Production dynamics of *Cenococcum geophilum* ectomycorrhizas in response to long-term elevated CO₂ and N fertilization

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Abstract

Ectomycorrhizal fungi are important in many forest ecosystems, yet their production dynamics and responses to environmental changes are poorly understood. *Cenococcum geophilum* is a common ectomycorrhizal fungus important to plant and forest soil biogeochemical cycles. The seasonal and inter-annual patterns of production and persistence of mycorrhizas formed by *C. geophilum* in a pine forest exposed to elevated atmospheric CO₂ and nitrogen fertilization were monitored using a 12 y minirhizotron dataset. Production of *C. geophilum* mycorrhizas was distinctly seasonal and peaked in late summer/autumn. Elevated CO₂ generally increased production while nitrogen fertilization strongly decreased production. Persistence times of *C. geophilum* mycorrhizas was *ca.* 2.7 y and was unaffected by CO₂ and nitrogen addition. Total production was greater in shallow soil (0-16 cm) but persistence was longer in deeper soil (17-32 cm). These observations provide insights into the autecology of *C. geophilum* and suggest that its tissues may be slow to decompose compared to other ectomycorrhizal species.

Key words Free-air-CO₂-enrichment (FACE), minirhizotron, lifespan, ectomycorrhizal fungi, phenology, melanin, global change, forest ecosystem, ascomycetes, soil ecology.

Introduction

Terrestrial ecosystems are currently experiencing unprecedented shifts in concentrations of atmospheric carbon dioxide (CO₂) and are receiving increased inputs of inorganic nitrogen (N) (Galloway *et al.*, 2004; Stocker *et al.*, 2013). Increasing atmospheric CO₂ concentrations and N deposition and fertilization impact plant productivity and resource allocation to aboveground and belowground processes leading to changes within mycorrhizal communities, altered root systems, and shifts in community composition (Parrent & Vilgalys, 2007; Thomas *et al.*, 2009; Drake *et al.*, 2011; De Kauwe *et al.*, 2014; Beidler *et al.*, 2015). Ectomycorrhizal (EM) fungi may act as mediators of ecosystem responses to these environmental changes in many terrestrial environments because they play active roles in soil biogeochemical cycles and account for a substantial portion of net ecosystem primary production (Godbold *et al.*, 2006; Hobbie & Hobbie, 2006; Courty *et al.*, 2010; Ekblad *et al.*, 2013; Fernandez *et al.*, 2016). Despite likely impacts of these broad scale environmental changes on the activity of EM fungi, we are just beginning to understand patterns of ectomycorrhiza (i.e. fine-root tips colonized by EM fungi) production and their likely responses to different regional and global environmental change factors.

Responses of ectomycorrhizal taxa to different environmental factors are often variable among species (Parrent & Vilgalys, 2007; Avis *et al.*, 2008), however, some general patterns have emerged. Nitrogen fertilization often reduces fungal biomass and abundance (Treseder, 2004; Wallenstein *et al.*, 2006; Ekblad *et al.*, 2016), and alters ectomycorrhizal community composition (Parrent & Vilgalys, 2007; Avis *et al.*, 2008). Broad changes to ectomycorrhizal community structure have also been observed at larger scales along N availability gradients (Lilleskov *et al.*, 2002). Still, given that few studies directly assess changes to standing biomass and rates of new biomass production together with rates of activity by individual ectomycorrhizas (Mohan *et al.*, 2014), we are limited in how much we may generalize these studies to determine common responses of ectomycorrhizas to N addition. Changes in ectomycorrhizal communities have also been commonly observed with elevated atmospheric CO₂ along with some increases in biomass and abundance (Treseder, 2004; Parrent & Vilgalys, 2007), but these changes have been less consistent and are often more nuanced (Andrew & Lilleskov, 2014; Ekblad *et al.*, 2016). Overall, ectomycorrhizas are likely to play important roles determining how individual plants and whole-forest ecosystems respond to N fertilization and elevated CO₂ (Pickles *et al.*, 2012) and further work is needed to better understand their responses in both single and multifactor contexts.

