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Functional Ecology

RESEARCH ARTICLE

# Soil fungal necromass in deciduous-dominated boreal forest after 13 years of inorganic nitrogen addition

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## Abstract

1. Ectomycorrhizal (ECM) fungi comprise a large proportion of the living and dead microbial-derived soil carbon (C) pool in boreal forests. Because soil nitrogen (N) and C cycles are closely interlinked, shifts in N availability and subsequent effects on dead fungal mass ("necromass") may influence C storage in soils. Several mechanisms could underlie the balance of fungal necromass production and stabilization, including fungal morphological traits and physiological traits and biochemical interactions between roots and ECM fungi.
2. We applied inorganic N (30 kg ha$^{-1}$ year$^{-1}$) for 13 years in a boreal forest dominated by *Populus tremuloides* Michx. and measured total fungal necromass concentrations and total C concentrations in organic and mineral soil. We also measured total fungal biomass concentrations in soil (representing changes in inputs), condensed tannin and chitin concentrations of mycorrhizal roots (representing changes in necromass stabilization), and the potential genetic capacity of the ECM fungal community to produce chitinases (indicating chitin degradation potential) and class II peroxidases (indicating polyphenol degradation potential).
3. We detected little effect of long-term N addition on soil fungal necromass concentration. Long-term N addition did not have a detectable effect on soil C concentration, standing fungal biomass, fine root tannin or chitin concentrations. Despite downward trends, there was also no detectable effect of N addition on the potential genetic capacity of the ECM fungal community to produce chitinases or class II peroxidases.
4. Our study indicates that 13 years of inorganic N addition in a deciduous broadleaf-dominated boreal forest has little detectable effect on soil fungal necromass concentrations or potential underlying mechanisms. However, morphological and physiological traits of the ECM community appear decoupled in response to inorganic N addition, representing key functional responses that warrant further investigation.

KEYWORDS
chitin, condensed tannins, deciduous, global change, mycorrhizal fungi, *Populus tremuloides*, soil carbon, soil organic matter

## 1 | INTRODUCTION

Approximately 65% of the dead microbial mass ("necromass") in forests is derived from fungi (Wang et al., 2021), particularly mycorrhizal fungi (Clemmensen et al., 2013; Frey, 2019), which transfer nutrients to the host plant in parallel with receiving photosynthetically derived carbon (C). Mycorrhizal fungi and the roots they colonize are a dominant pathway through which C enters the soil, and has been found to contribute up to 70% of stored soil C in boreal forests (Clemmensen et al., 2013). Ectomycorrhizal (ECM) fungi are efficient scavengers of nitrogen (N), particularly in boreal forests where N availability is constrained by low temperatures and decomposition rates (Read et al., 2004). Studies in forests and other ecosystems have also found that N fertilization increases soil C concentrations (Huang et al., 2011; Tipping et al., 2017). In boreal forests, however, nearly all N addition studies have been conducted in evergreen-dominated stands with ericaceous understories (Karst et al., 2021), despite deciduous broadleaf-dominated stands comprising a considerable fraction of boreal forests (Massey et al., 2023). Furthermore, few studies have investigated the potential shifts in fungal biomass and necromass concentrations following long-term N addition in boreal forests (Forsmark et al., 2024; Maaroufi et al., 2015). Since most microbial necromass is derived from fungi, understanding the mechanisms that influence soil fungal necromass formation or stabilization is important for identifying how N addition may affect soil C storage in deciduous broadleaf-dominated boreal forests.

