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Research

Priority report

Nitrogen fertilization reduces the standing biomass, abundance, and size of *Cenococcum* sclerotia: a ubiquitous but rarely quantified ectomycorrhizal soil carbon pool

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Summary

- Unlike most ectomycorrhizal (EM) fungi, *Cenococcum geophilum* is a prolific producer of sclerotia, which represent a large and persistent, yet rarely quantified pool of EM fungal biomass and carbon in soils. How biomass of these asexual propagules is impacted by global change factors, such as anthropogenic nitrogen (N) deposition, remains unquantified.
- This study examined the effects of long-term experimental N fertilization on the standing biomass, abundance, and size of *C. geophilum* sclerotia in an oak (*Quercus* spp.) savanna ecosystem at Cedar Creek Ecosystem Science Reserve in Minnesota, USA.
- Standing sclerotia biomass in the control treatment averaged 192 g m^{-2} (95% CI = 136–267 g m^{-2}) and declined sharply under N enrichment, by 44% (95% CI = −53–79%) and 66% (95% CI = 39–82%) in the low N (5.4 g N m^{-2} yr^{-1}) and high N (17 g N m^{-2} yr^{-1}) treatments, respectively. Sclerotia abundance also declined under both fertilization levels by 58% (95% CI: 8–81%) and 62% (95% CI: 12–84%), while sclerotia diameter was significantly reduced only under high N.
- Given their high carbon content, melanization, and long persistence, the observed declines in *C. geophilum* sclerotia (*c.* 84–127 g m^{-2}) represent substantial losses from belowground carbon (C) pools. These findings indicate that chronic N deposition suppresses the formation of a functionally important and recalcitrant fungal structure, likely impacting soil C storage and mycorrhizal functional diversity.

Introduction

Anthropogenic nitrogen (N) deposition has been widely demonstrated to fundamentally alter soil biogeochemistry, with cascading effects on belowground biota (Goulding *et al.*, 1998; Phoenix *et al.*, 2012; Borer & Stevens, 2022). In particular, there is mounting evidence suggesting that ectomycorrhizal (EM) fungi, which form widespread symbiotic relationships with many forest trees, are highly sensitive to the deposition of inorganic N (Averill *et al.*, 2018; Lilleskov *et al.*, 2019). Nitrophobic EM fungal taxa, characterized by their sensitivity to N enrichment, consistently decline in abundance with increasing N inputs, while nitrotolerant EM fungal taxa demonstrate resilience or even increased abundance under elevated N conditions (Lilleskov *et al.*, 2001, 2011; Avis *et al.*, 2003; Suz *et al.*, 2014). These differential responses often correspond to significant shifts in EM fungal community structure along with a general loss of EM fungal species with increased N inputs (Avis *et al.*, 2008). The relationship between N deposition and EM fungal abundance may be more complex than simple linear relationships. For instance, some EM fungal taxa have been shown to have unimodal responses where moderate N availability may initially stimulate certain EM taxa before inhibiting them at higher concentrations (e.g. *Paxillus involutus*; Lilleskov *et al.*, 2002). This complexity is further illustrated by strong context dependencies, as studies have found stronger negative effects on EM fungi from external nitrogen deposition than from endogenous sources of N made available through saprotrophic mineralization, even when nitrogen availability levels are similar (Jörgensen *et al.*, 2024).

Beyond taxonomic changes, N deposition can also induce significant functional shifts in EM symbioses. Elevated N availability generally decreases host plant carbon (C) allocation to mycorrhizal fungi (Hasselquist *et al.*, 2016), as enhanced N acquisition becomes less critical (Treseder, 2004). As soil N availability increases, EM fungal communities also typically exhibit reduced enzyme activities involved in organic N mobilization (Allison *et al.*, 2008) and taxa with peroxidase capabilities (i.e. some *Cortinarius* spp.) decline in abundance with increasing inorganic N availability (Argiroff *et al.*, 2022). These functional responses of EM fungi to N deposition likely have significant consequences for forest ecosystem processes. For example, reduced EM fungal biomass under elevated N conditions can affect soil carbon dynamics, as these fungi are significant contributors to soil C storage through both living biomass and necromass production (Ekblad *et al.*, 2013; Fernandez *et al.*, 2016). Further, reduced enzyme activities and altered C allocation patterns also disrupt the tight coupling between C and N cycles that characterize undisturbed forest ecosystems (Zak *et al.*, 2019a,b).

