are the source of the labile C based on findings from girdling (Weintraub et al., 2007), exudate addition (Landi et al., 2006; Drake et al., 2013), and isotopic labeling (Bengtson et al., 2012; Dijkstra et al., 2013) experiments. Such rhizosphere priming effects are believed to be especially relevant in forests exposed to elevated CO₂, as described by the rhizosphere-accelerated mineralization and priming (RAMP) hypothesis (Phillips et al., 2012). However, despite the emerging consensus about the importance of accelerated rhizosphere priming, a critical question remains: are increased priming effects the result of root-induced stimulation of free-living rhizosphere saprotrophs, or mycorrhizal fungi that use root-derived C to degrade SOM as ‘decomposers in disguise’ (sensu Talbot et al., 2008)?

The results of this study indicate that, while priming may occur in both the rhizosphere and hyphosphere, CO₂-induced stimulation of root and fungal activities may have different consequences for N availability and SOM turnover. On an annual basis, nonmycorrhizal and mycorrhizal roots accelerated N-
acquisition enzymes by four-fold in unfertilized plots exposed to elevated CO₂, whereas (medium- to long-distance) fungal hyphae increased NAG by only 2.5-fold in these same plots (significant effect of atmospheric CO₂; interactive effect of atmospheric CO₂ × N fertilization × soil microsite not significant; Fig. 5). However, mycorrhizal hyphae enhanced Perox in unfertilized and fertilized plots exposed to elevated CO₂. Previous studies have reported root-accelerated C and N cycling for trees (Langley et al., 2009; Bengtson et al., 2012), but did not distinguish between root and mycorrhizal contributions to this enhancement. Using a different method to estimate the rhizosphere fraction of soil, Phillips et al. (2011) found that CO₂-induced increases in exudation were positively correlated with rhizosphere NAG at the Duke Forest FACE site (Phillips et al., 2011), but this technique did not permit the isolation of rhizosphere (i.e. of nonmycorrhizal and mycorrhizal roots and associated saprotrophic fungi and rhizosphere bacteria) and hyphosphere (i.e. of medium- to long-distance fungal hyphae and associated saprotrophic fungi and hyphosphere bacteria) effects.

Enhanced activities of NAG under elevated CO₂ have been reported in numerous studies (cf. Kelley et al., 2011). NAG is produced by both fungi and bacteria (i.e. actinomyces; Billings & Ziegler, 2008), to degrade chitin from fungal cell walls and peptidoglycan from Gram-positive bacteria into smaller N-containing organic compounds which can then be further mineralized to inorganic N. Increased NAG activity may reflect increased demand for N as well as increased formation of N-acetylglucosamine (a component of chitin and peptidoglycan) by fungi and Gram-positive bacteria, respectively (Billings & Ziegler, 2008). In our investigation, NAG activity increased in the rhizosphere more than in the hyphosphere under elevated CO₂, which suggests that stimulated root priming of soil bacteria and saprotrophic fungi and the direct release of NAG by mycorrhizal roots added to greater total NAG activity in the rhizosphere than increased hyphal priming and the direct release by mycorrhizal hyphae in the hyphosphere. Of course, we cannot rule out the possibility that other measures of N mining from SOM that were not measured in this study (e.g. proteolytic enzyme activity) were stimulated in the hyphosphere, and future studies should investigate these dynamics.

The current study documents increased production of Perox in the hyphosphere of loblolly pine under elevated CO₂, suggesting greater decomposition of lignin by hyphosphere microbes. Lignin forms a polymeric cage around cellulose microfibrils and is generally slow to decompose. During the course of the decomposition process, depolymerization of lignin increasingly limits microbial C acquisition (Schimel & Weintraub, 2003; Allison, 2006; Herman et al., 2008) and therefore drives changes in microbial community composition (Moorhead & Sinsabaugh, 2006). The increased production of Perox by hyphae under elevated CO₂ in our study may indicate such a change in microbial community composition associated with fungal cords. Furthermore, oxidoreductases such as Perox can also degrade humics, detoxify phenolics and reactive metals, and act as an antimicrobial defense (Burke & Cairney, 2002; Claus, 2003; Rabinovich et al., 2004; Baldrian, 2006). Changes in the activity of this enzyme are therefore likely to exert significant effects on soil C processing.

