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Research paper

Root length, biomass, tissue chemistry and mycorrhizal colonization following 14 years of CO_2 enrichment and 6 years of N fertilization in a warm temperate forest

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Root systems serve important roles in carbon (C) storage and resource acquisition required for the increased photosynthesis expected in CO_2-enriched atmospheres. For these reasons, understanding the changes in size, distribution and tissue chemistry of roots is central to predicting the ability of forests to capture anthropogenic CO_2. We sampled 8000 cm^3 soil monoliths in a pine forest exposed to 14 years of free-air-CO_2-enrichment and 6 years of nitrogen (N) fertilization to determine changes in root length, biomass, tissue C : N and mycorrhizal colonization. CO_2 fumigation led to greater root length (98%) in unfertilized plots, but root biomass increases under elevated CO_2 were only found for roots <1 mm in diameter in unfertilized plots (59%). Neither fine root [C] nor [N] was significantly affected by increased CO_2. There was significantly less root biomass in N-fertilized plots (19%), but fine root [N] and [C] both increased under N fertilization (29 and 2%, respectively). Mycorrhizal root tip biomass responded positively to CO_2 fumigation in unfertilized plots, but was unaffected by CO_2 under N fertilization. Changes in fine root [N] and [C] call for further study of the effects of N fertilization on fine root function. Here, we show that the stimulation of pine roots by elevated CO_2 persisted after 14 years of fumigation, and that trees did not rely exclusively on increased mycorrhizal associations to acquire greater amounts of required N in CO_2-enriched plots. Stimulation of root systems by CO_2 enrichment was seen primarily for fine root length rather than biomass. This observation indicates that studies measuring only biomass might overlook shifts in root systems that better reflect treatment effects on the potential for soil resource uptake. These results suggest an increase in fine root exploration as a primary means for acquiring additional soil resources under elevated CO_2.

Keywords: elevated CO_2, FACE, mycorrhiza, *Pinus taeda*, root biomass, root tip.

Introduction

Plants serve as an important store of terrestrial carbon (C) and have the potential to capture increased C in response to rising atmospheric CO_2 concentrations. Root systems serve at least two major roles in the sequestration of C in plant biomass. First, trees can accumulate large amounts of C in their root systems. Estimates in mature southern pine forests show as much as 20% of total soil organic matter being stored in roots (Johnsen et al. 2001). Second, roots acquire the soil resources that are widely hypothesized to limit increases in C fixation via photosynthesis under elevated CO_2 (Luo et al. 2004). In light of the major contribution of root systems to the ability of plants to assimilate and store C, the responses of root systems to a changing global environment will likely play a key role in the ability of forest systems to mitigate anthropogenic increases in CO_2 (Norby and Jackson 2000).

The ability of increased plant biomass to serve as an effective long-term C sink will be largely dependent on the ability of root systems to provide adequate resources to accommodate higher photosynthetic rates. It has been suggested that plants will serve as an important C sink only to the point at which nitrogen (N) limits additional C fixation (Luo et al. 2004). The ability of plants to acquire sufficient amounts of water and other soil nutrients, such as phosphorus, may also serve as important limitations to increased net primary production (NPP) in response to higher atmospheric [CO_2]. For these reasons, knowledge of root responses to elevated CO_2 levels when soil resource availability varies spatially and temporally is critical to our assessment of the potential for forest biomass to serve as a significant C sink.

The complexity of below-ground interactions involved in plant responses to elevated CO_2 requires experimentation at the ecosystem scale. Moreover, these interactions may require substantial periods of time to become manifested, making long-term experiments necessary to understand the full effects of environmental change (Rillig et al. 2000, Norby et al. 2010). Currently, the most extensive effort to address both of these needs has been large-scale manipulation of [CO_2] using free-air-CO_2-enrichment (FACE) technology. The relatively large size of FACE plots allows for the monitoring of interactions between entire populations of roots, mycorrhizal fungi and soil microbes, providing a clearer understanding of how root systems in intact forests will function in a CO_2-enriched atmosphere. The relatively long timescale of FACE experiments has also been critical to understanding the adjustment that forests undergo to changing CO_2, highlighted by the finding that N limitation of NPP at the Oak Ridge FACE site occurred only after almost a decade of CO_2 enrichment (Norby et al. 2010).

At the Duke FACTS-1 FACE experiment, exposure of a loblolly pine forest to elevated CO_2 since 1996 has resulted in increasingly positive effects on NPP (Pritchard et al. 2008, Jackson et al. 2009, McCarthy et al. 2010). Prolonged increases in NPP at this site seem to be fueled by greater uptake of N by fine roots coupled with increased root exudates, which cause a priming effect on N-mineralizing soil microorganisms (Finzi et al. 2007, Phillips et al. 2012). Despite variation in temporal dynamics between sites, the three forested US FACE experiments have generally agreed on the positive response of fine root and mycorrhizal biomass to elevated CO_2 (Norby et al. 2004, Treseder 2004, de Graaff et al. 2006, Pregitzer et al. 2008, Pritchard 2011). The effect of elevated CO_2 on mycorrhizal fungi and fine roots observed at FACE experiments has, however, been smaller than predictions based on more controlled experiments conducted in growth chambers and open-top field chambers (Parrent and Vilgalys 2007, Garcia et al. 2008, Hofmockel et al. 2011, Pritchard et al. 2014). The stronger response of roots and mycorrhizal fungi to CO_2 enrichment observed in short-term container studies compared with long-term FACE experiments is likely attributable to more intense competition for resources, longer exposure durations and increasingly limiting soil resources characterized by FACE experiments. More work is needed to better characterize below-ground responses to long-term changes in atmospheric CO_2 availability.