Cenococcum geophilum Fr. is an ascomycete fungus and one of the most ubiquitous EM fungal species (or cryptic species complex (Obase *et al.*, 2016; Dauphin *et al.*, 2021); referred to as *Cenococcum* hereafter) on Earth. Like many EM fungal taxa, *Cenococcum* produces various extracellular enzymes that target organic nutrient pools (e.g. proteases, phosphatases) (Bae & Barton, 1989; Jones *et al.*, 2012), but shows significant reductions in genes encoding plant cell wall–degrading enzymes, a pattern that appears to be important in the evolutionary transition from a saprotrophic to an EM lifestyle (Peter *et al.*, 2016). Perhaps the most unique and defining feature of *Cenococcum* is its heavy melanization, which accounts for *c.* 20–30% of its dry mass (Fernandez & Koide, 2014). This amount is substantially higher than the average melanization level of EM fungi (typically well below 10%) and is important to its osmotic stress tolerance (Fernandez & Koide, 2013). High melanization strongly contributes to the exceptionally long-term recalcitrance of *Cenococcum* necromass, potentially contributing disproportionately to soil organic carbon (SOC) (Fernandez & Koide, 2014; Fernandez *et al.*, 2019). *Cenococcum* forms symbioses with a remarkably wide range of host plants – over 200 species across all EM host lineages – in boreal, temperate, subtropical, and even arid ecosystems (Trappe, 1962; Douhan & Rizzo, 2005; Dickie, 2007; Põlme *et al.*, 2018). Surveys of EM fungal communities, both older morphotype-based and modern DNA sequencing studies, consistently find *Cenococcum* as a major component of fungal communities across continents and forest types (Obase *et al.*, 2016). Beyond its ubiquity, *Cenococcum* has been shown to mobilize nutrients from soil organic matter (SOM) pools for its hosts (Ponge, 1990; Durall *et al.*, 1994; Wu *et al.*, 2003), making it an important symbiont in nutrient-poor soils.

Unlike many EM fungi, the sexual spore-producing sporocarps of *Cenococcum* are highly cryptic and/or rare and have yet to be described morphologically and confirmed in a convincing manner with molecular methods (Douhan *et al.*, 2007; but see Fernández-Toirán & Águeda, 2007). Instead, *Cenococcum* is a prolific producer of melanized sclerotia (Fig. S1) that serve as durable, nutrient-rich asexual propagules (Trappe, 1962; Obase *et al.*, 2016). Sclerotia are hardened masses of hyphae that serve as resilient resting structures, enabling fungi to withstand unfavorable environmental conditions (i.e. drought, nutrient scarcity, freezing). Ecologically, sclerotia buffer the fungus through stress periods, enabling seasonal regrowth and rapid colonization post-disturbance (Smith *et al.*, 2015). *Cenococcum* sclerotia persist for years – often decades – and may represent several hundred grams of biomass per m^2 (Fogel & Hunt, 1979; Vogt *et al.*, 1981; Nyamsanjaa *et al.*, 2022). Despite abundant studies on EM fungal community shifts, the specific impacts of inorganic N on *Cenococcum* sclerotia production and standing biomass remain unexamined.

