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Short communication

On the persistence of *Cenococcum geophilum* ectomycorrhizas and its implications for forest carbon and nutrient cycles

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ABSTRACT

The turnover of ectomycorrhizal (EM) fungal biomass represents an important litter input into forest biogeochemical cycles. *Cenococcum geophilum* is a nearly ubiquitous and often abundant EM fungus, making the turnover dynamics of this species relevant and important across forest ecosystems. To better understand the turnover dynamics of *C. geophilum* ectomycorrhizas we examined their persistence using minirhizotron imaging and vitality status using a fluorescein diacetate (FDA) stain and contrasted these results with ectomycorrhizas of other EM fungi. Ectomycorrhizas formed by *C. geophilum* persisted 4–10 times longer and exhibited contrasting seasonal patterns of vitality compared to ectomycorrhizas of other EM fungi. Together, this suggests that litter resulting from the death of *C. geophilum* ectomycorrhizas is relatively recalcitrant to decay and may disproportionately influence forest biogeochemical cycles by retarding the rate at which carbon and nutrients are cycled.

There is growing interest in understanding the turnover of the ectomycorrhizal (EM) fungi because of the ubiquity of this group of organisms and the large quantity of carbon (C) that is allocated to them by host trees (Hobbie, 2006). Thus, the death of EM fungal tissues represents a large litter input into forest ecosystem cycles (Langley and Hungate, 2003; Cairney, 2012; Clemmensen et al., 2013; Ekblad et al., 2013). Moreover, EM fungi envelop fine roots, forming a mantle on their exterior and modifying the biochemistry of litter inputs from fine roots (Langley et al., 2006; Koide et al., 2011), which are substantial (Jackson et al., 1997). Our knowledge of the decomposition dynamics of EM fungal litter is relatively poor, and there is likely a large amount of variation in the decomposition rates of tissues across species (Koide and Malcolm, 2009; Fernandez and Koide, 2012; Wilkinson et al., 2011).

The highly melanized asexual Ascomycete EM fungus, *Cenococcum geophilum*, has a global distribution with little host specificity (Trappe, 1962). *C. geophilum* is frequently abundant in EM communities (Dickie, 2007), thus making the turnover dynamics of this species relevant and important across forest ecosystems. Meyer (1964) noted the presence of a large fraction of *C. geophilum* ectomycorrhizas that appeared to be dead and hypothesized that this may be the result of an accumulation of these structures in the soil from slow decomposition rates. Corroborating this observation with vitality staining, Qian et al. (1998) found large proportions of dead *C. geophilum* ectomycorrhizas, relative to other morphotypes. The persistence of these structures in soil, however, has not been examined explicitly. Long persistence times of ectomycorrhizas can result from either long lifespans or from their resistance to decomposition after death. The decomposition of ectomycorrhizas can be faster (Koide et al., 2011) or slower (Langley et al., 2006) than non-mycorrhizal roots. The differential effects of EM colonization on root decomposition are likely the result of, among other factors, differences in the quality of the fungal litter (Koide and Malcolm, 2009; Fernandez and Koide, 2012). Indeed, there is reason to

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suspect that *C. geophilum* tissues are unusually resistant to decomposition because it deposits in the cell walls of its hyphae substantial amounts of melanin, a polymer that is highly resistant to decomposition (Martin et al., 1959; Kuo and Alexander, 1967; Hurst and Wagner, 1969; Malik and Haider, 1982).

Given its high abundance in many EM fungal communities and its global distribution, if *C. geophilum* is unusually resistant to decomposition, it may strongly influence biogeochemistry in forest ecosystems by sequestering large quantities of C and nutrients. We support this assertion with evidence of differential persistence and vitality of root tips colonized by *C. geophilum* compared with ectomycorrhizas of other EM fungal species assessed with minirhizotron observations and fluorescein diacetate (FDA) vitality staining.

Minirhizotron observations were conducted at the Duke FACE site in Durham, North Carolina, USA (detailed in Pritchard et al., 2008a, 2008b). We used images collected from control rings (i.e., ambient CO_2) but not experimental rings (i.e., elevated CO_2) from October 1998 to October 2004. Persistence of ectomycorrhizas was determined by noting the time of the birth (appearance), death (fragmentation and detachment), and subsequent decomposition (disappearance) of individual ectomycorrhizas. A total of 121 (10-right censored) ectomycorrhizas were tracked, 28 of which were identified as *C. geophilum* based on this fungus' distinctive morphology, which includes a jet-black mantle with emanating black, wiry hyphae. The data were subjected to Kaplan–Meier survival analysis (Kaplan and Meier, 1958) conducted in SAS JMP Pro 10 (SAS Institute, Cary, NC, USA).