Most terrestrial ecosystems experience significant seasonal and interannual variability in temperature and precipitation. These patterns are then expected to drive broader patterns in production dynamics of different EM fungi. Observation and frequent collections of fungal

sporocarps have suggested strong seasonal patterns of different fungal species and highlighted ongoing responses to climate change (Fogel, 1976; Kauserud *et al.*, 2012). However, most previous work sampling ectomycorrhizas has emphasized one or only a few periods of sample collection which may bias interpretation of which species are present and active during different time periods. Results from studies explicitly investigating temporal dynamics of EM communities belowground have indicated distinct differences in production patterns among species. Koide *et al.* (2007) conducted repeated sampling of both ectomycorrhizas and EM mycelium in a red pine (*Pinus resinosa*) stand in Pennsylvania, USA, over 2 y and observed that some species appeared to be more active in spring, others were more common in autumn, and several species exhibited little seasonality. Repeated samplings of ectomycorrhizas by Pickles *et al.* (2010) also indicated that the patchiness and distribution of different EM fungi changed between years. Still, given the challenges of repeatedly sampling or observing belowground fungal communities over time, it has been difficult to determine how stable production patterns of ectomycorrhizas are likely to be across multiple years and in response to changes in environmental conditions.

Minirhizotron camera systems provide a method to observe the belowground environment repeatedly and non-destructively using digital cameras and transparent tubes installed in the soil (Hendrick & Pregitzer, 1996). Using this approach, researchers can quantify long-term dynamics of EM fungi by observing the production and persistence of rhizomorphs and ectomycorrhizas *in situ* (Treseder *et al.*, 2005; McCormack *et al.*, 2010; Fernandez *et al.*, 2013; Pritchard *et al.*, 2014). Unfortunately, because observed fungal structures cannot be physically sampled and taxonomically identified using molecular techniques, most observed structures cannot be attributed to any single fungus. That said, *Cenococcum geophilum*, a common and often abundant EM fungal species (complex), produces distinct ectomycorrhizas that can be readily identified based on their jet-black mantle and emanating wiry black hyphae (Fig. 1). This provides an opportunity for researchers to determine long-term dynamics *C. geophilum* without repeatedly disturbing the soil environment.

The near-global distribution of *C. geophilum* in EM communities is thought to be the result of a combination of factors including an abnormally wide niche breadth, cryptic speciation, and the lack of host specificity (Trappe, 1962; Visser, 1995; Dickie, 2007; Matsuda *et al.*, 2009; Obase *et al.*, 2016). The species has been observed in essentially all forested biomes ranging from high latitude boreal forests, temperate forests, as well as wet and dry tropical forests (Trappe, 1962; Alexander & Högborg, 1986; Phosri *et al.*, 2012). *Cenococcum geophilum* produces hyphae with heavily melanized cell walls, which is thought to increase its resistance to water stress (Pigott, 1982; Fernandez & Koide, 2013). In addition to being abundant in many forest

ecosystems (Avis *et al.*, 2003; Pickles *et al.*, 2010; Trocha *et al.*, 2012), *C. geophilum* may disproportionately impact ecosystem C and nutrient cycling as a result of its high melanin concentrations, which may slow decomposition rates of fungal necromass and associated root tissues (Fernandez & Koide, 2014; Fernandez *et al.*, 2016). While *C. geophilum* is considered a relatively well-studied mycorrhizal fungus (Klironomos & Kendrick, 1993), its seasonal and interannual patterns of production phenology and long-term responses to elevated atmospheric CO₂ and N deposition are not well known.

In this study we took advantage of a 12 y minirhizotron data set collected from a loblolly pine (*Pinus taeda*) stand in central North Carolina, USA, exposed to elevated atmospheric CO₂ and N fertilization. Here, we focused on ectomycorrhizas formed by *C. geophilum* whose unique appearance allows for confident identification in minirhizotron images. While other species of EM fungi may produce darkly pigmented ectomycorrhizas, these species generally have very different morphological features and/or were uncommon at the study site. Using this extensive dataset we addressed multiple questions regarding the spatial and temporal dynamics of ectomycorrhizas produced by *C. geophilum* under ambient conditions and in response to elevated CO₂ and N treatments. We hypothesized that increased atmospheric CO₂ would increase production of *C. geophilum* ectomycorrhizas as plant productivity and total allocation belowground increased, while N fertilization would decrease production of *C. geophilum* ectomycorrhizas as proportional allocation to roots and EM fungi decreases (Pritchard *et al.*, 2008; Drake *et al.*, 2011; Pritchard *et al.*, 2014). We also hypothesized that more ectomycorrhizas of *C. geophilum* would be produced in shallower soil (0-16 cm) than in deeper soil (17-32 cm) as previous studies have associated higher abundances of *C. geophilum* ectomycorrhizas with more organic-rich soil layers (Fransson *et al.*, 2000; Rosling *et al.*, 2003). We then assessed seasonal and interannual patterns of production by *C. geophilum* across the site and in relation to interannual trends in temperature and precipitation. Finally, we determined the effect of CO₂, N fertilization, and soil depth on the persistence (lifespan and disappearance) of *C. geophilum* ectomycorrhizas.