N-induced changes in *Cenococcum* sclerotia stocks could have multiple ecosystem-scale implications. First, 'sclerotia banks' are the main means by which *Cenococcum* survives unfavorable conditions, seasonal or otherwise, allowing for rapid recolonization under more favorable conditions (Vogt *et al.*, 1982). If N deposition reduces the density of viable sclerotia, the ability of the fungus to persist through dormancy or recover from disturbance could be significantly compromised. Second, as a highly abundant and generalist symbiont, *Cenococcum* has been identified among EM fungi as a species with disproportionate influence on soil fungal community structure (Zhu *et al.*, 2024). As N deposition typically favors fast-turnover fungi at the expense of recalcitrant, carbon-demanding species (Lilleskov *et al.*, 2018; Fernandez *et al.*, 2023), EM interaction networks may become less stable (Zhu *et al.*, 2024). Finally, and perhaps most globally significant, *Cenococcum* sclerotia represent a major pool of sequestered SOC in forest soils (Vogt *et al.*, 1982). These dense, melanized structures have C contents ranging from 40% to 50% (Watanabe *et al.*, 2007; Nyamsanjaa *et al.*, 2022), and because of their large standing biomass and very slow decomposition, sclerotia effectively lock up plant-derived C for decades (Nyamsanjaa *et al.*, 2022). A reduction in sclerotia biomass due to N deposition would thus translate directly into a loss of *Cenococcum*'s function as a strong C sink. Over long timescales, this could contribute to higher soil C turnover and lower sequestration. Given the interest in mycorrhizal contributions to soil C dynamics (Zak *et al.*, 2018; Frey, 2019), understanding any N-driven loss of *Cenococcum*'s C sink function is crucial.

In this study, we examined the effects of N fertilization on the standing sclerotia biomass and morphology of *Cenococcum*. Specifically, by quantifying sclerotia in experimental plots at the Cedar Creek Ecosystem Science Reserve in Minnesota, USA, subjected to varying levels of N fertilization, we aimed to improve our understanding of whether inorganic N deposition alters the production, abundance, and size of a rarely quantified soil C pool. We tested three hypotheses. First, (H1) increased N fertilization will significantly reduce the standing *Cenococcum* sclerotia standing biomass. Since standing biomass can be affected by changes in both size and abundance of sclerotia with potentially different functional implications, we also examined both sclerotia abundance and diameter across the N-treated soils. The abundance (H2) and (H3) size of *Cenococcum* sclerotia will be negatively affected by N fertilization.

Materials and Methods

Site description

The study was conducted within a long-term nitrogen fertilization experiment established in 1983 in an oak savannah ecosystem at the Cedar Creek Ecosystem Science Reserve, Minnesota, USA, which has been maintained to the present day (Tilman, 1987). Soils are Typic Udipsamments, characterized as being sandy, well-drained, and infertile. Background wet nitrogen deposition at the Cedar Creek Ecosystem Science Reserve is *c.* 6 kg N ha^{-1} yr^{-1} (= 0.6 g N m^{-2} yr^{-1}) (Tilman, 1987; Clark & Tilman, 2008). Plant communities in the plots are dominated by mature (50–150 yr old) EM host trees, bur oak (*Quercus macrocarpa*) and pin oak (*Q. ellipsoidalis*), with tall-grass prairie plants, including the herbaceous EM plant *Crocanthemum bicknellii* (synonym: *Helianthemum bicknellii*; Dickie *et al.*, 2004), in the understory. Nine experimental plots (20 m × 50 m) were established in 1983 (Tilman, 1987) and were randomly assigned to one of three N fertilization treatments (Control: 0 g N m^{-2} yr^{-1}; Low: 5.4 g N m^{-2} yr^{-1}; High: 17 g N m^{-2} yr^{-1}). The fertilization treatments were applied twice annually in May and late June to the plots as slow-release NH_4NO_3 pellets. In addition, plots also received equal levels of P, K, Ca, Mg, S, and trace elements to offset the indirect effects of inorganic N addition. For more information about the experimental plots, see Tilman (1987).

Sampling

Four replicate soil cores (5 × 15 cm^2) were collected from each plot (Total $N = 36$) in a systematic sampling design in July 2014. Sampling points were located 4 m apart from each other and arranged in a grid to avoid spatial autocorrelation (Lilleskov *et al.*, 2004), but all were located in the closed canopy portion of the plots. Cores were brought back to the laboratory and allowed to air dry. Samples were then homogenized in the plastic bag before taking a 2-g subsample. Each subsample was then passed through a 2-mm sieve to remove large particulate organic matter and roots. From the sieved soil subsample, *Cenococcum* sclerotia were hand-picked with forceps under a dissecting microscope. Sclerotia produced by *Cenococcum* are easily identified by their unique spherical morphology, ranging from *c.* 0.05 to 7 mm in diameter and jet-black color (Trappe, 1962; Fig. S1). Sclerotia extracted from each subsample were then imaged against the white surface of weigh boats with a digital camera. Images were imported into ImageJ software, and the number of sclerotia and their diameters were determined. Finally, the total mass of the sclerotia from each sample was determined using a microbalance. From the sclerotia total mass data, we calculated standing sclerotia biomass with the following equation:

