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Research

Changes in root architecture under elevated concentrations of CO_2 and nitrogen reflect alternate soil exploration strategies

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Summary

- Predicting the response of fine roots to increased atmospheric CO_2 concentration has important implications for carbon (C) and nutrient cycling in forest ecosystems. Root architecture is known to play an important role in how trees acquire soil resources in changing environments. However, the effects of elevated CO_2 on the fine-root architecture of trees remain unclear.
- We investigated the architectural response of fine roots exposed to 14 yr of CO_2 enrichment and 6 yr of nitrogen (N) fertilization in a *Pinus taeda* (loblolly pine) forest. Root traits reflecting geometry, topology and uptake function were measured on intact fine-root branches removed from soil monoliths and the litter layer.
- CO_2 enrichment resulted in the development of a fine-root pool that was less dichotomous and more exploratory under N-limited conditions. The per cent mycorrhizal colonization did not differ among treatments, suggesting that root growth and acclimation to elevated CO_2 were quantitatively more important than increased mycorrhizal associations.
- Our findings emphasize the importance of architectural plasticity in response to environmental change and suggest that changes in root architecture may allow trees to effectively exploit larger volumes of soil, thereby pre-empting progressive nutrient limitations.

Introduction

Forests account for *c.* 50% of terrestrial net primary production (NPP) and consequently have a large impact on terrestrial carbon (C) cycling (Bonan, 2008; Norby & Zak, 2011). Assessing the contribution forests make to the global C cycle has become increasingly important in the face of climate change. Large-scale climate change studies have shown that CO_2 enrichment stimulates primary productivity in forests (Norby *et al.*, 2005). However, the ability of trees to sustain higher levels of NPP in response to elevated CO_2 will probably depend on the capacity of their fine roots to supply sufficient belowground resources (Luo *et al.*, 2004) . Fine roots, root orders one through four with a diameter < 2 mm, serve as the major pathway by which plants obtain soil nutrients. In addition to influencing C uptake of forest canopies by supplying soil resources, fine roots are also a quantitatively important component of the global C cycle; as much as one-third of total global primary productivity is allocated to fine-root construction and maintenance (Jackson *et al.*, 1997), and much of the C stored in soil is thought to be derived from roots (Schmidt *et al.*, 2011; Phillips *et al.*, 2012). Previous work indicates that fine-root systems are sensitive to changes in atmospheric CO_2 concentration ($[CO_2]$) (Lukac *et al.*, 2003; Pritchard, 2011). Thus, an understanding of the morphology, size and function of fine-root systems is essential to predicting the role of forests in a changing global climate.

The efficacy of fine-root function is directly related to root system architecture (Harper *et al.*, 1991; Lynch, 1995). Architecture encompasses both root system shape and structure, shape being the position and distribution of roots in the soil, and structure referring to individual root segments and their organization within the system (Hodge *et al.*, 2009). Just as the arrangement and location of leaves on a branch influence the capture of CO_2 and sunlight, the architecture of fine roots affects nutrient and water absorption (Bentley *et al.*, 2013). Because root systems are complex and perform multiple tasks simultaneously, it can be difficult to link root system architecture and function (Farnsworth & Niklas, 1995; Pregitzer *et al.*, 2002). Connectivity among root segments (branching pattern), as well as the position of a particular segment on the larger root branching system (root order), plays a major role in determining the function of an individual root (Pregitzer *et al.*, 2002). For example, whether a root segment is a distal, first-order root or an interior, fifth-order root can mean the difference between a function that is largely absorption or transport and a longevity that can range from days to decades (Pregitzer *et al.*, 2002; Eissenstat & Volder, 2005; Guo *et al.*,

2008). Differences in branching pattern can have differential effects on the ability of a fine-root system to capture relatively mobile versus immobile soil nutrients (Fitter, 1987; Fitter & Stickland, 1991; Williamson *et al.*, 2001).

Topology and geometry are functionally significant components of root architecture (Fitter, 1986). Because architecture itself is difficult to measure, measures of topology (branching pattern) and geometry (inter-branch distances and branching angles) are more commonly used to describe root system structure (Fitter, 1987; Harper *et al.*, 1991; Fitter *et al.*, 1991; Lynch, 1995). For a summary of commonly used topological characteristics, see Table 1. Roots can vary significantly in their topology between two extreme branching patterns, dichotomous and herringbone (Fitter, 1987). A herringbone pattern consists of root branches primarily confined to a main axis, whereas dichotomous structures are more randomly branched with each branch leading to a similar number of distal root segments (Fig. 1; Fitter *et al.*, 1991). Along with topology, inter-branch distances, branch trajectory and the frequency with which branching occurs (i.e. link length, branch angle, and branch density, respectively) affect nutrient interception and absorption (Fitter *et al.*, 1991; Harper *et al.*, 1991; Arredondo & Johnson, 1999). Link length and branching angle, for example, affect overlap of nutrient depletion zones (Fitter & Stickland, 1991).

Branching pattern is determined by genetic constraints, soil nutrient availability, interactions with other soil organisms, soil physical properties, and plant carbohydrate availability and allocation patterns (López-Bucio *et al.*, 2003; Rich & Watt, 2013). Herringbone systems are thought to be more efficient at intercepting mobile nutrients, such as nitrate (Fitter, 1987; Fitter *et al.*, 1991; Dunbabin *et al.*, 2003). Mobile nutrients move by mass flow and are better captured through coarse-scale foraging, or extensive soil exploration (Fitter *et al.*, 1991; Lynch, 2005). Because herringbone systems are thought to forage for nutrients on a larger scale they involve greater C costs (Fitter, 1985, 1987; Fitter *et al.*, 1991). By contrast, dichotomous systems are better at acquiring diffusion-limited resources, such as phosphorus, through fine-scale foraging or intensive soil exploration (Fitter, 1987). Thus, the exploitation efficiency associated with intensive versus extensive soil exploration depends on the resource needs of the plant and the conditions of the local soil environment (Taub & Goldberg, 1996; Lynch, 2005).