Materials and Methods

Site Description

The Duke FACTS-1 (Forest Atmosphere Carbon Transfer and Storage-1) site is located in central North Carolina, USA (35°58'N, 79°5'W). The site was established on an unmanaged, 15-y-old loblolly pine (*Pinus taeda*) plantation with local soils characterized as Enon loam (fine,

mixed, thermic Ultic Hapludalfs) (Oh & Richter, 2005). While the site remained dominated by loblolly pine throughout the experiment, a number of deciduous species established after the initial planting including *Acer rubrum*, *Ulmus alata*, *Liquidambar styraciflua*, and *Liriodendron tulipifera*, which collectively accounted for *ca.* 2% of stand basal area. The experimental design was previously described by Hendrey *et al.* (1999). Briefly, the experiment was established as a randomized block design with three replications of ambient and elevated atmospheric CO₂ in 30 m diameter plots. CO₂ enriched plots were maintained at *ca.* 200 ppm above ambient levels using Free-Air-CO₂-Enrichment (FACE) technology. CO₂ enrichment was initially administered at all times except when air temperatures fell below 5 °C or wind exceeded 5 m s⁻¹. Beginning in 2003, CO₂ fumigation was limited to daylight hours.

Prior to the 2005 growing season, the plots were split and a N-fertilization treatment was applied to half of each FACE ring. At the end of the growing season in 2004, each plot was bisected by a 70 cm deep trench, and impermeable plastic sheeting was then laid along the trench before refilling with soil to prevent lateral flow of water and growth of roots between the two halves. The fertilization treatment was then randomly assigned to one of the two halves in the form of NH₄NO₃ at a rate of 112 kg ha⁻¹ y⁻¹ which is approximately an order of magnitude in excess of background deposition rates (Finzi *et al.*, 2002).

Minirhizotron installations and image processing

The procedure for the installation and monitoring of minirhizotron tubes was previously described by Pritchard *et al.* (2001). 72 minirhizotron tubes were installed among the 6 FACE rings (12 tubes per ring) in June of 1998. These were distributed with six tubes in each half of the ring that would later be assigned to N fertilization or non-fertilized treatments. Each tube was installed at a 45° angle to a vertical depth of *ca.* 32 cm. The portion of the tube remaining aboveground was covered in a black foam sleeve which was then covered in a white PVC cap. The tubes were anchored using 40 cm stainless steel rods to prevent movement over time.

Image collection began in October 1998 and continued until October 2010. Images were generally collected at monthly or bi-monthly intervals, with a few extended periods where 3-4 months elapsed between imaging sessions. From 1998 to October 2004, a BTC-100x microvideo camera (Bartz Technologies Corp, Carpinteria, CA, USA) was used to record images along the upper surface of each tube. An indexing handle was used to precisely collect each image from the same soil location between sessions which allowed us to monitor the production and persistence of individual ectomycorrhizas through time. Video images were originally recorded for 32 images per tube. Once collected, video images were digitized in the lab for every other frame resulting in

16 frames per tube per session. In November 2004, the original camera system was replaced with a BTC-2 ICAP system (Bartz Technologies Corp, Carpinteria, CA, USA) which collected digital images for each indexed location along the full length of the tube. However, over the course of the experiment 11 tubes became inoperable and were omitted from the second half of the analysis. In total, the experiment consisted of two consecutive and complementary minirhizotron data sets with a total of *ca.* 200,000 analyzed images. Because the total number of tubes changed over the course of the experiment and because the total number of frames imaged per tube varied slightly, data were normalized to a per frame basis rather than a per tube or per plot basis.