In the current study, we sampled 8000 cm^3 soil monoliths to investigate the effects of 14 years of CO_2 fumigation and 6 years of N fertilization on root length, biomass, tissue chemistry and extent of mycorrhizal colonization. We predicted that total root length and biomass would increase in response to elevated CO_2 but decrease under N fertilization. We did not predict changes in [C] in root systems for either treatment, but expected higher [N] in roots grown in fertilized plots. We also expected mycorrhizal colonization of fine roots to increase under elevated CO_2 but decrease under N fertilization.

Methods

Site description

This study was conducted at the Duke FACE experiment in Orange County, NC, USA (35°97′N, 79°09′W). This site is comprised of a loblolly pine (*Pinus taeda* L.) plantation planted from 3-year-old half-sib seedlings in 1983 in which hardwood colonizers have not been excluded. Colonizing deciduous species include sweetgum (*Liquidambar styraciflua* L.), winged elm (*Ulmus alata* Michx.), dogwood (*Cornus florida* L.), red maple (*Acer rubrum* L.), redbud (*Cercis canadensis* L.), white ash (*Fraxinus americana* L.) and tulip poplar (*Liriodendron tulipifera* L.). More than 98% of basal area at this site is comprised of loblolly pine and the soils are predominantly clay loam of the Enon series.

Detailed descriptions of the experimental design employed at the Duke FACE site are available elsewhere (Hendrey et al. 1999, Matamala and Schlesinger 2000, Drake et al. 2011). Briefly, the Duke FACE experiment was comprised of six circular experimental plots measuring 30 m in diameter. Three of these plots were exposed to CO_2 at concentrations ~200 ppm above ambient air from August 1996 through October 2010, while the other three plots served as the control at ambient CO_2 concentrations. Each plot was divided in half in 2005 by an impermeable plastic sheet installed 70-cm deep into the soil, optimizing similarity in litter production between halves. An ammonium nitrate treatment was implemented by hand broadcasting 11.2 gN m^{-2} to one half of each plot twice in 2005 (March and April) and once per year (March) from 2006 to 2010. The resulting treatment combination represents a split-plot, randomized complete block design with 14 years, 2 months of CO_2 fumigation and 5 years, 8 months of N fertilization.

Monolith extraction

Fifteen soil monoliths measuring 20 × 20 × 20 cm (8000 cm^3) each were extracted following the end of CO_2 fumigation in early

November 2010. Sampling sites were placed approximately equidistant from all surrounding *P. taeda* trees, which were planted at a 2.4 × 2.4-m spacing. One monolith was taken from each split plot except for one fertilized ambient CO_2 plot, one fertilized elevated CO_2 plot and one unfertilized elevated CO_2 plot, for which two monoliths were sampled. This resulted in the number of replicates for each treatment represented in Table 1. A 5-cm wide trench was dug surrounding each sampling site to aid in monolith extraction, and all roots connecting the monolith to the surrounding soil matrix were severed using a reciprocating saw. Monoliths were then detached from the soil below and lifted using a pair of flat shovels taking care to retrieve any roots that were connecting the bottom of the monolith to the soil. Each monolith consisted of the O soil horizon and mineral soil to a depth of 20 cm, which has been shown to capture >90% of roots at this site (Matamala and Schlesinger 2000). Following extraction, monoliths were stored at –10 °C until processing. Although this sampling scheme did not allow for a high number of replicates within each plot (possible with soil cores or minirhizotrons), the intention of this sampling effort was to optimize total contiguous soil volume sampled to gain a complete suite of all small diameter roots in intact branching systems (Taylor et al. 2013).

Monolith processing

Roots were extracted from monoliths by washing thawed monoliths over a series of three screens with mesh sizes of 3, 1 and 0.5 mm. Root segments were exhaustively removed from the 3-mm screen by hand using forceps. One 5 cm^3 subsample was taken from the material caught by each of the 1 and 0.5 mm screens, and all root fragments were extracted from these subsamples using a dissecting microscope at 10× (SMZ-1, Nikon Inc., Melville, NY, USA). Data on the root fragments extracted from the subsamples were then multiplied by the total volume of material captured by each respective screen. This was done to account for the root segments that detach from large root branches during the washing of monoliths, which has been shown to be a substantial portion of the total root length (Pierret et al. 2005).

Following removal from the monolith soil, all root segments were cleaned of visible fungal mycelia and rhizosphere soil by hand using forceps. Each root segment was then floated in water to reduce branch overlap and scanned using an Epson expression 10000 XL scanner at 400 dpi to create a digital image of the root segment. Digital images were analyzed for length, diameter and root tip number using WinRhizo root analysis software (Regent Instruments, Quebec, Canada). Due to the size limitations of the scanner, multiple scans were required to image all root segments for a single monolith. The number of scans required for a monolith varied depending on total length and architecture of the roots in the monolith. Previous analyses (Taylor et al. 2013) suggest that the monolith size used in this study may not reliably sample the rare large roots occasionally observed in this sampling effort. For this reason, we limited analyses in this study to either roots ≤10 mm diameter, which we refer to as 'all roots' or a subset of those roots ≤2 mm, which we refer to as fine roots. Finally, based on the analysis of average diameters of fine roots by orders, we also analyzed treatment effects for fine roots <1.0 mm in diameter since the absorptive first-, second- and third-order roots at this site all have average diameters in this size range (K.V. Beidler, B.N. Taylor, A.E. Strand, E.R. Cooper, M. Schonholz, S.G. Pritchard, unpublished data).