The form and function of a root system should be adapted to use C effectively. This means striking a balance between the costs

Table 1 Definitions of individual root characteristics and root system topological characteristics

Individual root characteristics	Abbreviation	Definition
Link*	L	A segment of root between two nodes or a node and a tip
Base link*	BL	The link from which all other links descend
Internal link*	IL	A segment of root between two nodes (internodes)
External link*	EL	A terminal segment or tip

Topological characteristics	Symbol	Definition
Magnitude*	μ	The number of root tips (exterior links) in the system
Altitude*	a	The number of links in the longest single path from an external link to the base link
Maximum altitude*	max(a)	The theoretical altitude for a system of given magnitude that has a completely herringbone topology $\max(a) = \mu$
Minimum altitude*	min(a)	The theoretical altitude for a system of given magnitude that has a completely dichotomous topology $\min(a) = \lfloor \log_2(\mu - 1) \rfloor + 2$
External path length*	P_e	The sum of the number of links in all paths from each external link to the base link
Maximum external path length*	max(P_e)	The theoretical external path length for a system of given magnitude that has a completely herringbone topology $\max(P_e) = 0.5\ (\mu^2 + 3\mu - 2)$
Minimum external path length*	min(P_e)	The theoretical external path length for a system of given magnitude that has a completely dichotomous topology $\min(P_e) = \mu\ [\min(a) + 1] - 2^{\min(a)\ -\ 1}$
Topological index*	TI	A way to quantify branching pattern using the relationship between $\log_{10}$ altitude or $\log_{10}$ external path length and $\log_{10}$ magnitude. The larger the topological index the more herringbone a system. $TI = \log_{10}(a)/\log_{10}(\mu)$ or $TI = \log_{10}(P_e)/\log_{10}(\mu)$
Topological trend† or	TT	Estimates the degree to which a system is fully herringbone (TT = 1) or fully dichotomous (TT = 0) based on the value for total exterior path length relative to max(P_e) and min(P_e). TT values are on a scale between 0 and 1. $TT = [P_e - P_e(\min)]/[P_e(\max) - P_e(\min)]$
Dichotomous branching index‡	DBI	DBI is the same calculation as TT. DBI values also range from 1, a fully herringbone topology, to 0, a fully dichotomous topology. $DBI = [P_e - \min(P_e)]/[\max(P_e) - \min(P_e)]$

*Fitter (1985, 1987).
†Trencia (1995).
‡Šmilauerová & Šmilauer (2002).

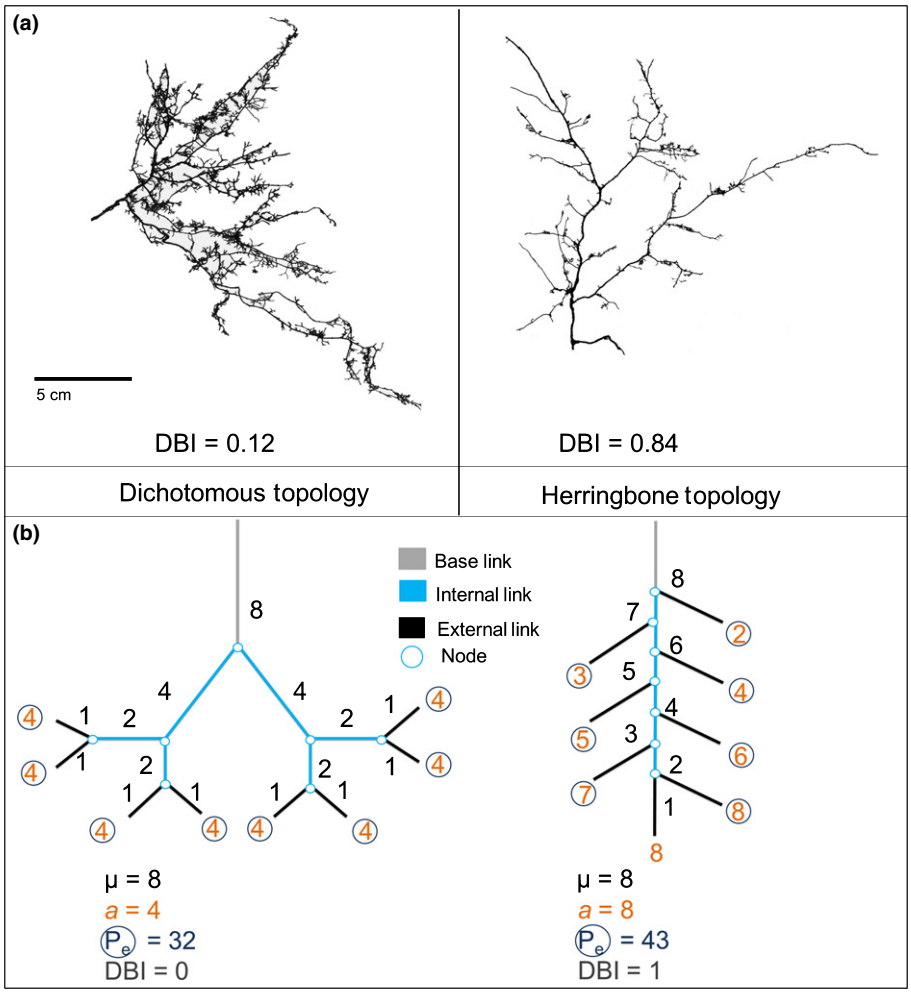

Fig. 1 Scanned images and schematic diagrams depicting dichotomous and herringbone topologies. (a) *Pinus taeda* intact fine-root branches with their respective dichotomous branching index (DBI) values. The root on the left side of the panel has a more dichotomous topology while the root on the right is more herringbone. DBI is the dichotomous branching index developed by Šmilauerová & Šmilauer (2002): DBI = (P_e − min(P_e)/((max (*Pe*) − min(*Pe*)), where P_e is external path length (see Table 1). (b) The link-based method for classifying root topology, introduced by Fitter (1987). μ is magnitude, or the number of external links; *a* is altitude, or the number of links in the longest path length. A path length is the number of links between a link and the base link. P_e or external path length is the sum of path lengths for all external links. A DBI of zero is characteristic of a perfectly dichotomous branching structure, whereas a DBI of one represents a perfectly herringbone branching structure. Note: this figure does not illustrate the classification scheme used to determine root order.