Ectomycorrhizas formed by *C. geophilum* were identified within each image based on their characteristic jet-black mantle and occurrence of wiry black hyphae emanating from the mantle surface (Fig. 1). While other EM species can produce ectomycorrhizas that are loosely similar in pigmentation, these species are morphologically distinct (e.g. Thelephoraceae species) or were generally uncommon or non-existent within the site (*Melinomyces bicolor*, some *Tuber* *sp.*, and *Geopora* *sp.*) (Parrent & Vilgalys, 2007). Once located in an image, each *C. geophilum* ectomycorrhiza was monitored from the initial time it was produced (birth) to the last session in which it was visible (death) and these dates were then recorded. Individual ectomycorrhizas present in the last session of a given data set (either October 2004 or October 2010) and whose date of disappearance could not be directly observed were noted and analyzed as being right censored in survivorship analyses (see below). Individual ectomycorrhizas that were present on the first image session of a given data set (either October 1998 or November 2004) were omitted from this analysis as their approximate date of birth could not be determined and, in the case of the second image set, there was the possibility of double counting ectomycorrhizas that had been previously recorded in the first image set.

Statistical analysis

The effects of each treatment were assessed separately for the first and second halves of the study (1999-2003 pre N addition; 2005-2009 post N addition) using Analysis of Variance with block treated as a random effect ($n=3$). Due to the strong effect of depth, these analyses were conducted separately for the shallow soil layer (0-16 cm) and the deeper soil layer (17-32 cm). As the blocking design was initially established to account for a N gradient across the site, block was also considered as a fixed factor in each test. The main effects of CO₂ and block were considered for the first portion of the study and CO₂, N fertilization, and block were considered along with the interaction between CO₂ and N fertilization in the second portion. Seasonal production was assessed by binning day of year production dates into 30 d windows across the year and summing

each bin across the full 12 y study. When observed separately, the two portions of the study (1999-2004 and 2005-2010) revealed similar overall patterns (data not shown). These patterns were observed together for all depths and treatments and were observed separately between the shallow (0-16 cm) and deep (17-32 cm) soil depths and separately for ambient and elevated CO₂ treatments.

Relationships between interannual patterns of production and climate factors were investigated using linear regression. Total annual production of *C. geophilum* ectomycorrhizas was compared with independent variables related to climate during the growing season: average temperature from March to October, average temperature from April to September, cumulative precipitation from March to October, and cumulative growing season from April to September.

Survivorship analysis was conducted to estimate the persistence of *C. geophilum* ectomycorrhizas at different depths and in response to the elevated CO₂ and N fertilization treatments. In this study we emphasize *persistence* of ectomycorrhizas formed by *C. geophilum* rather than lifespan as their slow decomposition makes it likely that these ectomycorrhizas persist long after their functional, metabolically-active lifespan (Fernandez *et al.*, 2013; Fernandez & Koide, 2014). We used Cox Proportional Hazards to determine the effect CO₂, N fertilization, and depth on the persistence of *C. geophilum* ectomycorrhizas (Cox, 1972). Because the N fertilization treatment did not run the length of the experiment we separated the dataset and analyzed persistence of ectomycorrhizas in relation to depth and CO₂ treatments for the full 12 years and then separately analyzed the effect of N fertilization on persistence for the years following initiation of the N treatment. Kaplan-Meier survival tests were then used to generate summary statistics and survivorship curves for the different treatment comparisons (Kaplan & Meier, 1958). Where the Cox Proportional Hazards test indicated significant treatment or depth effects, Log-Rank and Wilcoxon tests were used to test for dissimilarity of survivorship curves.

All statistical analyses were carried out using JMP Pro 12.0.1 (SAS Institute Inc.). Results were interpreted as significant with an *alpha* of 0.05. However, due to the low replication available in the study design and the large variation inherent in belowground and minirhizotron studies we also denoted a statistical trend following an *alpha* of 0.10 (Filion *et al.*, 2000; Pritchard *et al.*, 2008).

Results

Effects of CO₂, nitrogen fertilization, and depth on total production

There was a large increase in the production of *C. geophilum* ectomycorrhizas in the elevated atmospheric CO₂ treatment compared to the control during the study (Fig. 2). Despite the

largest absolute differences occurring in the first year of observation (1999; Fig. 3), this effect was only significant during the second half of the study and was constrained primarily to the shallow soil layer (Table 1). After the first few years of observation elevated CO₂ plots averaged around 40% greater production through the remainder of the experiment (Fig. 2 and 3). Nitrogen fertilization significantly decreased the production of *C. geophilum* ectomycorrhizas in both the shallow and deep soil layers ($P=0.002$ and $P=0.025$, respectively, Table 1) and led to an overall reduction of 83% compared to background levels (Fig. 2B). This effect was similar in both ambient and elevated CO₂ plots with no significant interaction between the CO₂ and N fertilization treatments (Table 1).