$$\text{Standing sclerotia mass } (\text{g m}^{-2}) = \frac{\left(\frac{\text{Sclerotia mass in subsample (g)}}{2\text{ g}} \times \text{Total dry mass of soil core (g)}\right)}{\text{Soil core area } (\text{m}^2)}$$

Statistics

To test the effects of N fertilization on sclerotia standing biomass, abundance, and diameter, we used generalized linear mixed models (GLMMs) implemented in the *glmmTMB* package in R. Because all three response variables exhibited right-skewed distributions, we specified a Gamma error distribution with a log link to better accommodate the observed heteroscedasticity and ensure positive model prediction values. For the sclerotia standing biomass and abundance data, plot was included as a random intercept to account for nested spatial structure. To account for the nested structure of the sclerotia diameter data (sclerotia sampled within soil cores and plots), we included a random intercept for each core nested within plot. Because sclerotia abundance varied across treatments, this hierarchical structure controls for pseudoreplication and prevents unequal weighting of plots with more observations. To evaluate whether N treatments altered not just the mean response but also its variability, we modeled the residual dispersion as a function of treatment using the 'dispformula' argument. This heteroskedastic modeling framework allowed us to test whether N addition influenced both the central tendency and heterogeneity of sclerotia biomass, abundance, and diameter, providing greater insight into potential threshold or convergence dynamics in response to nutrient enrichment (e.g. whether N fertilization leads to more uniform (convergent) or more variable (divergent) sclerotia metrics). Treatment effects from GLMMs were converted to percent changes relative to controls by back-transforming log-scale coefficients (% change = $(1 - \exp(\beta)) \times 100$). Confidence intervals (95%) were calculated on the log scale ($\beta \pm 1.96 \times \text{SE}$) before back-transformation, accounting for asymmetry when transforming between scales.

Results

We found strong support for H1: *Cenococcum* sclerotia biomass was highest in the control plots and progressively declined with increasing N fertilization rate (Fig. 1). The high N treatment (17 N g m^{-2} yr^{-1}) caused a 66% (95% CI: 39–82%) reduction in mean sclerotia standing biomass ($P < 0.001$), while the low N treatment (5.4 N g m^{-2} yr^{-1}) reduced standing biomass mean by 44% (95% CI = −53–79%), but was not statistically significant ($P = 0.26$). However, the low N treatment significantly reduced variability in biomass ($P = 0.0099$; Table S1), indicating that N addition can also impact heterogeneity in sclerotia production.

Supporting H2, we found that N fertilization significantly reduced sclerotia abundance (Fig. 2a), with low and high N treatments showing *c.* 59% ($P = 0.03$; 95% CI: 8–81%) and 63% ($P = 0.025$; 95% CI: 12–84%) declines relative to the control, respectively. However, no significant differences in variability were detected among treatments (Table S2). Finally, we also found partial support for H3, where high N fertilization marginally reduced mean sclerotia diameter compared with the control ($P = 0.066$), corresponding to a *c.* 10% decrease in diameter (Fig. 2b; Table S3). However, the low N treatment did not