The formation and stabilization of fungal necromass may be driven by morphological traits of ECM fungi, biochemical interactions between roots and ECM fungi, and the physiological traits of ECM fungi. Ectomycorrhizas are often characterized by morphological traits such as exploration type, referring to the abundance, length, and branching patterns of emanating hyphae (Agerer, 2001). Exploration types may indicate different foraging strategies among ECM fungal species, and might also be linked with soil fungal biomass inputs (Weigt et al., 2012). For example, 'distance' exploration types may add approximately 15 times more mycelium biomass to soils than 'contact' or 'smooth' exploration types (Weigt et al., 2012), though other work has questioned this link (Jörgensen et al., 2022). Previous work in a deciduous-dominated boreal forest has found that inorganic N addition increased the relative abundance of distance exploration types (Karst et al., 2021), suggesting more ECM biomass under N addition. Along with influencing the input of soil fungal necromass, exploration types may also affect necromass stabilization. Dead rhizomorphs may decompose slower than diffuse hyphae belonging to the same fungal species (Certano et al., 2018), and cord-forming fungi may persist in soils longer than other types (Treseder et al., 2005). However, other research has found that cord-forming fungi turnover more rapidly than other exploration types, limiting the size of this necromass pool (Clemmensen et al., 2015). Collectively, these studies suggest that morphological traits of ECM fungi can play a key role in the formation and persistence of fungal necromass.

Biochemical properties of the mycorrhiza (i.e. root and fungus together) may also play a role in stabilizing necromass. Chitin is a N-rich, labile part of fungal cell walls. However, it can form hydrogen bonds with root tannins, resulting in chitin-tannin complexes that are resistant to decomposition and that contribute to soil organic matter accumulation (Adamczyk et al., 2019). Condensed tannins are a group of secondary metabolites in plants, and intimate physical contact between chitin and tannins (i.e. conditions favoured by ECM fungal colonization of roots) is necessary for complexes to form (Adamczyk et al., 2019). However, complex formation may be disrupted by N addition. Chitin concentrations of ECM roots have been linked with root degradability (Fernandez & Koide, 2012; Koide et al., 2014), and other work has found that ECM chitin concentrations across several ECM taxa decreased from early summer to autumn, despite large differences among taxa (Wallander et al., 1997). This could reflect seasonal changes in soil inorganic N availability and tree N demand that have also been found in boreal aspen forest (Huang & Schoenau, 1997), suggesting that N fertilization may increase ECM chitin concentrations and therefore increase root degradability. Furthermore, condensed tannin concentrations of roots have been shown to decline under N fertilization (Gebauer et al., 1997; Kraus et al., 2004), thus potentially limiting chitin-tannin complex formation. These processes combined may ultimately decrease the fungal necromass pool in soils if the necromass becomes more labile.

Along with morphological and biochemical traits, the physiological traits of ECM fungi may further influence the accumulation of soil fungal necromass and soil C concentration. Fungi deploy extracellular chitinases to hydrolyse chitin and acquire N, and the ability to produce chitinases varies widely among ectomycorrhizal fungal taxa (Lindahl & Taylor, 2001; Maillard et al., 2023). The abundance and expression of chitinase genes among ECM taxa has recently been found to predict N uptake from chitin (Maillard et al., 2023), suggesting a direct link between chitinase gene abundance and instantaneous N uptake from chitin. Higher inorganic N availability may therefore reduce the need for chitinase, consequently selecting against ECM fungi with higher genetic potential to produce chitinase and ultimately favouring fungal necromass accumulation. However, the potential accumulation of fungal necromass may be offset by overall lower abundance of standing fungal biomass and more labile fungal necromass inputs, leading to negligible changes in soil fungal necromass and total soil C concentration, but this balance is poorly understood. Peroxidase activity is another key physiological trait of some ECM fungi