There was a general pattern of greater production of *C. geophilum* ectomycorrhizas in the shallow soil layers (0-16 cm depth) compared with deeper soil (17-32 cm). Across the full study, there was roughly 3-fold greater production in shallower depth compared to deeper depth (Fig. 4). While the effect was most evident in the elevated CO₂ plots, the pattern was generally consistent in both ambient and elevated treatments and was consistent throughout the study. Generally the highest levels of production were found in the upper 6 to 8 cm and decreased gradually until around 16 cm depth, after which there was little difference in production across the 17 to 32 cm range (data not shown).

Seasonal and interannual patterns of production

There was a distinct seasonal pattern of production with more *C. geophilum* ectomycorrhizas produced in the second half of the year between day 180 and 300 with an apparent peak around September (Fig. 4). However, this pattern only occurred in the shallower soil depth (0-16 cm). While there was less production in deeper soils overall, ectomycorrhizas of *C. geophilum* were generally produced evenly throughout the year in the deeper depths (Fig. 4).

In addition to distinct seasonal variation, there was also strong interannual variation in production *C. geophilum* ectomycorrhizas (Fig. 3). Overall, production tended to be greater in years with cooler, wetter growing seasons. However, these relationships were relatively weak (Fig. 5). There was a significant trend indicating a negative relationship for mycorrhiza production and average growing season temperature from April through September ($R^2=0.32$, $P=0.07$). The relationship between cumulative precipitation across the same period was not significant ($R^2=0.2$, $P=0.19$). Relationships between production and climate variables assessed from March to October were non-significant in both cases (data not shown).

Persistence of Cenococcum geophilum ectomycorrhizas

Ectomycorrhizas produced by *C. geophilum* persisted for an average of 999 d (ca. 2.7 years) across all treatments and soil depths. Ectomycorrhizas produced in deeper soil persisted significantly longer than those in shallow soils with mean persistence of 1144 and 928 d, respectively (Log-Rank $P=0.03$ and Wilcoxon $P=0.06$; Fig. 6). There were no significant interactions or main effects of elevated CO₂ or N fertilization on the persistence of *C. geophilum* ectomycorrhizas ($P>0.10$, all tests).

Discussion

The spatial and temporal partitioning of EM fungi in forest ecosystems and responses to increased atmospheric CO₂ and N addition are not well known. In this study we monitored patterns of production and persistence of ectomycorrhizas formed by a cosmopolitan and often dominant EM fungus, *Cenococcum geophilum*, using a 12 y minirhizotron dataset collected in an intact loblolly pine forest exposed to elevated atmospheric CO₂ and N addition. Averaged across the entire study, there was roughly 50% greater production of *C. geophilum* ectomycorrhizas with elevated CO₂ compared to control plots but this difference was only significant during the second half of the study despite large initial differences (Fig. 2 and 3). The overall increase largely mirrored increases in coarse- and fine-root biomass also observed at the site (Pritchard *et al.*, 2008; Jackson *et al.*, 2009) and is likely associated with systematic increases in whole-ecosystem productivity in response to elevated CO₂ which were sustained throughout the study (Drake *et al.*, 2011), rather than specific increases in allocation to *C. geophilum* ectomycorrhizas. However, Parrent and Vilgalys (2007) did observe an increase in the frequency of sampled root tips colonized by *C. geophilum* relative to other species from 1.1% in ambient plots to 3.7% in elevated CO₂ plots at the site.

With the addition of N fertilizer at the beginning of 2005, we observed a strong decline in the production of *C. geophilum* ectomycorrhizas (Fig. 2B). Reductions in root colonization rates and mycorrhizal tip production in response to N fertilization are commonly reported in other studies (Treseder, 2004), but the specific, relative effects on *C. geophilum* ectomycorrhizas have been mixed with some reporting significant decreases in the occurrence of *C. geophilum* similar to our observations (Clemmensen & Michelsen, 2006), and others noting significant increases (Fransson *et al.*, 2000). The reduced frequency of *C. geophilum* ectomycorrhizas observed in the present study may have been the result of decreased carbohydrate allocation to *C. geophilum* ectomycorrhizas or reductions to all EM fungi as N becomes more available and plants become less reliant on their fungal symbionts. These changes may also have been associated with reductions in belowground allocation to fine roots and mycorrhizal fungi in general. The

magnitude of the fertilization effect observed for *C. geophilum* ectomycorrhizas was substantially larger than that observed for fine-root biomass (~12% reduction) and for all other ectomycorrhizas (~20% reduction) but was similar in magnitude for the reduction in EM extramatrical mycelia (57% reduction) previously reported at our study site (Jackson *et al.*, 2009; Pritchard *et al.*, 2014; Taylor *et al.*, 2014; Ekblad *et al.*, 2016). This suggests that production of *C. geophilum* ectomycorrhizas was more sensitive to changes in site fertility than other aspects of belowground production ostensibly associated with soil resource acquisition.