Specific root length

Specific root length (SRL) was calculated from >300 cm of *P. taeda* roots removed from trenches dug around each monolith during extraction. For this analysis, roots were used from four monolith trenches in each treatment including trenches dug around monoliths in the two prototype plots at the Duke FACE site that were excluded from the analysis of monolith roots. Roots were separated into five diameter categories and each category was scanned and analyzed using WinRhizo to obtain total length and average root diameter for that category. Average diameter of basic size categories ranged from 0.3 to 3.5 mm. The set of roots in each category was then dried to a constant mass at 65 °C and weighed. Specific root length was calculated as the total fresh length of all roots in a size

Table 1. Mean root length, biomass, root C and root N values (±SE) for each experimental treatment. Values designated as fine roots are for only roots <2 mm diameter. Values designated as all roots include all roots <10 mm diameter. All values are scaled to represent 1 m^2 to a depth of 20 cm.

		Ambient/unfertilized	Ambient/fertilized	Elevated/unfertilized	Elevated/fertilized
	N	3	4	4	4
All roots	Length (m m^{-2})	7628.81 (1616.07)	8286.59 (2316.36)	15,099.43 (3266.21)	10,599.48 (4395.30)
	Biomass (g m^{-2})	905.91 (324.63)	831.65 (301.96)	1124.14 (331.01)	804.86 (346.41)
	C (g m^{-2})	428.15 (53.83)	392.83 (39.28)	530.49 (53.91)	379.52 (60.06)
	N (g m^{-2})	3.01 (0.26)	5.47 (0.58)	3.69 (0.35)	5.38 (0.95)
Fine roots	Length (m m^{-2})	7522.85 (1582.93)	8191.09 (2282.88)	15,003.29 (3236.25)	10,528.20 (4363.52)
	Biomass (g m^{-2})	422.90 (97.90)	410.68 (91.27)	587.64 (92.37)	433.01 (132.13)
	C (g m^{-2})	196.29 (33.64)	192.91 (15.86)	273.87 (22.09)	203.02 (43.06)
	N (g m^{-2})	1.63 (0.26)	2.82 (0.25)	2.15 (0.18)	3.04 (0.71)

category divided by the total dry mass of the roots in that category, and was used to establish a continuous relationship between average diameter and SRL as in Iversen et al. (2008). Specific root mass (SRM) was calculated as the inverse of SRL, and the relationship between SRM and diameter was used to calculate total biomass for roots at this site.

Carbon and nitrogen analysis

The subset of *P. taeda* roots collected for SRL analysis was also used to obtain information on root tissue [C] and [N]. Roots were sorted into diameter categories, scanned and dried as described above. Once dried, these samples were ground using a Wig-L-Bug ball grinding mill (REFLEX Analytical Corp., Ridgewood, NJ, USA). Ground samples were analyzed for C and N content using an NA 1500 elemental analyzer (Carlo Erba, Milano, IT, USA).

Mycorrhizal root tip estimates

The number of total ectomycorrhizal (EcM) root tips (short roots) in bulk soil at this site was estimated using a combination of two approaches. To estimate the number of mycorrhizal root tips on root branches, tips were hand counted on all monolith root segments that were fully intact and contained at least three root orders according to Strahler's stream ordering classification system (Pregitzer and DeForest 2002). Total root length analyzed in this manner averaged 242 cm per monolith. This analysis yielded the number of mycorrhizal root tips per centimeter of root length for each branching system in a given monolith, which was then averaged for an individual monolith and multiplied by the total fine root length for each monolith. Due to an insufficient number of suitable intact branching systems in some monoliths (i.e., containing at least the most terminal three to five intact root orders), only 12 monoliths (three per treatment) were used in EcM tip estimates.

In order to account for the relatively fragile mycorrhizal root tips that detach during monolith processing, we estimated the number of mycorrhizal tips in the 0.5 and 1 mm screen catchings. To do this, we calculated the percentage of root fragments that were mycorrhizal root tips in the 5-ml screen material subsamples based on the characteristic bifurcating morphology of pine EcM tips. We then applied this percentage to the total number of root tips counted by the WinRhizo analysis for each subsample scan and scaled this number to the total amount of material caught by each screen. This resulted in an estimate of the total number of mycorrhizal root tips caught by each screen. As we felt that designations between live and dead roots and root fragments were unreliable, these estimates account for both live and dead structures.

Statistical analyses

The analyses for this study were conducted as a split-plot design with two levels of CO_2 treatment and two levels of N treatment. Treatment effects were tested using the analysis of covariance with a main effect of CO_2 and a split-level effect of fertilization treatment. Differences in the relationship between tissue C and N concentration and root diameter across treatments were tested using linear regression. A covariate of N-mineralization was included in the original models but was not significant in any model and so was removed. Due to the small number of replicated plots, it has been suggested that an $\alpha = 0.1$ is appropriate when analyzing data from FACE studies (Filion et al. 2000). We therefore designate statistical significance as $P < 0.1$. All statistical analyses were conducted in R version 2.15.0 (R Development Core Team 2012).

Results

A total of 635,313 cm of root length ranging in diameter from 0.025 to 10 mm was sampled from 15 monoliths. Responses of total root length to CO_2 enrichment were N fertilization-dependent, with a 98% increase in root length under elevated CO_2 in unfertilized plots, but no significant CO_2 response in fertilized plots ($CO_2 \times N$ interaction, $P = 0.09$; Figure 1b). This effect was primarily driven by changes in fine roots <2.0 mm, which made up >99% of all root length encountered. Average root length per monolith was 32,018 and 51,397 cm for ambient and elevated