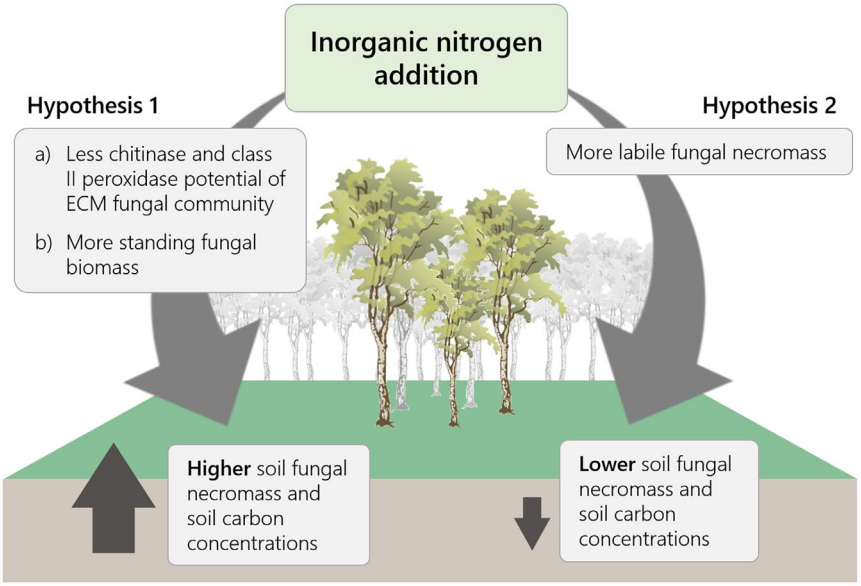


**FIGURE 1** Conceptual diagram of hypotheses for the effects of inorganic nitrogen addition on soil fungal necromass and carbon concentration. We hypothesized that the effects of Hypothesis 1 would outweigh the effects of Hypothesis 2, resulting in a net increase in soil fungal necromass and soil carbon concentrations with inorganic nitrogen addition. Tree images retrieved from Vecta (vecta.io).

that could influence soil C (Kyaschenko, Clemmensen, Karltun, et al., 2017; Lindahl et al., 2021). Due to their dependence on host plants for C, most ECM fungi have lost the ancestral ability to produce oxidative enzymes, such as class II peroxidases, for degrading complex organic matter (Kohler et al., 2015). However, several lineages of ECM fungi have retained this critical trait, allowing them to liberate nutrients locked within complex organic matter (Kohler et al., 2015). Previous work has demonstrated that higher N availability downregulates the activity of class II peroxidases and reduces the relative abundance of ECM taxa that possess this trait, as inorganic N is more readily available (Kyaschenko, Clemmensen, Hagenbo, et al., 2017). With higher inorganic N availability and consequently lower class II peroxidase production, organic matter may then accumulate.

We applied inorganic N for 13 years at a relatively low rate to simulate the impact of atmospheric N deposition in a Canadian boreal forest dominated by *Populus tremuloides* Michx. We investigated the influence of N addition on the mechanisms underlying the formation and stabilization of fungal necromass, and consequences for soil C concentration. Our main hypothesis was that inorganic N addition would increase soil fungal necromass concentrations and subsequently increase soil C concentrations (Figure 1). This main hypothesis was driven by the net outcome of competing hypotheses. First, we hypothesized (**H1a**) that inorganic N addition would lower the chitinase and class II peroxidase potential of the ECM fungal community, which would favour soil fungal necromass accumulation and soil C concentration; and (**H1b**) that inorganic N addition would increase standing fungal biomass (driven by a higher proportion of distance-type ECM fungi as reported by Karst et al. (2021)), therefore increasing soil fungal necromass and soil C concentration. Our competing hypothesis, (**H2**) was that inorganic N addition would make fungal necromass more labile (driven by lower condensed tannin concentrations and higher chitin concentrations of ectomycorrhizas, and shifting abundance of some ECM fungal taxa as reported by Karst et al. (2021)), therefore decreasing soil fungal necromass and soil C concentration. We hypothesized that the effect of H1 will outweigh the competing effects of H2, resulting in a net increase in soil fungal necromass and soil C concentration with inorganic N addition. Testing these hypotheses will further our mechanistic understanding of how inorganic N addition influences the formation and stabilization of soil fungal necromass and soil C concentration, particularly in deciduous broadleaf-dominated boreal forests.