Repeated belowground observations can reveal distinct patterns of production phenology among different species of EM fungi as well as among fine roots of many of their host plants (Koide *et al.*, 2007; McCormack *et al.*, 2014). We observed a strong peak in the production of *C. geophilum* in autumn months in the upper soil horizon (0-16 cm). This is consistent with findings from Vogt *et al.* (1980) and Hupperts *et al.* (2016) who observed a greater proportion of *C. geophilum* ectomycorrhizas on root samples collected in autumn and winter months and declining abundance in spring and summer. Rygielwicz *et al.* (2000) also found distinct seasonality of *C. geophilum* ectomycorrhiza abundance on *Pseudotsuga menziesii* seedlings in a glasshouse experiment examining the effects of warming and elevated CO₂. However, in their study the autumn/winter production peak of *C. geophilum* ectomycorrhizas only occurred in the ambient CO₂ treatment while they instead observed a summer peak in production with elevated CO₂. This may indicate potential flexibility for production across a range of seasons which is similar to the broadly distributed, though low relatively low levels of production we observed in the deeper soil depth in this study (Fig. 4).

In contrast to our study, repeated sampling by Koide *et al.* (2007) revealed that the frequency of *C. geophilum* ectomycorrhizas was roughly consistent across all seasons, even in relatively shallow soil (e.g. <10 cm depth). At the same time, Koide *et al.* (2007) observed distinctly seasonal patterns in other ectomycorrhizal fungi including spring dominant species like *Amanita vaginata* and *Ramaria concolor*, and species more common in the fall such as *Lactarius oculatus* and *Suillus intermedius*. In the case *C. geophilum*, prolonged persistence, or the amount of time that individual ectomycorrhizas of *C. geophilum* remain in the soil before decomposing may explain the different results. We previously observed that while most ectomycorrhizas have relatively short lifespans and show visible signs of decomposition within a few months, ectomycorrhizas produced by *C. geophilum* often persist and remain intact for multiple years (Fernandez *et al.*, 2013). Commonly used destructive sampling methods would not be sensitive to differences in persistence times of ectomycorrhizas among EM fungi. Instead, previous studies (Koide *et al.* 2007; Pickles *et al.*, 2010) showing no seasonal patterns may be the result of

sampling undecomposed dead ectomycorrhizas and the relic DNA held within these tissues. The tendency for greater production in the autumn is also consistent with findings from our previous study, that used fluorescein diacetate vitality stain, showing that a greater proportion of *C. geophilum* ectomycorrhizas were vital in the autumn than in spring and summer seasons (Fernandez *et al.*, 2013). However, we cannot rule out the possibility that differences between our observations and previous studies may be the result of different plant host species and site conditions. Observations with minirhizotron cameras enable a more direct assessment of the production phenology of ectomycorrhizas and applying this tool to observe temporal patterns of production by *C. geophilum* in additional sites would be valuable and help determine the generality of the patterns reported here.