and benefits associated with different architectural schemes (Fitter, 1994). Plants exposed to higher concentrations of CO_2 are predicted to use the more readily available C in a way that optimizes the return of additional resources by exploiting nutrients on a larger scale (Eissenstat, 1992; McCarthy *et al.*, 2010; Kong *et al.*, 2014). Although the need for greater N uptake coupled with a more abundant supply of carbohydrates might lead one to predict a shift toward more herringbone root branching patterns in CO_2-enriched trees, this prediction is not supported by existing data on herbaceous plants. The few previous studies on root architectural responses to elevated [CO_2] suggest that plants build more dichotomous root systems with an increased number of laterals (Fitter & Stickland, 1991; Berntson, 1994; DeLucia *et al.*, 1997; Tingey *et al.*, 2005). These data mainly reflect responses of container-grown herbaceous species, however, and there is some evidence that root responses of trees and herbaceous plants differ (Pritchard *et al.*, 1999). To fully understand how fine roots forage for nutrients under elevated concentrations of CO_2, root architecture must be studied in ecologically relevant conditions in plant species of different functional types.

Effects of CO_2 enrichment on fine roots within intact ecosystems dominated by older, more mature woody plants have rarely been characterized. Free-air CO_2 enrichment (FACE) technology provides a unique opportunity to study plant responses to CO_2 manipulation *in situ*, on a large spatial scale, and for long time periods. Throughout the 14-yr duration of the Duke FACE experiment, increased stimulation of NPP in enriched CO_2 plots persisted despite predicted drop-offs attributable to nutrient limitations (Luo *et al.*, 2004; Hofmockel *et al.*, 2011; Norby & Zak, 2011). It is believed that the sustained increase in NPP at Duke FACE is partly the result of continued increases in nitrogen (N) uptake by plant roots (Finzi *et al.*, 2007; Drake *et al.*, 2011). In this study we quantified the long-term effects of CO_2 enrichment and soil N fertilization on fine-root architecture in an intact loblolly pine forest. We hypothesized that the known increase in N uptake by fine roots in CO_2-enriched plots corresponds with a change in fine-root topology at the Duke FACE site. In addition to root system architecture we assessed a number of root traits linked to uptake function, including root diameter, diameter as a function of branching order, specific root length (SRL) and per cent mycorrhizal colonization.

Materials and Methods

Site description

The Duke free-air carbon transfer and storage (FACTS-1) experiment was implemented in a 15-yr-old loblolly pine (*Pinus taeda*

L.) plantation in the Blackwood division of the Duke Forest, Orange County, North Carolina (35°58′N, 79°5′W) in 1996 (Schlesinger *et al.*, 2006). The pine stand was derived from 3-yr-old seedlings spaced at 2.4 × 2 m. The site was left unmanaged throughout the duration of the experiment and, as a result, a number of hardwood species became established, including sweetgum (*Liquidambar styraciflua*), red maple (*Acer rubrum*), dogwood (*Cornus florida*), and winged elm (*Ulmus alata*). Nonetheless, loblolly pine made up 98% of the forest's total basal area. Soil at the site is an Alfisol with a clay loam texture and low fertility (Schlesinger & Lichter, 2001; Lukac *et al.*, 2009).

The Duke FACE experimental has been described in detail in a number of studies (Hendrey *et al.*, 1999; Finzi *et al.*, 2007). In short, the site consists of six experimental plots, a prototype and reference plot, each 30 m in diameter. Plots were fumigated with either elevated CO_2 (200 ppm above ambient) or ambient air. The CO_2 treatment began in 1994 in the prototype and reference plots and in 1996 in the six experimental plots. CO_2 fumigation ended in November of 2010. In 1995, an NH_4NO_3 treatment was implemented in the prototype and reference plots and in 2005, the six remaining plots were split and half the subplots were fertilized with N. Plots were split by an impermeable barrier installed in soil to a depth of 70 cm; half of each plot was fertilized by annual hand-casting at a rate of 112 kg N ha^{-1} yr^{-1} (Pritchard *et al.*, 2014). Subplots were fertilized twice in 2005 (March and April) and annually (March) from 2006 through 2010 (Schlesinger *et al.*, 2006).

Root sampling

To obtain intact branching systems, fine roots were sampled from 20 cm × 20 cm × 20 cm (8000 cm^3) soil monoliths excavated from the Duke FACE site during the first week of November 2010. For the complete method of monolith extraction, see the companion study by Taylor *et al.* (2014). Generally, one monolith was sampled from the N-fertilized and unfertilized halves of each of the eight plots. For five subplots, we sampled fine-root systems from one additional monolith and data for the two subsamples for a given plot were averaged (total of 21 monoliths). Monoliths were stored at −10°C before being washed over three superimposed screens with the following mesh sizes: 3, 1 and 0.5 mm. All visible roots were removed from the 3-mm screen by hand, rinsed with deionized water and stored at 4°C until cleaned. The material caught by each of the 1- and 0.5-mm screens was recovered and stored at 4°C. Forceps were used to carefully clean roots of organic debris, fungal hyphae and rhizosphere soil. Cleaned roots were floated in a transparent tray with deionized water and imaged using an Epson Expression 10000XL scanner at 400 dpi (Epson, Long Beach, CA, USA). Multiple scans were required for each monolith. Scanned images were digitized and analyzed for length, diameter, and topology using WinRhizo image analysis software (Regent Instruments Inc., Québec, Canada).