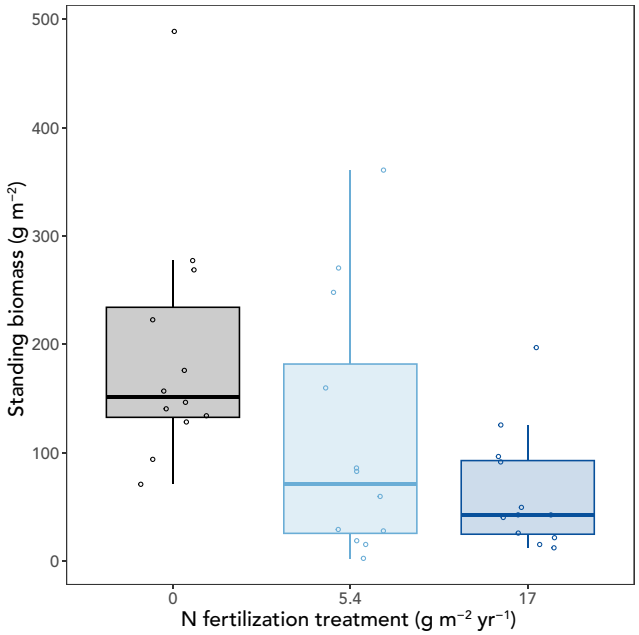

Fig. 1 Standing *Cenococcum geophilum* sclerotia biomass in the upper 15 cm of the soil profile in response to nitrogen fertilization (g N m^{-2} yr^{-1}). N fertilization levels are 0 (control), 5.4, and 17 g N m^{-2} yr^{-1}. Boxplots represent the median (line in box), 25th percentile (lower hinge), and 75th percentile (upper hinge) ranges, and whiskers represent 1.5× interquartile range. Points represent sample estimates from each treatment ($n = 36$). Nitrogen fertilization significantly reduced standing biomass, with biomass losses of up to 66% under high N addition relative to the control (see Supporting Information Table S1).

significantly differ from the control ($P = 0.91$). Modeling treatment-specific dispersion revealed that N fertilization also influenced sclerotia size variability, where the low N treatment significantly decreased diameter variability relative to the control ($P = 0.038$), while the high N treatment showed a marginal increase in variability ($P = 0.080$). These results suggest that sclerotia size trait convergence may occur at moderate enrichment levels.

Discussion

The 66% decline in standing biomass of *Cenococcum* sclerotia in response to an N deposition rate of 17 g m^{-2} yr^{-1} represents a substantial reduction of *c.* 127 g m^{-2} of persistent fungal biomass. This loss, which is on the same order as typical fine root biomass (*c.* 240–500 g m^{-2}; Jackson *et al.*, 1997), exceeds typical soil microbial biomass (*c.* 30–140 g m^{-2} total biomass, equivalent to 15–70 g C m^{-2}) in most forest soils globally (Xu *et al.*, 2013). The few existing quantifications of *Cenococcum* sclerotia biomass in forest ecosystems demonstrate that these structures represent substantial standing pools, with Fogel & Hunt (1979) documenting 279 g m^{-2} of *Cenococcum* sclerotia in soils of a *Pseudotsuga menziesii* forest and Vogt *et al.* (1981) reporting 230–300 g m^{-2} in *Abies amabilis* stands. Given that *Cenococcum* sclerotia are heavily melanized and can persist for decades in forest soils (Vogt *et al.*, 1981; Fernandez *et al.*, 2013; Clemmensen *et al.*, 2015), these observed declines under N enrichment levels typical of many parts of the eastern United States likely reflect strong shifts in belowground fungal C pools. This pattern also aligns with previous findings showing suppression of *Cenococcum* among EM root tips and in soil DNA under elevated N (Morrison *et al.*, 2016; De Witte *et al.*, 2017). Given the potential role of EM structures in stabilizing SOM and immobilizing nutrients (Ekblad *et al.*, 2013; Clemmensen *et al.*, 2015), the loss of *Cenococcum* root tips, mycelium, and sclerotia under chronic N loading may reduce both fungal C sequestration and belowground functional diversity.