Table 3 Soil volume and estimated activities of acid phosphatase (AP), β-1,4-N-acetyl-glucosaminidase (NAG), peroxidase (Perox), and phenol oxidase (Phenox) in bulk soil, rhizosphere or hyphosphere of four loblolly pine treatments at the Duke free-air carbon enrichment (FACE) site during the year 2010

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bulk Soil</th>
<th>Rhizosphere</th>
<th>Hyphosphere</th>
<th>Hyphosphere</th>
<th>Rhizosphere</th>
<th>Bulk Soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil Volume (%) of Total</td>
<td>60.4 (3.1)</td>
<td>23.3 (3.3)</td>
<td>16.3 (1.9)</td>
<td>15.9 (1.6)</td>
<td>56.5 (1.7)</td>
<td>30.0 (2.2)</td>
</tr>
<tr>
<td>AP (%) of Total</td>
<td>60.3 (3.2)</td>
<td>24.5 (3.3)</td>
<td>15.2 (2.1)</td>
<td>18.9 (1.5)</td>
<td>56.3 (1.7)</td>
<td>23.3 (1.8)</td>
</tr>
<tr>
<td>NAG (%) of Total</td>
<td>59.0 (1.4)</td>
<td>26.0 (9.7)</td>
<td>15.0 (2.1)</td>
<td>28.7 (1.0)</td>
<td>53.6 (2.2)</td>
<td>36.2 (2.2)</td>
</tr>
<tr>
<td>Perox (%) of Total</td>
<td>53.4 (4.4)</td>
<td>25.0 (0.6)</td>
<td>21.7 (4.9)</td>
<td>25.5 (0.6)</td>
<td>56.0 (6.1)</td>
<td>30.2 (4.2)</td>
</tr>
<tr>
<td>Phenox (%) of Total</td>
<td>51.8 (4.8)</td>
<td>23.6 (2.5)</td>
<td>24.7 (3.0)</td>
<td>18.9 (1.5)</td>
<td>51.7 (8.3)</td>
<td>23.3 (1.8)</td>
</tr>
</tbody>
</table>

Treatments: Amb, ambient CO₂; Elev, elevated CO₂; Amb+N, fertilized; Elev+N, elevated CO₂ and fertilized. The rhizosphere is influenced by roots and mycorrhizal fungi. Values are mean ± 1 SE. n = 4.

Fig. 5 Estimated ecosystem-scale activity of (a) β-1,4-N-acetyl-glucosaminidase (NAG) and (b) peroxidase (Perox) in the rhizosphere or hyphosphere of four loblolly pine (Pinus taeda) treatments at the Duke free-air carbon enrichment (FACE) site during the year 2010 (treatments: Amb, ambient CO₂; Elev, elevated CO₂; Amb+N, fertilized; Elev+N, elevated CO₂ and fertilized). Error bars, ± 1 SE. Significance: *P ≤ 0.1; **P ≤ 0.05; ***P ≤ 0.01; ns, not significant.
Environmental controls on extracellular enzyme activities

The activity of enzymes was highest in June 2010 when temperature and soil moisture increased after a warm and dry spring in 2010 (Table S1). Baldrian et al. (2013) found a similar increase in enzyme activities during warm periods when soil moisture was not limiting. Finzi et al. (2006) interpreted the significant temporal variations of enzyme activities at the Duke FACE site as a complex response of soil microbes to changes in the timing, quantity, and chemistry of plant-derived substrates under elevated CO2. They found little direct effect of elevated CO2 on the activity of N- and P-releasing enzymes during this earlier stage of the experiment, but the activity of nutrient-releasing enzymes relative to the degradation of recalcitrant C substrates suggested that progressive nutrient limitation of primary producers was increasingly shifting the ecosystem response of the Duke FACE site from production to decomposition. Several factors influence enzyme production and activity in the soil: most important are the availability of plant photosynthates (Courty et al., 2007, 2010) and soil resource abundance, that is, total soil C, N, and moisture (Sinsabaugh et al., 2008; Talbot et al., 2013).