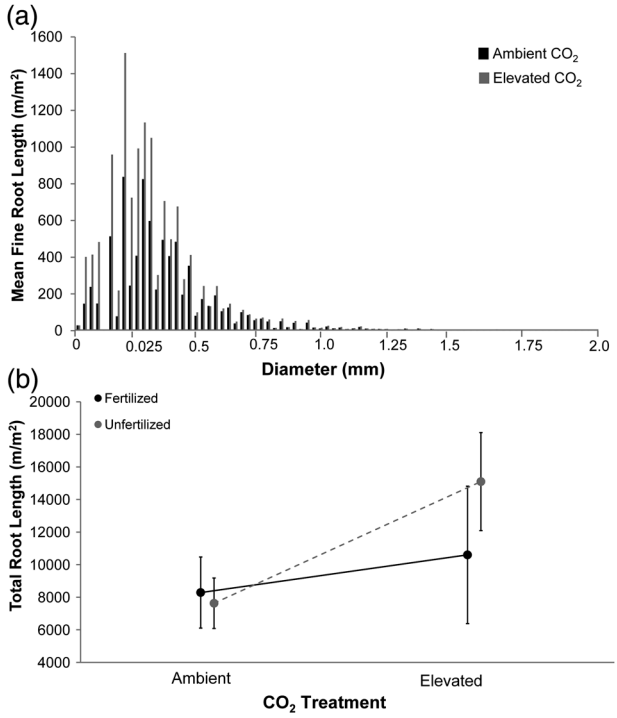

Figure 1. (a) The distribution of mean root length by diameter for fine roots. Carbon dioxide treatments are paired for each diameter category. Length values are the average length present in a treatment scaled to represent 1 m^2 to a depth of 20 cm. (b) Total root length per square meter as it is affected by the interaction between CO_2 and N fertilization treatments ($CO_2 \times N$ interaction: $P = 0.09$).

CO_2 treatments, respectively. For all roots ≤10 mm, average root diameter was significantly smaller in elevated CO_2 plots ($P < 0.001$). Average root diameters were 0.72 and 0.57 mm in ambient and elevated CO_2 plots, respectively (data not shown).

Specific root length varied significantly by root diameter ($P < 0.001$) in a non-linear fashion. The relationship between SRL and root diameter was influenced by the interaction between CO_2 and N treatments ($P = 0.05$). Nitrogen fertilization significantly decreased SRL in ambient CO_2 plots but not in elevated CO_2 (Figure 2).

Total standing crop biomass for roots ≤10 mm averaged across treatments was estimated to be 917.36 g m^{-2} to a depth of 20 cm. Standing crop biomass of roots <2.0 mm in diameter was 416.79 and 510.33 g m^{-2} for ambient and elevated CO_2, respectively (Table 1). Although this represents a 24% increase in fine root biomass under elevated CO_2, this increase was not statistically significant (Figure 3a). There was, however, significantly greater standing crop biomass for roots <1 mm in response to CO_2 fumigation in unfertilized (59%) but not fertilized plots ($CO_2 \times N$ interaction; $P = 0.09$). Root biomass was significantly lower in N-fertilized plots for both all roots ($P = 0.03$) and fine roots ($P = 0.07$). Total root biomass did not vary significantly by CO_2 treatment ($P > 0.1$).

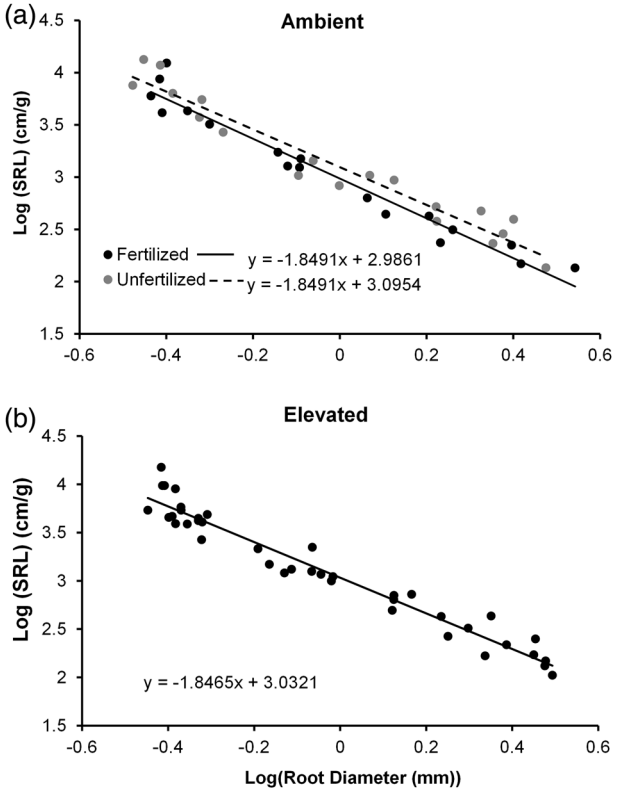

Figure 2. (a) The relationship between log(SRL) and log(Root Diameter) for (a) ambient CO_2 plots and (b) elevated CO_2 plots. Equations describing the relationship between SRL and diameter are provided. In elevated CO_2 plots, there was no effect of N fertilization on the relationship between SRL and diameter so all data are shown together.

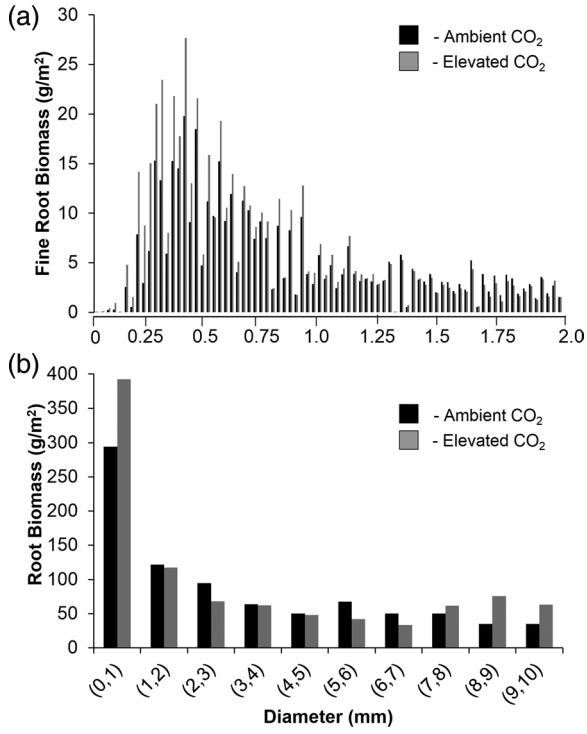

Figure 3. The distribution of root biomass by diameter for (a) fine roots, and (b) the entire root pool sampled. Carbon dioxide treatments are paired for each diameter category. Biomass values are the average biomass present in a monolith of a given treatment.