While both sclerotia size and abundance decreased significantly, the negative response in standing *Cenococcum* sclerotia biomass was primarily driven by reduced abundance rather than changes in size (Fig. S2). This pattern suggests that N deposition limits the initiation or persistence of new sclerotia. While increased N availability could reduce the persistence of sclerotia and thereby contribute to declines in standing biomass pools, given the well-documented longevity of *Cenococcum* sclerotia (Watanabe *et al.*, 2007; Nyamsanjaa *et al.*, 2022), this explanation appears less likely than a reduction in production. We can envision several nonmutually exclusive mechanisms that may alter sclerotia production following N deposition. First, fungi may shift resource allocation away from sclerotia production in favor of other functions under changing nutrient status. Experimental work on other fungal species has shown that sclerotia are energetically costly structures, and their production may be downregulated under nutrient-enriched conditions (Punja, 1986). Another likely mechanism for this response is through N-induced reductions in host plant root production and C allocation to EM fungi. N addition often reduces fine root production (Kjøller *et al.*, 2012; Zhu *et al.*, 2013) and EM root tip colonization rates (Treseder, 2004), and long-term fertilization can significantly decrease belowground C allocation from host trees (Janssens *et al.*, 2010). McCormack *et al.* (2017) tracked the production of *Cenococcum* root tips in a *Pinus*-dominated forest over a 12-yr period using minirhizotron imaging and found an 83% decline in response to N fertilization. Collectively, these changes reduce the flow of photosynthate to the fungal symbiont, likely limiting its capacity to form energy-intensive structures like sclerotia. Previously, Avis *et al.* (2003) characterized the EM fungal communities colonizing oak roots in the same plots at Cedar Creek and found that *Cenococcum* percent colonization responded inconsistently to the N treatments across two years of sampling – one year showed a slight but nonsignificant decline with N fertilization, while the next showed increased colonization in the N treatment plots compared with controls. Those findings suggest that colonization rates may be maintained under N enrichment, but C allocation to *Cenococcum* (and other EM fungi) may still be negatively affected.

The observed declines in *Cenococcum* sclerotia standing biomass are also likely to have important implications for both population- and community-level dynamics. Sclerotia serve as long-lived resting propagules that buffer *Cenococcum* populations