To account for roots that detached during washing, one 5-ml subsample from the material caught by the 1- and 0.5-mm screens was collected and all roots were removed from the subsample with forceps using a dissecting microscope at ×10 magnification (SMZ-1; Nikon Inc., Melville, NY, USA). The data generated from the screen subsamples were then multiplied by the total volume of material left on each respective screen (Pierret *et al.*, 2005; Taylor *et al.*, 2014) and combined with data on intact root systems to derive average monolith root diameter and distribution of root lengths for roots with diameters < 1.0 mm.

Architectural and mycorrhizal analysis

Continuous, unbroken sections of pine roots comprising at least the three most distal root orders were considered 'intact fine-root branches' and chosen for topological analysis. The following measurements were made in WinRhizo: total length, number of root tips, external path length (P_e), altitude (a), average branching angle, number of forks and average link length. Maximum altitude (max(a)), minimum altitude (min(a)), maximum external path length (max(P_e)), minimum external path length (min(P_e)), and topological index (TI) were calculated using methods outlined in Fitter (1987) (for explanations, see Table 1). The number of root tips was divided by total intact fine-root branch length to account for differences in branch size and provide a measure of tip density (tips cm^{-1}). Branching angle is defined here as the angle formed by a root and the root that it is attached to (Fitter, 1987). A root perpendicular to its higher order root would form a large angle while smaller angles would reflect a more acute angle connecting a given root to the higher root to which it is connected.

Root topology was quantified using the dichotomous branching index (DBI) presented by Šmilauerová & Šmilauer (2002), which is the same measure as topological trend (TT) first introduced by Trencia (1995; see Table 1). DBI was chosen over TT, because it has been used recently to assess root topology under elevated CO_2 (Lee-Ho *et al.*, 2007). TI and DBI are similar ways of representing the branching pattern of a root system; however, DBI is reported to be scale independent (Šmilauerová & Šmilauer, 2002). Further description of architectural traits measured can be found in Table 1. Mycorrhizal tip counts were obtained on a subset of the intact fine-root branches used in the topological analyses (20 intact fine-root branches from each treatment type). Characteristic bifurcating mycorrhizal root tips were marked on scanned images and counted by hand.

Distribution of root length of different diameters and specific root length (SRL)

All roots < 1.0 mm in diameter were assigned to diameter bins created using 0.025-mm increments and root length in each diameter bin was then summed. The total length in each of these bins was then divided by the total fine-root length in the 0–1.0-mm root pool for each monolith. This allowed us to report the proportion of total fine-root length by diameter (Fig. 4c). SRL was calculated from a subset of *P. taeda* roots removed from trenches dug around monoliths during extraction (four monolith trenches per treatment). Subsequently roots were cleaned and sorted into five diameter classes ranging from 0.3 to 6 mm in

order to establish a continuous relationship between SRL and diameter as in Iversen *et al.* (2008). Roots in each diameter class were scanned as a unit and dried to a constant mass at 65°C. Total length and average diameter were measured in WinRhizo. Following the drying period, the roots in the different diameter classes were weighed and SRL was calculated by dividing the total fresh length for each diameter class by the dry mass of roots in that class.

The average diameter of roots by order was determined for roots sampled from the litter layer in November 2010, accompanying monolith extraction. To obtain intact branching systems, roots were carefully extracted from organic matter and tips were traced back to the fifth branching order. During sampling events, branching systems were collected from four different locations in each plot. Roots were stored at −10°C until cleaned with forceps and dissected under ×10 magnification. Root branches were dissected according to the ordering scheme and methods used by Fitter (1982) and Pregitzer *et al.* (2002). Dissected roots were grouped by order for each plot and scanned for length and diameter using WinRhizo.

Data analysis

The Duke FACE experiment was established as a split-plot design ($n = 3$), with statistical blocks created consisting of an elevated and ambient CO_2 plot. Plots were blocked according to natural soil N mineralization rates in each plot (Finzi & Schlesinger, 2002). Statistical models tested for the effects of two levels of N treatment, two levels of CO_2 treatment, and interactions between the treatments. Analysis of covariance was used to test for effects of CO_2 and N fertilization treatments on architectural characteristics. The natural soil N-mineralization rate was originally included as a covariate in all statistical models, but was removed when nonsignificant. To control for scale effects, intact branch length was used as a covariate for the following variables: DBI, *P*e, average link length, average tip density, and average branching angle. DBI and branch length were log-transformed to improve the model fit. A regression of log P_e on log magnitude was performed and an analysis of covariance was used to remove scale effects and test for differences in slope (topological index) among treatment groups. Because FACE experiments have a small number of replicated plots, previous studies have proposed using an $\alpha = 0.1$ (Filion *et al.*, 2000; Taylor *et al.*, 2014). Consequently, statistical significance was assigned when $P < 0.1$. All statistics were run in R version 2.15.0 (R Core Development Team, 2012).