Nitrogen concentration was strongly dependent on root diameter ($P < 0.001$), and this relationship was significantly affected by N fertilization ($P < 0.001$) but not CO_2 ($P = 0.7$) treatment (Figure 4a). Together, root diameter and N treatment explained 74% of the variation in [N] in roots at this site. Carbon concentration increased significantly with root diameter in a non-linear fashion ($P < 0.001$), and [C] for a given diameter was significantly increased by N fertilization ($P = 0.02$), but was unaffected by CO_2 treatment ($P = 0.2$; Figure 4b). Carbon: nitrogen ratios increased with increasing root diameter ($P < 0.001$) and were significantly higher in the absence of N fertilization ($P < 0.001$; Figure 4c). Average C held in root systems at this site was estimated to be 432.75 and 216.52 gC m^{-2} for all roots and fine roots, respectively, with more total C held in root systems in unfertilized plots than in fertilized plots ($P = 0.03$; Table 1). Root N was 4.39 and 2.41 gN m^{-2} for all roots and fine roots, respectively (Table 1). Total N held in root systems was significantly higher in both fertilized ($P = 0.01$) and elevated CO_2 ($P = 0.04$) plots.

The total number of mycorrhizal tips estimated using the monolith processing methods employed for this experiment ranged from 6 to 14 million tips per meter square to a depth of 20 cm (Table 2). Previous estimates of mycorrhizal tip

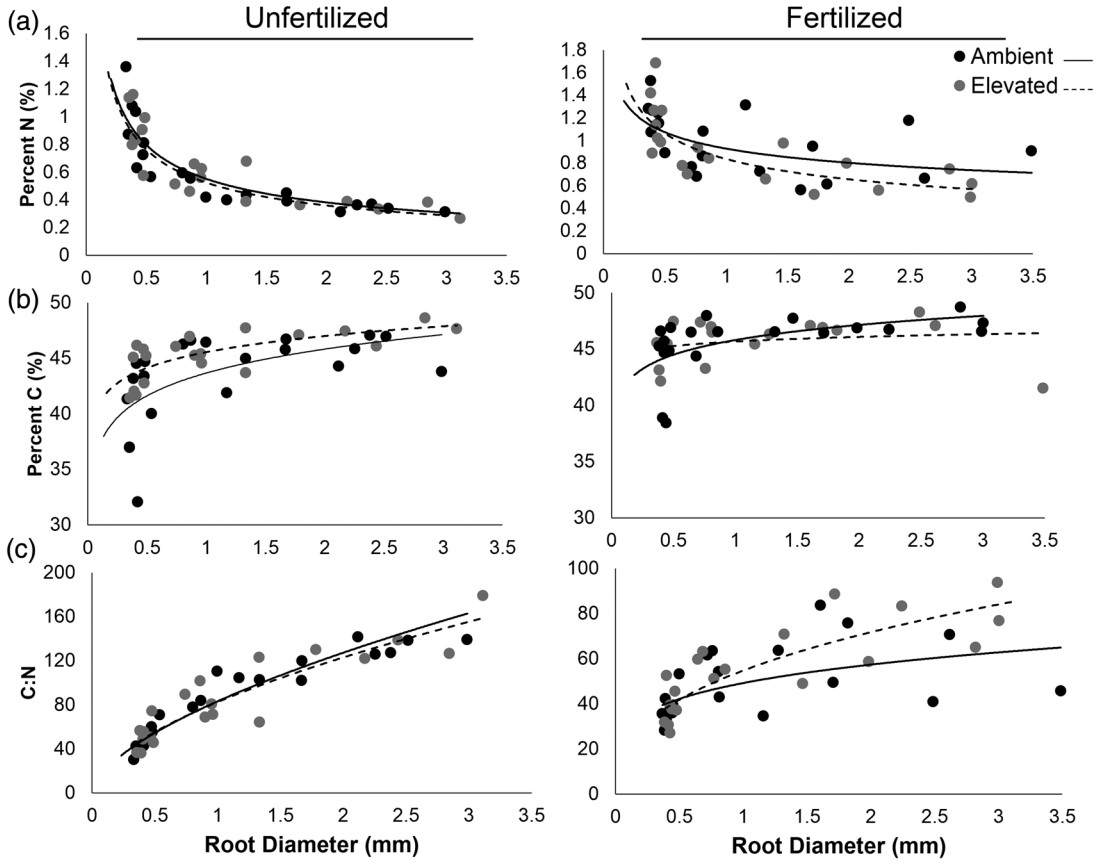

Figure 4. The relationship between (a) percent N, (b) percent C and (c) C : N ratio and diameter for each treatment group. Left and right panels represent unfertilized and fertilized plots, respectively, and CO_2 treatments are represented by different colors. Equations describing these relationships can be found in Table S1 available as Supplementary Data at *Tree Physiology* Online.

Table 2. EcM tip number and biomass (±SE) for each experimental treatment. All values are scaled to represent 1 m^2 to a depth of 20 cm.