Seasonal patterns in fungal production may be directly related to changes in climate or associated with patterns of carbohydrate availability supplied from EM host plants. Many species of EM fungi exhibit seasonal patterns of sporocarp production (Fogel, 1976), and observed changes in the timing of fungal activity may provide important clues to understand fungal and ecosystem responses to environmental change (Kausarud *et al.*, 2008; Buntgen *et al.*, 2011). At shorter timescales, belowground hyphal production has also been linked to periods of increased soil moisture in a water limited, mixed-species forest in California, USA (Allen & Kitajima, 2014). However, it is unclear what, if any, direct climate factors would contribute to the distinct pattern of *C. geophilum* ectomycorrhizas being produced in autumn in the loblolly pine plantation studied here as it generally experiences warm, mesic conditions throughout the growing season. Additionally, fine roots with which the EM fungi associate are generally produced across the growing season at this site (Pritchard *et al.*, 2008). More broadly, many species of EM host plants that commonly associate with *C. geophilum* are capable of producing new roots broadly throughout the growing season or can express multiple pulses of root production across spring, summer, and autumn (Reich *et al.*, 1980; McCormack *et al.*, 2014). Given that neither climate nor the availability of potential host roots are likely to be limiting factors throughout most of the growing season, it seems possible that the plant and/or fungus favor and exert some control over the production of different ectomycorrhizas at particular times of the year. These preferences may be related to differences in fungal traits that make individual species more or less beneficial at different points during the growing season. For example, differences in exploration type (e.g. short vs. long distance) or capacity to break down organic matter and utilize organic vs. inorganic nutrients may be important as there are often distinct seasonal patterns in the inputs of fresh plant litter and availability of inorganic nutrients (Finlay *et al.*, 1992; Agerer, 2001; Talbot *et al.*, 2008). Similarly, plants could favor EM species with high capacity for nutrient gain early in the season

and then later favor EM species that may enhance the overwintering success of fine roots. The short distance exploration strategy and relatively low respiration rates may indicate a lesser role for *C. geophilum* in nutrient acquisition compared to other species (Agerer, 2001; Malcolm *et al.*, 2008). Instead, colonization by *C. geophilum* may help roots survive less favorable growing conditions and successfully overwinter (Jany *et al.*, 2003; Hasselquist *et al.*, 2005).

On an annual basis, climate can affect both the amount and relative timing of fungal production as has been well documented for mushrooms aboveground (Büntgen *et al.*, 2011; Kauserud *et al.*, 2012). In the present study, total production generally appeared to increase in cooler, wetter years that resulted in less water stress and generally favored greater overall ecosystem net primary productivity (McCarthy *et al.*, 2010) (Fig. 5). However, we were unable to detect a strong relationship between total annual production and interannual differences in growing season temperature and precipitation, which may reflect the ability of *C. geophilum* to remain active irrespective of recent climate trends including moderate drought. Conversely, the weak relationship may simply be the result of high inherent variability within the belowground system making it difficult to tease out a direct relationship without additional observations despite the already robust minirhizotron dataset used in this study.

A final important detail highlighted in the present study is the extremely long persistence times observed for *C. geophilum* compared to all other ectomycorrhizas reported previously from this site and in other studies (Downes *et al.*, 1992; McCormack *et al.*, 2010; Pritchard *et al.*, 2014; Kou *et al.*, 2016). Estimates of median and mean persistence (i.e. lifespan) for bulk, non-*Cenococcum* type species generally range from 150 to 400 d, with only a few cases approaching 2 y. Averaged across all treatments, mycorrhizas produced by *C. geophilum* persisted for *ca.* 2.7 y. While few other studies have reported long-term dynamics for *C. geophilum*, these results are consistent with our previous study which reported mean persistence of *C. geophilum* using a more limited minirhizotron dataset (Fernandez *et al.*, 2013), and is consistent with the long carbon turnover times of *C. geophilum* reported by Treseder *et al.* (2004) based on radiocarbon signatures. We emphasize persistence rather than lifespan as it is unlikely that these individual mycorrhizas are functional and metabolically active during this extended period (Fernandez *et al.*, 2013). Rather, it is more likely that the highly melanized tissues of this species are relatively resistant to decay resulting in slow decomposition and disappearance (Fernandez & Koide, 2014).

Using an unprecedented 12 y minirhizotron dataset, our study provides insights into the patterns of production and persistence of ectomycorrhizas produced by *C. geophilum* in a pine forest exposed to ambient and elevated levels of atmospheric CO₂ as well as N fertilization. While there was an initial increase in production with elevated CO₂, this increase diminished

through time resulting in only modest differences taken across the entire study. Conversely, N fertilization significantly reduced the production of *C. geophilum* ectomycorrhizas. We observed no treatment effects on the persistence of *C. geophilum* ectomycorrhizas but there was a significant increase in persistence in the deeper soil depth. Finally, our observations revealed that while *C. geophilum* ectomycorrhizas are produced throughout the year and across the 0 to 32 cm depth profile, there was distinctly more production in the upper soil depth and a pattern of increased production during late summer and autumn months. As researchers continue to link trait variation among species of EM fungi to plant and ecosystem processes it will be important to continue to quantify temporal and spatial patterns of production among different EM fungi from which we may glean a better understanding of their functional roles in forest ecosystems.