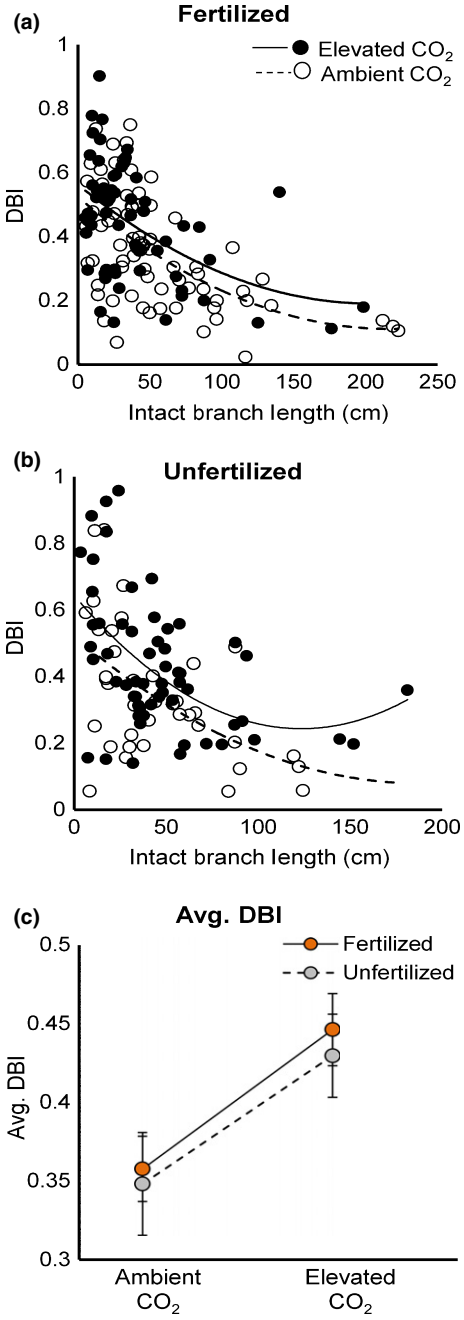

Fig. 2 The significant relationship for *Pinus taeda* between dichotomous branching index (DBI) and intact fine-root branch length ($P < 0.05$) for both (a) fertilized and (b) unfertilized subplots. Equations and r^2 values are presented in Supporting Information Table S1. (a, c) The effects of CO_2 and nitrogen (N) fertilization treatments on DBI. DBI increased significantly (less dichotomous) under elevated CO_2 in unfertilized subplots ($P < 0.05$).

Results

Architectural traits and mycorrhizal analysis

In total, 230 intact fine-root branches (10 800 cm of root length) were included in the architectural analysis. Intact branches ranged in length from 3.5 to 223 cm. DBI, P_e, link length, tip density and branching angle were weakly correlated with total branch length (see Fig. 2a,b and Supporting Information Fig. S1). For the equations and r^2 values describing the relationship between architectural traits and intact branch length, see Table S1.

DBI measures the degree to which a root is perfectly herringbone (DBI equal to 1), or dichotomously branched (DBI equal to 0). DBI decreased with increasing length and complexity of fine-root branches analyzed ($P < 0.05$). The relationship between log of DBI and log of branch length was significantly affected by a CO_2 × N treatment interaction ($P < 0.1$; see Fig. S1a). In both

N-fertilized and unfertilized subplots, DBI tended to be lower (more dichotomously branched) with increasing total branch length. Controlling for branch length, DBI increased significantly (less dichotomous) under elevated CO_2 in unfertilized subplots ($P < 0.05$; Fig. 2c). On average, DBI was 23% higher in unfertilized CO_2-enriched plots compared with unfertilized ambient plots (Table 2). In fertilized subplots, DBI did not differ significantly between CO_2 treatments ($P > 0.1$).

The calculated values for topological index confirm this change in branching pattern; the regression of $\log(P_e)$ versus $\log(\mu)$ showed marked differences in slope with respect to CO_2 treatment in unfertilized plots ($P < 0.05$) (Table 1, 2). Roots in elevated CO_2, unfertilized plots had more positive slopes, meaning they tended to have a less dichotomous, or more herringbone topology. Not surprisingly, external path length increased with increasing length of the analyzed root branches ($P < 0.001$). External path length was controlled by a complex interaction between CO_2 treatment, N fertilization, and soil N-mineralization rate. In general, external path length increased more rapidly with the length of the branch analyzed under CO_2-enriched conditions than under ambient conditions.

Average root tip density and link length decreased as intact branch length increased ($P < 0.05$), while average branching angle increased with branch length ($P < 0.05$; Fig. S1). The interaction between CO_2 and N treatments had a significant effect on average root tip density (number of root tips per cm of fine-root length; $P < 0.001$) and average link length ($P < 0.05$). Tip density was reduced by CO_2 enrichment in unfertilized subplots but was increased in fertilized plots compared with the ambient treatment (Fig. 3a). We observed the opposite for average link length; link length was greater in elevated compared with ambient CO_2 conditions under unfertilized conditions, whereas link length was lower in CO_2-enriched fertilized subplots (Fig. 3b). Finally, a $CO_2 \times N$ interaction was also noted for average branching angle ($P < 0.05$; Fig. 3c). In unfertilized plots, average branching angle decreased by 3% in response to elevated CO_2 ($P < 0.05$). The average number of mycorrhizal tips per cm did not differ significantly between treatments ($P > 0.1$; Table 2).

Per cent total root length, average diameter and SRL

Relationships between per cent total root length and diameter differed significantly between CO_2 treatments and between fertilizer treatments in ambient CO_2 plots ($P < 0.05$; Fig. 4c). In CO_2-enriched plots, there was a shift toward finer diameter roots compared with the distribution of root length in ambient plots (Fig. 4c). Of the total root length sampled from the soil monoliths, 99% had a diameter < 2 mm and a substantial portion of the length came from the screen samples (60%; Taylor *et al.*, 2014). The average diameter of fine roots (orders one through five) ranged from 0.2 to 1.8 mm. Mean root diameter increased with branching order (Fig. 4b), but did not differ among treatments ($P > 0.1$). The average diameter of the entire pool of roots present in monoliths was significantly smaller in elevated CO_2 plots ($P < 0.001$; Fig. 4a). SRL decreased with increasing root order but was not affected by main effects of CO_2 or N fertilization, or their interactions ($P > 0.1$; Table 2).