		Ambient/unfertilized	Ambient/fertilized	Elevated/unfertilized	Elevated/fertilized
	N	3	3	3	3
With screens	Tip number (tips m^{-2})	6,008,724 (1,897,161)	6,573,078 (991,684)	14,098,375 (2,072,377)	7,832,746 (4,262,779)
	Biomass (g m^{-2})	233.74 (73.80)	347.06 (52.36)	620.33 (91.18)	287.46 (156.44)
Without screens	Tip number (tips m^{-2})	1,139,859 (275,708)	1,277,891 (426,094)	1,862,279 (416,448)	1,341,490 (327,173)
	Biomass (g m^{-2})	44.34 (10.73)	67.47 (22.50)	81.94 (18.32)	49.23 (12.01)

numbers in bulk soil for *Pinus* species have not considered the tips that detach from branches during processing (Helmisaari et al. 2009). To provide EcM tip count estimates comparable to previous studies, we also calculated the total number of EcM tips without including those caught by the 0.5- and 1-mm screens (Table 2; Figure 5a). These estimates ranged from 1.1 to 1.9 million tips m^{-2} and were not significantly affected by CO_2 or N treatment. Total biomass held in EcM tips attached to root branches ranged from 44.3 to 81.9 g m^{-2}, which constituted an average of 12.8% of fine root biomass in the monoliths. Biomass estimates for mycorrhizal tips when the 0.5- and 1-mm screens were included ranged between 233.7 and 620.3 g m^{-2}. Although no main effects of CO_2 or N treatment were found for EcM tip biomass, a significant $CO_2 \times N$ interaction existed for mycorrhizal tip biomass when screen material was not included; EcM biomass tended to increase in response to elevated CO_2 in unfertilized plots (85%), but the opposite trend was seen in fertilized plots (–27%; $CO_2 \times N$ interaction, $P = 0.09$; Figure 5b).

Discussion

The data presented here represent root systems exposed to the longest running field-based CO_2 enrichment experiment to date, showing the cumulative effects of 14 years of CO_2 manipulation and 6 years of N fertilization. Due to limitations in destructive sampling during the experiment's active period, this study also employed a large sample volume relative to destructive sampling estimates previously made at this site.

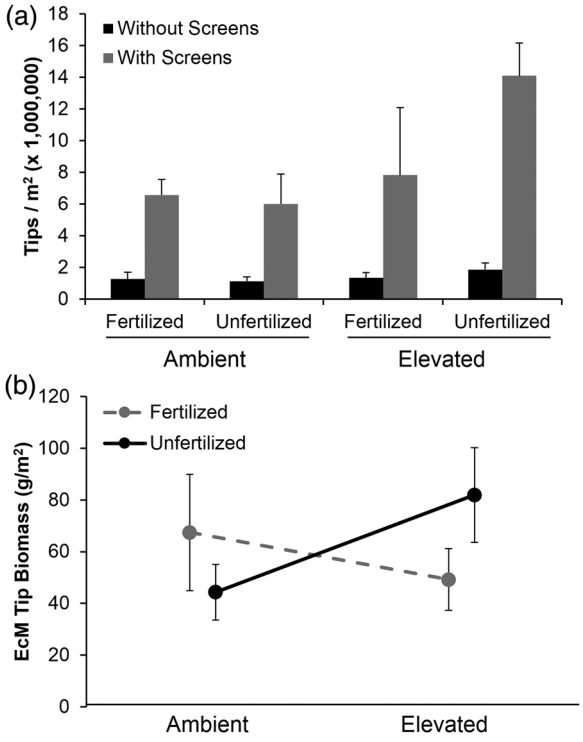

Figure 5. (a) The number of EcM root tips estimated per square meter to a depth of 20 cm for each experimental treatment. Different colors represent estimates made either including or disregarding the material caught in 0.5 and 1 mm mesh screens during monolith processing. (b) Reaction norm plot illustrating the interaction of CO_2 and fertilization on total biomass of EcM root tips. Material caught in screens during monolith processing was not included in this analysis.

Given the long treatment exposure period, the level of detail employed in sample processing and the relatively large sample volume, this study serves as the most complete assessment of roots within this diameter range at Duke FACE.

Increases in total root length and in biomass of fine roots <1.0 mm in diameter support the common finding that increased fixation of atmospheric C results in greater allocation of C to root systems, allowing roots to more effectively forage for additional water and nutrients to support the increase in leaf area and photosynthesis under elevated CO_2 (McCarthy et al. 2007, Ellsworth et al. 2012). This increase in root length under elevated CO_2 was largely driven by an increase in fine root length, which has also been observed using minirhizotrons (Pritchard et al. 2008). Although root length using destructive methods has not been previously reported, soil cores at this site (Matamala and Schlesinger 2000, Jackson et al. 2009, Hofmockel et al. 2011) and other FACE and open-top chamber CO_2 manipulation experiments indicate an increase in fine root biomass (Nie et al. 2013).

Models of C allocation predict that trees more limited by N are expected to maintain a larger population of fine roots compared with fertilized trees (Lacointe 2000, Nadelhoffer 2000). Empirical evidence for effects of fertilization on root production are more mixed (Brassard et al. 2009), with studies conducted on *P. taeda* trees finding either negative responses (Albaugh et al. 2004, Will et al. 2006) or no response (Lee and Jose 2003, Coyle et al. 2008) of fine roots to fertilization. Here, we found a negative response of root biomass to N fertilization for all roots <10 mm. The influence of N availability on root length, however, was more complicated. Our results indicate that CO_2 enrichment increased fine root length in unfertilized plots while fine root length was unaffected by CO_2 enrichment in fertilized plots. A similar interaction was noted for the response of mycorrhizal tips in a companion study by Pritchard et al. (2014) and by a study on root exudation by Phillips et al. (2011).