At the Duke FACE site, fine-root production under elevated CO2 peaked late in the growing season of 2009, followed by increased root mortality in spring 2010 and low levels of production for the remainder of the growing season (S. G. Pritchard, unpublished). Given that most fine roots at the site are heavily colonized by mycorrhizal fungi, the turnover of the fine-root pool may explain the increased NAG activity in the rhizosphere observed in this study. Moreover, carbon allocation in loblolly pine generally shifts from leaf and wood production to mycorrhizal tip production (Pritchard et al., 2008a,b, 2014) and exudation (Phillips et al., 2008) in late spring. At the same time, root (Drake et al., 2008) and soil respiration (Jackson et al., 2009; Oishi et al., 2014) increase; the latter is dominated by the decomposition of SOM (Taneva & Gonzalez-Meler, 2011) and explains most of the missing C in the annual C budget (Schäfer et al., 2003). Thus, the high NAG activity in June probably reflects both root- and mycorrhizal-enhanced demand for N, consistent with the RAMP hypothesis (Phillips et al., 2012). Both roots and to some extent mycorrhizas are priming rhizosphere saprotrophs to break down dead mycorrhizal hyphae. Compared with the soil matrix, mycorrhizal hyphae are enriched in N as a consequence of N mining from SOM and N scavenging and are, thus, a preferred resource.

Sensitivity of the sampling and scaling method

Responses of enzyme activity to elevated CO2 vary among studies and ecosystems. In bulk soil, increased activities of NAG or AP caused by elevated CO2 have been found previously (Larson et al., 2002; Ebersberger et al., 2003; Dorodnikov et al., 2009) while other studies in northern fens (Kang, 2001) or temperate forests (Larson et al., 2002) did not find an effect of elevated CO2 on activities of AP, Phenox or Perox in bulk soil. A study by Henry et al. (2005) reported a negative effect of elevated CO2 on the activities of hydrolases (polysaccharide-degrading enzymes and AP), while oxidoreductases were unaffected. To some extent these contradictory results may arise from differences in environmental factors and time of the year, as discussed above, but they may also result from differences in sampling scale. For example, in our previous study conducted in the Duke FACE site using the adhering soil separation method (Phillips et al., 2011), we found that CO2-induced increases in exudation led to a 24% increase in rhizosphere NAG at the peak of the growing season, while enzyme activities in bulk soil were unaffected. In the current root box sampling, we found that elevated CO2 led to a 215% increase in rhizosphere NAG, which may primarily be a result of less soil dilution by bulk soil in the root box sampling. A major advantage of the current method is the analysis of extracellular enzyme activities in the hyphosphere, which unveiled specific influences of rhizomorphs and hyphal tips on the activity of Perox.

This study scaled extracellular enzyme activities to the ecosystem scale of a forest exposed to elevated atmospheric CO2. It combines the advantages of in situ small-scale soil sampling of undisturbed soil from root box windows with the temporal observation of root and fungal cord growth from mini-rhizotron tubes. Our ecosystem scaling exercise was based on several assumptions: (1) fungal cords are representative of rhizomorphs and diffuse hyphae, (2) the zone of influence of exudation is a 1-mm cylindrical sphere from the root and hyphal surface, as found in previous studies (Herman et al., 2006; Schenck zu Schweinsberg-Mickan et al., 2010; Marschner et al., 2012), (3) all spheres are independent and additive, and (4) the activity of enzymes is similar across total root and hyphal length. While the first two assumptions may underestimate to some extent the zone of influence of rhizospheres and hyphospheres, the latter two assumptions probably overestimate sphere volumes and enzyme activities. Future research should focus on the development of novel techniques for a more detailed estimate of the spatial extent of rhizosphere and hyphosphere volumes in ecosystems.

Conclusions

We isolated rhizosphere and hyphosphere effects on C and N cycling by comparing enzymatic activities within soil microsites, accessed via a ‘root box’ buried in soils at the FACE site. Using this approach, we found that the observed patterns of accelerated C and N cycling under elevated CO2 at the Duke Forest FACE site are driven by both root-induced changes in microbial activity and soil fungal activity. Notably, the rhizosphere appears to be involved in the recycling of N from dead microbes, whereas the fungal hyphosphere appears to be involved in the breakdown of more recalcitrant SOM. Our findings indicate that belowground processes are likely to increase in importance as atmospheric CO2 concentrations rise, and highlight the need to further our mechanistic understanding of the interactions between roots, soil microbes (including mycorrhizal fungi) and SOM.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Collection of soil from soil microsites of the root window: soil adjacent to root tips (rhizosphere) and bulk soil.

Fig. S2 Collection of soil from soil microsites of the root window: soil adjacent to hyphal tips (the hyphosphere) and bulk soil.

Table S1 Climatic conditions during the four sampling periods from May to October 2010

Table S2 Correlations between the activities of different extracellular enzymes and variation in the activities of single enzymes

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