Table 2 Means and standard errors of specific root length (SRL), architectural traits, and mycorrhizal colonization for *Pinus taeda* exposed to elevated and ambient CO_2 and fertilized and unfertilized conditions

	Ambient CO_2		Elevated CO_2	
	Unfertilized	Fertilized	Unfertilized	Fertilized
N[1]	72	39	63	60
Intact branch length (cm)	55.6 (5.5)	44.9 (5.4)	39.3 (5.0)	47 (4.7)
SRL (cm g^{-1})	2144 (99)	1814 (228)	2288 (303)	2357 (210)
Architectural traits				
Number of links	498 (64)	371 (45)	369 (50)	400 (47)
Number of tips[2]	151 (16)	136 (19)	115 (15)	123 (13)
P_e	5085 (833)	3706 (671)	4072 (831)	4250 (730)
Tip density (tips cm^{-1})	2.82 (0.10)	3.24 (0.19)	3.07 (0.12)	2.65 (0.11)
Link length (cm)	0.139 (0.006)	0.125 (0.005)	0.128 (0.006)	0.141 (0.005)
Link branching angle[3]	50.3 (0.34)	54.1 (0.39)	50.7 (0.29)	49.6 (0.30)
DBI	0.358 (0.02)	0.349 (0.03)	0.446 (0.02)	0.429 (0.03)
TI[4]	1.50	1.41	1.58	1.59
Mycorrhizal colonization				
N[1]	20	20	20	20
Mycorrhizal tips (number cm^{-1})	4.66 (0.73)	4.39 (0.34)	4.38 (0.29)	4.82 (0.63)

Means (1 SE).
[1]The number of intact fine-root branches used in each analysis.
[2]WinRhizo recognizes all root endings as root tips.
[3]Link branching angle is the angle between the link and the extension of the preceding link; for a visual representation, refer to branch angle in Fitter (1987).
[4]The slope of the linear regression of $\log_{10}(P_e)$ versus $\log_{10}(\mu)$, where P_e is external path length and μ is magnitude. The slope of the relationship is used as a topological index. For definitions of architectural traits, see Table 1.

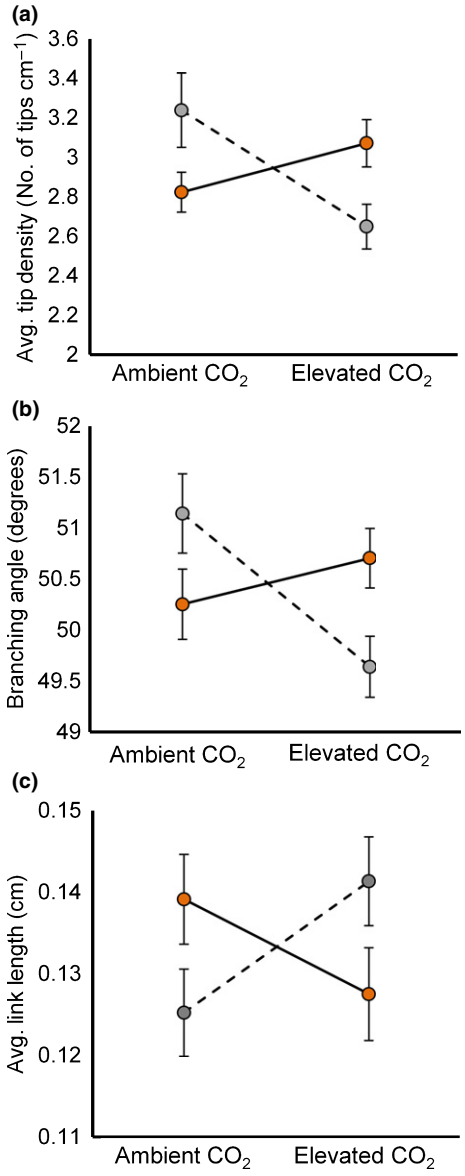

Fig. 3 The effect of CO_2 and nitrogen (N) fertilization treatments on *Pinus taeda* (a) average tip density (b) average link length and (c) average branching angle. Red circles, fertilized; gray circles, unfertilized. The interaction between CO_2 and N treatments had a significant effect on average tip density (number of tips cm^{-1}; $P < 0.001$), link length ($P < 0.05$) and branching angle ($P < 0.05$) after correcting for the effects of branch length.

Discussion

Major findings

The results reported in this study indicate that 14 yr of CO_2 enrichment altered fine-root architecture at the Duke FACE site. In the absence of N fertilization, roots grown under elevated CO_2 adopted a higher DBI, suggesting a shift toward more extensive soil exploration. The interaction between CO_2 and N treatments often had contrasting effects on root system architecture, a trend consistent with the findings of Tingey *et al.* (2005). Roots in N-fertilized, CO_2-enriched plots had shorter links and greater root tip densities compared with roots in N-fertilized ambient plots. Inversely, roots in unfertilized, CO_2-enriched plots exhibited longer links with smaller branching angles and fewer tips per cm of fine-root length compared with unfertilized ambient plots. These findings suggest that trees exposed to elevated concentrations of CO_2 can produce alternate fine-root foraging strategies and that these foraging strategies are heavily dependent upon soil N fertility. The average diameter of roots of a given order, in contrast, did not vary significantly with CO_2 and N fertilization. Similarly, no treatment effects were found for SRL or mycorrhizal colonization. These results emphasize the importance of architectural plasticity in response to environmental change and have implications for understanding feedbacks between rising atmospheric CO_2 and N biogeochemistry.

Root architecture

Trees are known to exhibit plasticity in the deployment of fine roots and mycorrhizal structures in order to maximize resource acquisition in variable environments (Lõhmus *et al.*, 2006; Ostonen *et al.*, 2011). Lõhmus *et al.* (2006) suggested that the two dominant modes of acclimation by fine roots are either: (1) to become more extensive by increasing fine-root length and biomass and adopting a more herringbone rooting habit, or (2) to mine soil more intensively by increasing mycorrhizal tip proliferation and exploiting a given volume of soil more thoroughly (i.e. by adopting a more dichotomous branching habit). In our study, fine-root branches became less dichotomous (had a higher DBI) and had longer links, reduced root tip density, and smaller branching angles under CO_2 enrichment (in unfertilized subplots), suggesting a shift toward a more extensive rooting habit.