Because minirhizotron imaging is only able discern the persistence of these structures and not their lifespan (i.e. the period of time when they are alive), we utilized vitality staining to help examine whether or not *C. geophilum* ectomycorrhizas were more likely to be dead relative to ectomycorrhizas of other species. Ectomycorrhizas were randomly sampled in the spring (12 May, 2011; $n = 102$), summer (22 July, 2011; $n = 52$), and autumn (5 October, 2011; $n = 51$) in a red pine (*Pinus resinosa* Aiton) plantation located in central Pennsylvania, USA (for site details see Koide et al., 2007). Ectomycorrhiza vitality was assessed with fluorescein diacetate (FDA) staining (based on methods from Qian et al., 1998; Noland and Mohammed, 1997) where the FDA is assumed to be metabolized by living cells, which causes it to fluoresce under ultraviolet light (see Rotman and Papermaster, 1966). Cross sections were examined under an Olympus SZ40 fluorescence microscope equipped with a 420–490 nm excitation filter and a 500 nm barrier filter and were given a vitality ranking (1–3) based on the level of fluorescence from the cortical cells in the cross sections (0% = dead(1), 1–30% = partially vital(2), >30% = vital(3)). We used a cumulative link model with a logit link to examine differences in the odds of vitality scores ≥ 2 between ectomycorrhizas (1 = *C. geophilum*; 0 = all other EM fungal species pooled) across season (spring, summer and autumn) in Program R (R Foundation for Statistical Computing, Vienna, Austria). We fit a single model with ectomycorrhiza morphotype, season and an interaction term using the ordinal package (Christensen, 2012) in R. Coefficients ($\widehat{\beta} \pm$ SE) are presented on the logit scale.

Observations from minirhizotrons indicated that *C. geophilum* ectomycorrhizas persisted 4–10 times longer than ectomycorrhizas of other EM fungi (Log-Rank: $\chi^2 = 66.2$, $P < 0.0001$; Wilcoxon: $\chi^2 = 48.5$, $P < 0.0001$). Median persistence of *C. geophilum* ectomycorrhizas was 831 d compared to 129 d for all other ectomycorrhizas (Fig. 1). The large difference in persistence (i.e. the amount of time the individual ectomycorrhizas remained visible in the soil) between *C. geophilum* and all other EM species suggests that either lifespan or resistance to decomposition following death, or both, is very different.

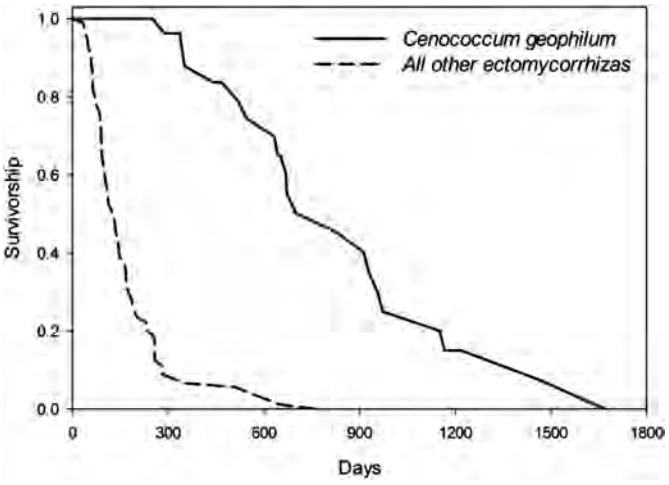

Fig. 1. Kaplan Meier estimates of survivorship for ectomycorrhizas formed by *Cenococcum geophilum* (solid line, $n = 28$, 8 censored) and all other species (dashed line, $n = 93$, 2 censored). *Cenococcum geophilum* had a significantly greater lifespan (median = 821 d) compared to all other species (median = 129 d).