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Figures

Figure 1. Typical ectomycorrhizas produced by *Cenococcum geophilum* on roots of pine (*Pinus* sp.). (A) a cleaned and washed mycorrhiza collected from a pine plantation under a dissecting scope at 10x magnification (white scale bar = 0.2 mm). (B) presents a sequence of minirhizotron images collected from the Duke FACE site and used in this study showing bare soil (1), initial production of a pine root (2), and the subsequent production of a characteristic pine—*Cenococcum geophilum* mycorrhiza with black mantle and wiry black hyphae (3). Photo in (A) by CW Fernandez.

Figure 2. Production of *Cenococcum geophilum* ectomycorrhizas in response to elevated atmospheric CO₂ (A) and nitrogen fertilizer (B) in shallow soil (0-16 cm) at the Duke FACE site in central North Carolina, USA. The effect of CO₂ in the first portion of the study was not significant during 1999-2003 ($P=0.13$), but was significant during the later portion of the study (1999-2003, $P=0.04$). Nitrogen fertilization began in year 2005 and significantly decreased production of *C. geophilum* ectomycorrhizas ($P=0.002$). Error bars represent standard error (n=3). Note that data shown for the CO₂ response in later years only presents data from subplots that did not receive nitrogen fertilization.

Figure 3. Interannual variation in production across the full study in ambient and elevated CO₂ plots. Production in (C) represents the average number of *C. geophilum* ectomycorrhizas produced per frame in each given year. Production that occurred in plots exposed to elevated atmospheric CO₂ are shown in by the dashed orange lines and production in ambient plots are shown in solid blue lines. Year 2004 was excluded due to and equipment change and incomplete observations during that year.

Figure 4. Seasonal production patterns of *Cenococcum geophilum* ectomycorrhizas in shallow (0-16 cm) (A) and deeper (17-32 cm) (B) soil layers. Proportional production across all years, depths

and CO₂ treatment of observation were determined and allocated to individual months across the year based on the day of year for each session in which each mycorrhiza was first observed. Production in plots exposed to elevated atmospheric CO₂ are shown in by the dashed orange lines and production in ambient plots are shown in solid blue lines.

Figure 5. Relationship between annual production of *Cenococcum geophilum* mycorrhizas and interannual variation in climate. There was a significant trend of decreasing production with increasing temperature averaged from April to September ($P=0.07$), indicated by the solid line on the left panel. However, there was not a significant relationship between cumulative precipitation from April to September ($P=0.19$). Note that x-axes in panels (A) and (B) do not reach zero.

Figure 6. Persistence of *Cenococcum geophilum* ectomycorrhizas in response to elevated atmospheric CO₂ (A), nitrogen fertilization (B), and within shallow (0-16 cm) vs. deeper (17-32 cm) soil depths (c). Log-Rank and Wilcoxon tests of homogeneity based on Kaplan-Meier analysis indicated that CO₂ and nitrogen did not result in significant differences in persistence ($P>0.10$ all tests) but persistence was significantly longer in deeper than shallow soil (Log-rank $P=0.03$, Wilcoxon $P=0.06$).

Tables

Table 1. Summary of analysis of variance for effects of CO₂ (ambient vs. elevated) and N fertilization (background vs. fertilized) on the production of ectomycorrhizas formed by *C. geophilum* for the first half of the experiment (years 1999-2003, with no N fertilization) and for the second half of the experiment (years 2005-2010). Block was considered a fixed effect for each analysis. Analyses were run separately for shallow and deep soil layers. Year 2004 was not included in analysis due to a change incomplete observation.

Tables

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Source	Shallow soil (0-16 cm)		Deep soil (17-32 cm)	
	<i>F</i> ratio	<i>P</i> -value	<i>F</i> ratio	<i>P</i> -value
<i>Years 1999-2003</i>				
CO ₂	6.17	0.131	3.08	0.222
Block	12.2	0.076*	8.64	0.104
<i>Years 2005-2010</i>				
CO ₂	6.45	0.044**	0.006	0.939
N fertilization	26.9	0.002**	8.84	0.025**
CO ₂ x N fertilization	0.34	0.577	0.006	0.939
Block	6.11	0.357**	3.41	0.103

Results considered significant with $P \leq 0.05$, indicated by **, or were noted as a statistical trend at $P \leq 0.10$ indicated by *.