Because the smallest pool of fine roots makes up almost all of the length of a root system but very little of its biomass (King et al. 2002, Figure 1), our interpretations of the effects of experimental treatments may be very different depending on the root trait measured. The difference in the response of length and biomass in this study highlights the need for researchers to measure both of these traits when assessing the effects of experimental treatments on root systems, as these two metrics may indicate very different physiological and ecological responses such as a shift in allocation from permanent structural tissue pools to more ephemeral absorptive roots (King et al. 2002, Albaugh et al. 2004). The majority of fine root studies measure biomass, rather than root length density, due to its relative ease of measurement. It is important to keep in mind that root function is determined primarily by surface area—a metric best captured by length, not biomass. Had the current study focused only on root biomass, a large increase in root exploration, surface area and uptake capacity in response to experimental treatment would have been missed. For example, the shift toward a smaller diameter fine root population likely explains the observation by Drake et al. (2011) of greater N uptake per unit of fine root biomass production in CO_2 enriched compared with ambient plots at this site. Therefore, the contribution of greater fine root surface area production to the delay of progressive N limitations to long-term CO_2-enrichment, at Duke FACE and elsewhere, should not be overlooked. The issue of measuring biomass vs. length may prove critical for forest and global C-modeling exercises that should seek to incorporate feedbacks regarding tree growth responses to atmospheric CO_2-enrichment and access to soil resource pools by those trees.

Specific root length at this site was dependent on the interaction of both experimental treatments. Under ambient CO_2 conditions, SRL was lower in N-fertilized compared with unfertilized plots, consistent with the findings of a meta-analysis by Ostonen et al. (2007). However, there was no fertilization effect on SRL of roots grown under elevated CO_2. The increase in SRL for a root of a given diameter indicates a decrease in root tissue density that is likely attributable to a change in root cell wall thickness, alterations in root cell sizes or to shifts in the anatomical arrangement of root tissue types (i.e., relative allocation to xylem, phloem or cortex tissue).

Although not statistically significant in this study, the general increases in fine root biomass under elevated CO_2 are consistent with previous findings at the Duke FACE site (Matamala and Schlesinger 2000, Jackson et al. 2009, Drake et al. 2011), and agree with the results of increased below-ground plant biomass from other CO_2 manipulation studies (Ainsworth and Long 2005, de Graaff et al. 2006, Norby and Zak 2011). For example, the 24% increase in biomass of fine roots <2.0 mm in diameter under elevated CO_2 seen here closely resembles the increase in fine root biomass seen by Jackson et al. (2009) using soil cores (24%) and Pritchard et al. (2008) using minirhizotrons (23%). Our data are partially validated by these similar overall estimates of fine root biomass at this site, despite the markedly different sampling methods. The significant (59%) increase in biomass of the finest root pool (<1.0 mm) under elevated CO_2 in unfertilized plots indicates greater allocation to absorptive tissue within the root system. The first three root orders for *P. taeda* trees at this site are all <1 mm in diameter (K.V. Beidler, B.N. Taylor, A.E. Strand, E.R. Cooper, M. Schonholz, S.G. Pritchard, unpublished), and it is these three root orders that are thought to account for the majority of resource uptake within the root system (Guo et al. 2008). Although this response represents a modest contribution to the C budget of this forest (Drake et al. 2011), it could be critical over long time periods for balancing the ecosystem-scale stoichiometry of carbohydrates with availability of mineral nutrients. Focusing only on the traditional definition of fine roots (<2.0 mm) may, therefore, be too coarse a metric for assessing changes in biomass allocation to root uptake capacity in response to experimental treatments.

The similarity in magnitude of the CO_2 effect on root biomass between our results and previous studies, coupled with the large increase in root length density and biomass of the finest root pool in CO_2-enriched compared with ambient plots under unfertilized conditions, indicates a sustained stimulatory effect on fine roots through the entire period of CO_2 fumigation. Early assessments of fine root biomass at this site showed a much larger effect of elevated CO_2 (86%; Matamala and Schlesinger 2000) prior to full canopy closure. The decreased but temporally consistent effect of CO_2 on fine root standing crop following canopy closure is likely due to the consistent 18–21% increase in NPP at this site (McCarthy et al. 2006). In contrast, Day et al. (2013) reported an increase in fine root length in a CO_2-enriched scrub-oak ecosystem compared with control plots prior to what they termed 'root closure.' After root closure in this Florida scrub system, the positive CO_2 effect disappeared. Similarly, fine root standing crop at ORNL FACE showed a large response to elevated CO_2 for a period early in the experimental treatment, but this response disappeared by the end of the experiment (Norby et al. 2004, Ledford et al. 2008). At Rhinelander FACE, increases in root biomass were sustained throughout the experiment (King et al. 2001, Pregitzer et al. 2008), although it is important to note that this forest was relatively young with rapidly expanding root systems. It has been suggested that positive responses of root biomass to elevated CO_2 may be diminished or absent in mature forests, as was reported by Bader et al. (2009). Thus, it seems that fine root responses to elevated CO_2 may be dependent on forest age, specifically relative to canopy and root closure.

Our data showed a decrease in [N] with increasing root diameter, consistent with previous studies (e.g., Iversen et al. 2008, Jackson et al. 2009). Also consistent with previous work was the increase in [N] in roots exposed to fertilization treatment. Interestingly, we also found a slight but significant increase in [C] in N-fertilized plots. First-order absorptive root tips have been shown to contain significantly lower concentrations of C and cellulose than root orders two and three (Guo et al. 2004, 2008, Valenzuela-Estrada et al. 2008), despite having similar diameters (K.V. Beidler, B.N. Taylor, A.E. Strand, E.R. Cooper, M. Schonholz, S.G. Pritchard, unpublished). Thus, it is possible that the increase in [C] under N fertilization seen in this study is due to a reduction in the abundance of first-order root tips relative to second- and third-order roots of the same diameter in fertilized plots. Nitrogen fertilization apparently shifted allocation toward larger diameter structural roots.