Overall patterns of vitality among ectomycorrhizas indicated that *C. geophilum* ectomycorrhizas likely decomposed more slowly than other species leading to the increased persistence of *C. geophilum* in soil. Compared to other species, *C. geophilum* ectomycorrhizas were significantly less vital in spring ($\widehat{\beta} \pm$ SE = -0.88 ± 0.40, $P = 0.03$), but not significantly different in vitality in summer ($\widehat{\beta} \pm$ SE = -0.74 ± 0.55, $P = 0.18$) (Fig. 2). *C. geophilum* vitality did not significantly change between spring and summer ($\widehat{\beta} \pm$ SE = 0.14 ± 0.68, $P = 0.84$), when only a small fraction (10.0% in spring, 5.8% in summer) of *C. geophilum* ectomycorrhizas were classified as vital. By comparison, a much larger fraction of ectomycorrhizas formed by all other species was vital in spring (32.0%) and summer (28.6%). In autumn, *C. geophilum* ectomycorrhizas were >6 times more likely ($=e^{1.80}$) to be partially vital or vital than in the spring ($\widehat{\beta} \pm$ SE = 1.80 ± 0.73, $P = 0.014$) and >5 times more likely ($=e^{1.66}$) to be partially vital or vital than in the summer ($\widehat{\beta} \pm$ SE = 1.66 ± 0.82, $P = 0.044$). In autumn, the percent of vital *C. geophilum* ectomycorrhizas increased to 38.5% while the percent

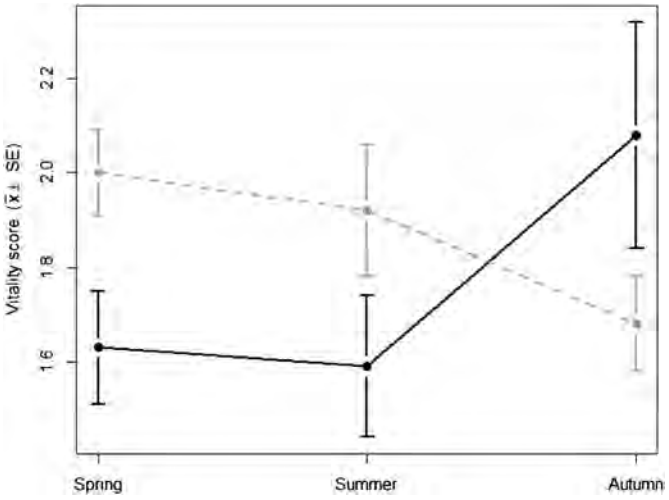

Fig. 2. Mean vitality scores (1 = dead, 2 = partially vital, 3 = vital) ± SE of *Cenococcum geophilum* (black solid line) and other ectomycorrhizas (dashed grey line) as measured during 2011 from a red pine (*Pinus resinosa* Aiton) plantation located in central Pennsylvania, USA.

of vital ectomycorrhizas for all other species decreased to 7.9%, but this result was non-significant ($\widehat{\beta} \pm SE = 0.92 \pm 0.61$, $P = 0.13$). These contrasting trends in vitality are partially explained by differential seasonality among *C. geophilum* and other EM species which is supported by observations of patterns of ectomycorrhiza production made with minirhizotrons (McCormack *Unpublished data*).

The long persistence of *C. geophilum* ectomycorrhizas coupled with their frequently low vitality during the growing season suggests that a substantial proportion of *C. geophilum* ectomycorrhizas found in forest soils are dead and resistant to decomposition. This recalcitrance may be the result of the heavy deposition of melanin in the cell walls of this fungus. Melanins are a group of polymers that are composed of phenolic or indolic monomers which are complexed with other components of the cell wall (Feofilova, 2010) and provide the cell protection from various environmental stressors (Bell and Wheeler, 1986; Butler and Day, 1998). Because of its aromatic and complex chemical structure, the polymer likely has an analogous effect on the decomposability of fungal tissues, as lignin has on plant litters, but further confirmation is needed.

The persistence of *C. geophilum* ectomycorrhizas in the environment may significantly retard the rate of C, N, and phosphorous cycling as they are sequestered in recalcitrant *C. geophilum* litter. This not only applies to ectomycorrhizas of *C. geophilum*, but also to the large amounts of extramatrical mycelia and sclerotia (resting structures) that it produces (Hunt and Fogel, 1983; Dahlberg et al., 1997). Together, our findings suggest that *C. geophilum* may play a disproportionate role in the sequestration of C and nutrients in many forest ecosystems and highlight the differences in decomposition dynamics of litter produced across EM fungal species. Future assessments of EM fungi should work to better link community structure and dynamics of both live and dead EM tissues to patterns of belowground and whole-ecosystem cycling of C and nutrients.

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