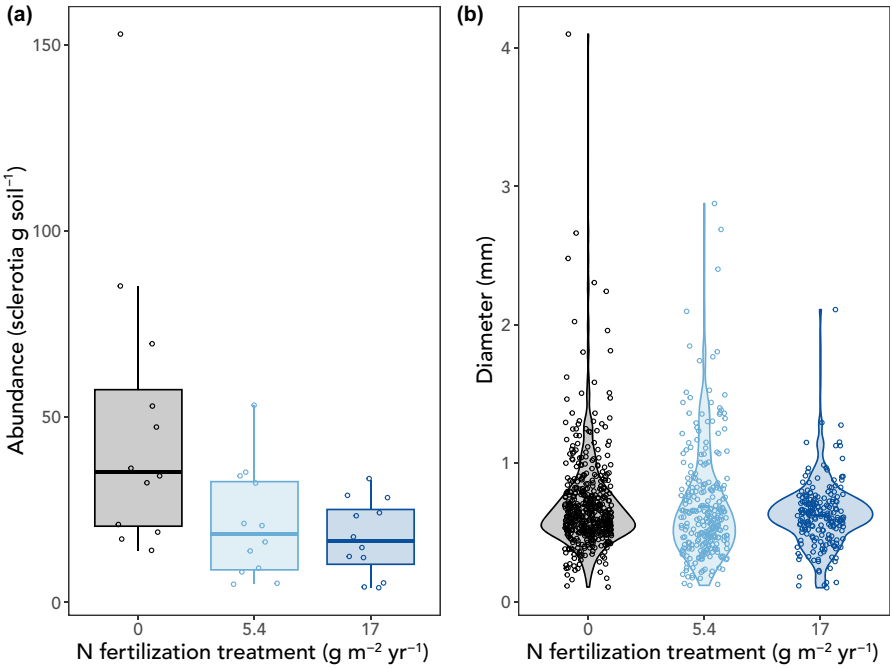

Fig. 2 *Cenococcum geophilum* sclerotia abundance per g soil (a) and diameter (b) in the upper 15 cm of the soil profile in response to nitrogen fertilization (g N m^{-2} yr^{-1}). N fertilization levels are 0 (control), 5.4, and 17 g N m^{-2} yr^{-1}. (a) Boxplots represent the median sclerotia abundance (line in box), 25th percentile (lower hinge), and 75th percentile (upper hinge) ranges, and whiskers represent 1.5× interquartile range for each treatment. Points represent the abundance from each subsample ($n = 36$). Nitrogen addition significantly reduced sclerotia abundance, with the greatest decline observed under high N inputs (see Supporting Information Table S2 for model results). This pattern suggests a suppression of sclerotia production in response to increased nitrogen fertilization. (b) The width of the violin reflects the relative frequency of sclerotia diameter observations, and points represent individual sclerotia diameters ($n = 1148$) taken from each treatment. Nitrogen addition significantly reduced sclerotia diameter (see Table S3 for statistical results), with a pronounced shift in the distribution toward smaller size classes under elevated N availability.

through adverse conditions and enable rapid recolonization when favorable conditions return (Trappe, 1962; Read & Haselwandter, 1981; Obase *et al.*, 2014). A sustained reduction in production may increase the risk of local extinction and reduced genetic diversity by limiting the pool of surviving genets (Taylor & Alexander, 2005; Obase *et al.*, 2018). In systems with chronic N deposition, this could translate to smaller, more fragmented *Cenococcum* populations that are less able to recover after disturbance or drought. At the community level, reductions in *Cenococcum* sclerotia banks are likely to cause changes to both EM fungal assemblages and host tree interactions. As a drought-tolerant, generalist EM fungal species, *Cenococcum* often dominates in nutrient-poor soils and can facilitate the establishment of a broad range of tree seedlings (Nara, 2006). Fewer sclerotia may therefore open niches for less stress-tolerant EM taxa, potentially shifting community composition toward species with faster turnover and lower resistance to environmental extremes (Lilleskov *et al.*, 2002). Such turnover can alter nutrient foraging strategies at the stand scale and affect seedling survival, with potential implications for forest regeneration trajectories under global change (Lilleskov *et al.*, 2011). Finally, *Cenococcum* sclerotia have been demonstrated to harbor unique assemblages of fungi (Obase *et al.*, 2014) and bacteria (Nonoyama & Narisawa, 2021), and reductions in standing biomass may have negative consequences on the microbial communities relying on these structures as substrate and/or refugia.

Conclusion

Sclerotia produced by the ubiquitous EM fungus *Cenococcum geophilum* represent substantial but rarely quantified soil C pools in EM-dominated ecosystems. This underrepresentation in the literature is likely due to the technical difficulty, time investment, and taxonomic expertise required to isolate, identify, and quantify *Cenococcum* sclerotia. Given the size of these pools and their sensitivity to global change drivers such as N deposition, quantifying and understanding these structures is critical—not only for advancing fungal ecological understanding but also for assessing their role in soil C dynamics and broader ecosystem functioning. Their extreme persistence for decades or longer suggests that *Cenococcum* sclerotia may play a disproportionate role in long-term C sequestration relative to other fungal structures. As such, incorporating them into conceptual and quantitative frameworks may improve predictions of how forest ecosystems respond to environmental change.

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Competing interests

None declared.

Author contributions

CWF designed and conducted the research. CWF and PK analyzed the data and wrote the manuscript.

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Data availability

Supporting data is available in Dataset S1.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Dataset S1 *Cenococcum geophilum* sclerotia standing biomass, abundance, and size measurements across nitrogen fertilization treatments.

Fig. S1 Photograph of *Cenococcum geophilum* sclerotia.

Fig. S2 The relationship between *Cenococcum geophilum* sclerotia standing biomass and abundance, and mean diameter.

Table S1 Output from a generalized linear mixed-effects model (GLMM) with a gamma error distribution and log link function predicting standing biomass of *Cenococcum geophilum* sclerotia.

Table S2 Output from a generalized linear mixed-effects model (GLMM) with a gamma error distribution and log link function predicting abundance of *Cenococcum geophilum* sclerotia from soil subsamples.

Table S3 Output from a generalized linear mixed-effects model (GLMM) with a gamma error distribution and log link function predicting diameter (mm) of *Cenococcum geophilum* sclerotia from soil subsamples.

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