In contrast to our results, prior studies have shown that exposure to elevated CO_2 resulted in a shift from herringbone to more dichotomously branched root systems in *Arabidopsis thaliana* (Lee-Ho *et al.*, 2007), *Senecio vulgaris* (Berntson & Woodward, 1992), and *Phaseolus vulgaris* (Nielsen *et al.*, 1994). This inconsistency could be attributable to a difference in methodology. Previously, the effects of CO_2 on topology were tested on individually grown seedlings in controlled environments (Nielsen *et al.*, 1994; Lee-Ho *et al.*, 2007). While these studies provide valuable insight into root growth patterns, they omit the effects of neighboring root systems. Herringbone topologies are thought to reduce inter- and intra-plant competition (Fitter *et al.*, 1991). Small growth containers can also obscure how roots grow and are deployed in response to elevated CO_2 (Berntson *et al.*, 1993). Richards & Rowe (1977) found that restricted soil volumes resulted in shorter, more densely branched root systems. Increased rooting volume and competition could explain the discrepancy between our results and previous findings.

Alternatively, most previous studies reporting more dichotomous roots were conducted on herbaceous species that have been shown to respond differently to elevated [CO_2] compared with trees. A review of root responses of crops indicated that CO_2 enrichment results in increased root proliferation near the soil surface, and more horizontal root systems (Pritchard & Rogers, 2000), while a recent review of trees indicated that CO_2

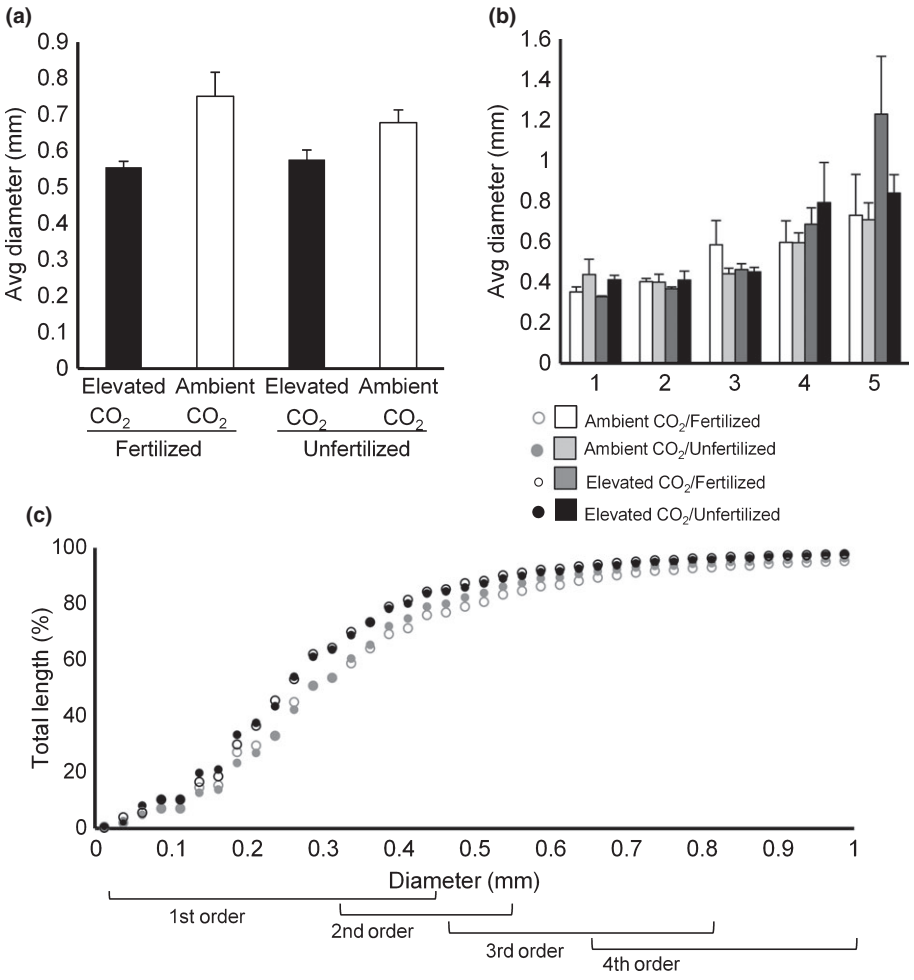

Fig. 4 The effect of CO_2 and nitrogen (N) fertilization treatments on *Pinus taeda* (a) average root diameter (mm) for all roots extracted from 20 × 20 × 20 cm monoliths (average values include both fine and coarse roots found in soil, and, although mostly pine, may also include species other than pine); (b) average diameter of the five most distal root orders of pine (intact root branches were extracted from the litter layer at the same time that the monoliths were collected); and (c) the relationship between percentage total length and root diameter for all roots with a diameter < 1.0 mm sampled from monoliths (these data represent all roots extracted from soil and therefore contain pine in addition to roots of other species.) Average diameter of all roots (a) was significantly smaller in elevated CO_2 plots ($P < 0.001$), average diameter of pine by order (b) was not affected by CO_2 or N fertilization ($P > 0.1$), and relationships between per cent total root length and diameter (c) differed significantly between CO_2 treatments and between fertilizer treatments in ambient CO_2 plots ($P < 0.05$). Error bars, + 1 SE of the mean.

enrichment of forest systems leads to deeper rooting distributions (Iversen, 2010). The shift toward a more exploratory root growth habit observed here is a mechanism that may explain the finding of Iversen (2010) that tree roots are distributed in deeper soil when grown with atmospheric CO_2 enrichment.

Our results could also simply have stemmed from changes in overall root system size in the different treatments. CO_2 enrichment is known to increase fine-root production and the overall size of root systems (Berntson & Bazzaz, 1998; Norby & Zak, 2011). A companion study from the Duke FACE site reported a 98% increase in length of fine roots in unfertilized, elevated CO_2 subplots compared with unfertilized ambient plots (Taylor *et al.*, 2014). As root systems become larger and require more resources, a less dichotomous branching pattern may enhance the uptake of diffuse nutrients through more extensive soil exploration, as discussed previously. Therefore, shifts in topology in unfertilized, CO_2-enriched subplots might be driven by the dilution of soil nutrients in a larger and faster growing ecosystem biomass or by greater root competition caused by the substantial increase in fine-root length.