## 2 | MATERIALS AND METHODS

### 2.1 | Field experiment

An ammonium-nitrate addition experiment was established in 2006 in a boreal forest dominated by *P. tremuloides* and located in Alberta, Canada (56.14 N, −110.87 W). No permits were required for fieldwork. *Populus tremuloides* comprised 71% of stand basal area, while another 22% comprised of *Picea glauca* Moench (Jung & Chang, 2012). The forest naturally regenerated, likely after a stand-replacing wildfire in the 1930s (Karst et al., 2021). The experiment had four blocks, each containing two 20 × 20 m plots surrounded by a 5–10 m forest buffer. Each of the two plots within a block was randomly assigned to either control or N addition (30 kg N $ha^{-1}$ $year^{-1}$) treatment. The N addition treatment is within the lower range of other N addition studies in boreal and temperate forests (Karst et al., 2021). Background N deposition rates are approximately 1.5 kg $ha^{-1}$ $year^{-1}$ (Wieder et al., 2019). Granular $NH_4NO_3$ was broadcasted by hand once per year from 2006 to 2008 in early summer, and starting in 2009, applied three times per year in early (June), mid (July) and late summer (August) to better simulate N deposition. In 2019, the year of the current survey, the early summer N addition was missed. Soils and roots were collected on August 24, 2019. See Karst et al. (2021) for further details on site description, and Table 1 for a summary of previous

TABLE 1 Summary of responses to inorganic N addition (30 kg N $ha^{-1}$ $year^{-1}$) found in earlier studies from the same experiment.

| Measurement | Years of treatment | Response | Reference |
|---|---|---|---|
| *Populus tremuloides* growth | 13 | Initial increase in annual stem radial growth (first 4 years) followed by convergence with control plots | Karst et al. (2021) |
| Soil pH | 10 | No difference | Kwak et al. (2018) |
| Soil C concentration | 4 | No difference | Jung and Chang (2012) |
| ECM distance exploration types | 13 | Higher relative abundance | Karst et al. (2021) |
| ECM fungal community composition | 13 | No difference | Karst et al. (2021) |
| ECM fungal diversity | 13 | Higher Shannon diversity | Karst et al. (2021) |
| ECM fungal richness | 13 | No difference | Karst et al. (2021) |

Abbreviations: C, carbon; ECM, ectomycorrhizal.

findings from this experiment including *P. tremuloides* growth, soil pH, and the ECM fungal community.

## 2.2 | Measuring concentrations of microbial biomass and necromass

To estimate standing soil fungal biomass, we measured soil ergosterol concentrations. A step soil probe (1.9 cm diameter × 23 cm depth) was used to core soils at three random locations within each plot. Vegetation, litter, and fibric material was removed, then organic and mineral soil was placed in separate bags. Soils were then put on ice for approximately 24 h before storing at −20°C. Soils frozen for ergosterol were analysed within 2 weeks of field collection. We extracted ergosterol from 125 mg of freeze-dried organic and mineral soil, respectively, using 10% KOH in methanol, according to Sterkenburg et al. (2015). Extracts were filtered through glass wool and a 20 μL injection volume was analysed using an Ultra High Performance Liquid Chromatography/Diode-Array Detector (1290 Infinity, Agilent Tech., Santa Clara, CA) fitted with a Poroshell 120 EC-C18 column (2.1 mm × 150 mm, 2.7 μm; Agilent Tech.). The mobile phase consisted of an isocratic binary system of 25% methanol (HPLC-grade) and 75% HPLC-grade acetonitrile flowing at 0.4 mL $min^{-1}$ for 10 min. We detected an ergosterol peak at 282 nm wavelength and quantified its concentrations (mg $g^{-1}$ dry soil) using a concentration standard curve comprised of four serial dilutions of an ergosterol standard (≥95%; Sigma-Aldrich, St. Louis, MO).