The higher [C] of N-fertilized roots notwithstanding, the C : N ratio of root tissue averaged across root diameters was greater in roots grown under unfertilized conditions than those exposed to N fertilizer, an effect driven by the proportionately large increase in [N] in large-diameter fertilized roots (Figure 4c). Roots in this study did not, however, exhibit higher C : N ratios in plots exposed to elevated CO_2, which is consistent with several other studies at this site (Matamala and Schlesinger 2000, Jackson et al. 2009).

We approached the estimation of EcM root tips in two ways, each with its own strengths and limitations. We estimated the mycorrhizal tip number and biomass by either including or omitting the many tips that were detached from root branches during root extraction from soil, which were subsequently caught on fine mesh screens. Our method of estimating mycorrhizal root tips without considering tips that were detached during washing most closely matches previous methods of estimating EcM root tips in bulk soil (Helmisaari et al. 2009). On the other hand, our estimates of mycorrhizal tips that include the tips caught in the 0.5- and 1-mm screens are substantially larger than previous estimates at sites dominated by EcM tree species (Helmisaari et al. 2009). Many root studies that differentiate live from dead roots do so based on tensile strength, and it is possible that primarily dead or dying roots with reduced tensile strength were detached from root branches and caught in the screens during processing in the current study. Furthermore, our estimates of the contribution of mycorrhizal tips to total

fine root biomass, based on estimates derived by omitting detached tips, closely agree with those made using minirhizotrons at this site (Pritchard et al. 2014). On the other hand, we recognize that this method likely underestimates the total live mycorrhizal tip pool by disregarding the live but fragile mycorrhizal tips that separate from root branches during monolith processing. These two estimates likely represent the range of values for total mycorrhizal tips and their biomass at this site.

Reports of treatment effects on mycorrhizae based on different sampling methods for this study site have proven variable. Although Garcia et al. (2008) showed a significant 14% increase in EcM colonization of pine roots at Duke FACE, more recent analyses of mycorrhizal root tips using minirhizotron images indicated that increases in mycorrhizal tip production at this site are likely transient in nature and more closely linked to climatic conditions than [CO_2] (Pritchard et al. 2014). Non-significant CO_2 effects on ^{15}N fractions in plant material at this site reported by Hofmockel et al. (2011) lead to the conclusion that a large, consistent stimulatory effect of CO_2 enrichment on mycorrhizal fungi is unlikely given that greater isotopic fractionation would be expected if increasing CO_2 were increasing the amount of mycorrhizal-derived plant N. We did, however, observe a significant $CO_2 \times N$ interaction (Figure 5b) for mycorrhizal tip biomass in the current study. In unfertilized plots, elevated CO_2 was associated with higher mycorrhizal tip biomass than ambient CO_2, whereas in fertilized plots, mycorrhizal tip biomass was higher under ambient rather than elevated CO_2. Our results obtained from monolith sampling that are reported here contrast with our recent minirhizotron study by Pritchard et al. (2014) which, although also reporting a similar $CO_2 \times N$ interaction, found that the CO_2 response in unfertilized plots noted in 2008 had dissipated by 2010 due to mycorrhizal tip mortality. The differing results in these two studies is likely attributable to the inability of minirhizotrons to sufficiently sample structures from the organic soil horizon and shallow layers of mineral soil (Hendrick and Pregitzer 1996); we observed a high density of fine roots and mycorrhizal structures in these layers when processing monoliths (personal observation). Increases in mycorrhizal tip biomass and fine root length in response to elevated CO_2 in unfertilized plots were 85 and 98%, respectively, indicating that increases in mycorrhizal colonization are not the only source of increased N under elevated CO_2, but rather that these two mechanisms may be working simultaneously to increase N uptake. The significant interaction effect on mycorrhizal tip biomass but insignificant main treatment effects on total tip number also suggest changes in the size and dimensions of mycorrhizal root tips in response to CO_2 and N manipulation as seen in Pritchard et al. (2014). This may be the result of a shift in mycorrhizal community structure, as has been suggested to occur at the Rhinelander FACE site (Andrew and Lilleskov 2009, Pregitzer and Talhelm 2013).

Conclusions

The findings of this study are qualitatively consistent with previous work showing increased root length (Pritchard et al. 2008) and an increase in small diameter root biomass (Jackson et al. 2009) under elevated CO_2 at Duke FACE. Despite a non-significant CO_2 effect on the traditional fine root biomass category (<2.0 mm), the similarity between our current estimates and those made previously at this site, coupled with the significant increase in root length density and biomass of roots <1.0 mm, indicates that the effects of elevated CO_2 on fine roots have been sustained to some degree even after 14 years of fumigation. Increases in mycorrhizal tip biomass, but not tip number in unfertilized, elevated CO_2 plots, suggest a shift in tip dimensions likely due to changes in the mycorrhizal fungal community. In addition to the shift towards a finer fine root population, data on SRL, [N] and [C] also suggest changes in how root systems forage for nutrients and utilize C and N resources at the individual root level under elevated CO_2. The significant stimulation of fine root length density, biomass of fine roots <1.0 mm in diameter and the decrease in average root diameter, in the absence of significant effects on biomass of fine roots <2.0 mm in diameter, indicate an adjustment in the architecture of fine root systems at the forest scale that likely contributed significantly to greater N accumulation in biomass of CO_2-enriched trees at this research site (Finzi et al. 2007, Drake et al. 2011). Finally, it should be pointed out that studies that measure fine root biomass, rather than root length density, might be unable to detect an important acclimation response to long-term exposure to CO_2 enrichment—a response that has the potential to facilitate increased soil resource uptake allowing for a sustained response to elevated CO_2.

Supplementary data

Supplementary data are available at *Tree Physiology* online.

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Conflict of interest

None declared.

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