In addition to root system size, branching patterns are functionally dependent upon the geometric properties of branching structures (Berntson, 1995). In our study, link length decreased as branching angle and root tip density increased. Our results are consistent with the findings of Berntson & Woodward (1992), who found a relationship between interior link lengths and branching angle in *S. vulgaris*; shorter links were accompanied by larger branching angles, and a more horizontal growth trajectory. As link length increased, the proliferation of root tips (tip density) decreased, which may be related to exploitation efficiency, or the volume of soil exploited per unit volume of root (Berntson, 1994). Longer links are thought to have higher exploitation efficiencies, because depletion zones are not as likely to overlap (Fitter *et al.*, 1991). Decreased frequency of root tip proliferation events along a length of root may be indicative of a more exploratory growth strategy.

We observed complicated interactive effects of CO_2 enrichment and N fertilization (Fig. 3). Roots in elevated CO_2, unfertilized plots had the highest average link length and the smallest branching angles and root tip density. Similarly, Fitter & Stickland (1991) found that, under low nutrient availability, both dicots and grasses had more herringbone roots with longer interior links. The increased availability of C coupled with a greater need for soil resources could mean that roots in unfertilized, elevated CO_2 plots are taking on a more exploratory architecture as this is the treatment that should experience the most acute soil N

limitations (Finzi *et al.*, 2007; Drake *et al.*, 2011). The smaller branching angles probably reflect more vertical growth trajectories as roots exploit deeper soil to better seek out mineral nutrients. On the other hand, fertilization caused roots in elevated CO_2 plots to have shorter links, larger branching angles and higher tip densities. This observation is in agreement with a previous study by Cruz *et al.* (1997) which found that well-fertilized carob trees (*Ceratonia siliqua* L.) had shorter, thicker roots in addition to having more lateral roots under elevated CO_2. Moreover, interior link lengths decreased and branching angles increased in *S. vulgaris* under high CO_2/high water conditions in Berntson & Woodward's (1992) study. With an abundance of C and mobile resources (i.e. N), roots seem to be utilizing a more intensive foraging strategy. The effects of N on root geometry were reversed in ambient CO_2 plots.

In addition to root system architecture, nutrient absorption is also influenced by the presence and status of mycorrhizas. In fact, increased mycorrhizal colonization has been the leading theory behind the delay in progressive N limitation at Duke FACE (Finzi *et al.*, 2007; Drake *et al.*, 2011). This idea, however, might need to be reconsidered based on our current data which indicated that colonization of fine roots by mycorrhizas was unaffected by elevated CO_2. A small or undetectable effect of elevated CO_2 on mycorrhizas is consistent with several other studies conducted at Duke FACE showing either minor or inconsistent effects through time (Hofmockel *et al.*, 2011; Pritchard *et al.*, 2014; Taylor *et al.*, 2014). It is important to note that, although an increase in mycorrhizal tip abundance was reported for Duke FACE by Taylor *et al.* (2014), this increase was smaller in magnitude compared with the increase in total fine-root length. This explains the lack of an effect on patterns of mycorrhizal colonization reported here as well as the observation that the natural abundance of ^{15}N in plant tissues in CO_2-enriched plots also does not indicate an enhanced role of ectomycorrhizas in uptake of additional N at Duke FACE (Hofmockel *et al.*, 2011). So, while the absolute number and biomass of mycorrhizal root tips were increased by elevated CO_2 in this experiment, that increase was smaller in magnitude than the stimulation of fine-root proliferation, the shift toward a smaller diameter fine-root pool, and the adjustments in fine-root branch system topology. Clearly, such changes in structure of fine roots combined with increased exudation and accelerated soil organic matter processing also reported (Phillips *et al.*, 2012; Meier *et al.*, 2015) could explain enhanced N uptake in CO_2-enriched plots in the absence of changes in the per cent mycorrhizal colonization of fine roots.

Conclusions

There is significant speculation about the potential for forest NPP to increase as atmospheric CO_2 concentrations continue to rise. While this issue remains unresolved for different forest types, we are now beginning to grasp the important role of canopy–root–mycorrhizal–soil organic matter linkages for controlling both the size of the soil C pool and the potential for elevated atmospheric CO_2 to stimulate ecosystem NPP over the long term (Calfapietra *et al.*, 2010; Drake *et al.*, 2011; Phillips *et al.*, 2013). Because fine roots are the chief interface of plants with soil (and all the nutrients it contains), the importance of this connection to forest productivity and C cycling cannot be overstated. To date, however, information on long-term fine-root responses to global changes are limited and data on fine-scale growth habits (architecture) of the most distal, smallest diameter, and most metabolically active pool of roots are virtually absent.

Here we have shown that exposure of an intact loblolly pine forest to 14 yr of atmospheric CO_2 enrichment has resulted in the development of a fine-root pool that is less dichotomous and more exploratory under N-limited conditions. These architectural changes are commonly considered more efficient in nutrient acquisition and may be allowing plants to effectively exploit larger volumes of soil, thereby pre-empting progressive nutrient limitations (Larigauderie *et al.*, 1994; BassiriRad *et al.*, 2001). The smaller effect of CO_2 enrichment on fine-root biomass, relative to the larger increase in fine-root length density in soil, coupled with a shift toward a fine-root pool dominated by smaller diameter roots, may partly explain the enhanced N uptake efficiency observed in CO_2-enriched plots in this study (Fig. 2; Drake *et al.*, 2011). These changes in fine roots may reflect a plastic response to the dilution of soil nutrients at the ecosystem scale in a larger amount of forest biomass. Alternatively, such architectural adjustments might also follow from greater belowground competition by a substantially larger population of fine roots. Similar analyses conducted within the context of long-term environmental change experiments will help to resolve this issue. Finally, our study also indicates that soil N availability will strongly interact with atmospheric CO_2 enrichment to dictate the growth habits, and corresponding function, of fine-root systems in the future.

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Supporting Information

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

Fig. S1 The relationships between topological and architectural attributes.

Table S1 Table of equations and r^2 values describing the relationship between architectural traits and length of intact fine-root branches (equations apply to relationships illustrated in Fig. S1)

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