To quantify soil fungal necromass concentrations, we measured the amino sugar glucosamine (Amelung, 2000). We concurrently measured muramic acid to quantify soil bacterial necromass concentrations. We used a slide hammer (5 cm diameter × 18 cm length; AMS American Falls, Idaho, USA) to core soils in three random locations within each plot. Vegetation, litter, and fibric material were removed, then organic and mineral soil was placed in separate bags. Soils were put on ice for approximately 24 h before further processing on August 25–26, 2019. Roots were then removed, and soil was sieved through a 2 mm mesh. Approximately 2 g of homogenized soil was subsequently air-dried following Zhang and Amelung (1996). Soils for amino sugar analysis were air-dried for 5 days and then ground on a TissueLyser II (Qiagen Inc., Mississauga, ON, CAN). Hydrochloric acid was used to extract amino sugars (Zhang & Amelung, 1996). After removing interfering substances, the amino sugar was derivatized to aldononitrile acetates. Amino sugar analysis was subsequently performed on a Gas Chromatograph/Mass Spectrometer (GC/MS, Agilent 7890A/5975C, Agilent Tech.), equipped with a DB-5MS UI column (I.D. 0.250 mm × 0.25 μm × 30 m; Agilent Tech.). Helium was used as a carrier gas with a flow rate of 0.7 mL $min^{-1}$, and the volume of sample extract injection was 1 μL with a 20:1 split ratio mode. The initial temperature was 120°C held for 1 minute, increased to 300°C by 10°C per minute, and finally held for 10 min. Data were acquired using the Scan mode. We quantified the compounds based on the standards D-Glucosamine hydrochloride (chemical purity: ≥99%) and muramic acid (chemical purity: ≥95%) (Sigma-Aldrich). Fungal and bacterial necromass (mg $g^{-1}$ dry soil) was calculated following Liang et al. (2019, see Supporting Information).

## 2.3 | Soil C concentration

To evaluate the relationship between inorganic N addition and soil C concentration, we sampled soil from organic and mineral soil, respectively, in October 2015 using a shovel. Ten subsamples were pooled for each plot (final $n = 3$ per treatment). Soil samples were air-dried, ground using a ball mill, and analysed for total C concentrations (mg $g^{-1}$ dry soil) with a Carlo Erba NA 1500 elemental analyser (Carlo Erba Instruments, Milano, Italy). Further details regarding

processing of soil samples for measuring soil C concentration are described in Kwak et al. (2018).

## 2.4 | Evaluating tannins and chitin concentrations of *P. tremuloides* fine roots

We measured condensed tannins of fine roots (i.e. <1 mm diameter) that were traced from three *P. tremuloides* trees within each plot. We also measured condensed tannins of fine roots of mixed species extracted from the same soils that were cored with a slide hammer (described above). Roots were freeze-dried, ground, and condensed tannins were extracted with 70% acidified acetone, followed by the addition of a butanol-HCL solution and a ferric reagent, following the methods described in Dettlaff et al. (2018). The absorbance of the resulting solution was measured at 550 nm. The spectrophotometer readings were converted into mg of tannins $g^{-1}$ dry root using a non-linear standard curve. We used a single standard for condensed tannins, which were purified from the leaves of *P. tremuloides* following the method of Hagerman and Butler (1980).

To quantify the effects of N addition on chitin concentrations of ECM roots, from each subsample of traced roots ($n = 24$) we pooled 50 root tips colonized by *Cenococcum geophilum*. This species forms easily discernible morphotypes that are jet black and is an abundant ECM fungal species in forest soils (LoBuglio, 1999). The species is also relatively recalcitrant to decay, and may thus disproportionately influence biogeochemical cycles (Fernandez & Koide, 2012). We pooled another 50 ECM root tips selected randomly; this latter collection was done to isolate changes in chitin concentration from that owing to possible shifts in the community composition of ECM fungi at the stand level. We followed Kim et al. (2015) for sample extraction and analysis, which was slightly modified due to limited sample amounts and instrumental availability (see Supporting Information). The concentration of chitin is reported as mg $g^{-1}$ dry root.

## 2.5 | Estimating the relative abundance of chitinase and class II peroxidase gene copies

Concurrently with soil sampling, we also sampled roots (about 30–50 cm in length) from three *P. tremuloides* trees within each plot at the interface of organic and mineral soil. After collection, confirmed *P. tremuloides* roots were placed on ice for approximately 24 h and stored at −20°C at the University of Alberta, Edmonton, Canada. Afterwards, roots were thawed and washed free of adhering soil. Fine roots (<1 mm in diameter) were retained, cut into 1 cm fragments, and mixed thoroughly. Samples were then pooled within each plot for further DNA analysis (4 plots × 2 treatments = 8 samples total; see Karst et al., 2021). Briefly, we extracted DNA from the washed *P. tremuloides* roots, amplified the ITS1 region using a two-step PCR with primers ITS1F and ITS2 (Gardes & Bruns, 1993; White et al., 1990), and sequenced on Illumina MiSeq platform. We obtained amplicon sequence variants (ASVs), assigned taxonomy using the UNITE dynamic database (Nilsson et al., 2019), converted amplicon read-counts for ECM fungi to a proportion within a sample, and then scaled the resulting proportions to the largest observed proportion across samples for ECM fungi. Further sequencing and bioinformatics details are found in the Supporting Information.

To estimate the relative abundance of chitinase and class II peroxidase gene copies in the ECM fungal community, we calculated the average number of glycoside hydrolase family 18 (GH18) and auxiliary activity family 2 (AA2) genes, respectively, among fungal genera using published values in the Mycocosm database (Grigoriev et al., 2014), as done in previous studies (Defrenne et al., 2023; Hupperts & Lilleskov, 2022). See Table S1 for a summary of GH18 and AA2 gene copies among genera in this study. Genera with published GH18 and AA2 data for at least one species comprised 91.6% of sequence reads in the present study. We recognize that this is not a comprehensive analysis of gene abundance but represents our best estimate given the breadth of genome sequencing. The average numbers of GH18 and AA2 gene copies within each genus were then multiplied by the abundance of the genus in each sample.

## 2.6 | Replication statement

| Scale of inference | Scale at which the factor of interest is being applied | Number of replicates at the appropriate scale |
|---|---|---|
| Plot | Plot (20 × 20 m) | 4 (3 for soil C concentration) |

## 2.7 | Data analysis

Subsamples were pooled within respective plots prior to analysis. We fit linear mixed effects models in all analyses using the lme function in the 'nlme' package (version 3.1–162) of R v.4.2 (Pinheiro et al., 2018; R Core Team, 2022) in R Studio v.2023.12.1.402 (Posit team, 2024). Residuals were visually inspected to ensure normal distribution and homoscedasticity. Data were natural log or square-root transformed as necessary to meet assumptions (indicated in statistical results tables). All figures were made with the 'ggplot2' (version 3.4.4) and 'cowplot' (version 1.1.1) packages in R (Wickham, 2016; Wilke, 2020). Note that all figures display non-transformed data.

To test our main hypothesis that N addition would increase soil fungal necromass concentration, our initial model used N treatment, soil layer (i.e. organic or mineral soil), and their interaction as fixed effects, and block as a random effect. Soil layer was included because soil microbial diversity and composition may strongly differ between these soil layers (Mundra et al., 2021), and necromass inputs may therefore respond differently to inorganic N addition. Soil fungal necromass might also be a legacy of tree growth because root biomass has been positively linked with mycelial biomass (Xie et al., 2024). To control for this potential variation in

soil fungal necromass due to differences in average annual tree growth among plots, we tested an alternate model that included average annual tree stem radial growth during treatment years as a fixed effect, but was otherwise identical to the initial model. Tree stem radial growth was measured from increment cores collected in 2020, as described in Karst et al. (2021). We repeated the same analysis for soil C concentrations, without controlling for average annual radial tree growth.

To test hypothesis H1a that N addition would lower chitinase and class II peroxidase gene abundance of the ECM fungal community, we used N treatment as a fixed effect and block as a random effect. To investigate the relationship between gene abundance and fungal necromass or soil C concentration, we again used linear mixed effect models, with block as a random effect. We first modelled fungal necromass as the response variable and chitinase or class II peroxidase gene abundance as the predictor. We then repeated this analysis using soil C concentration in the organic layer as the response variable. We acknowledge that soil fungi and soil C were sampled approximately 3.8 years apart, which may add noise to our results. To test hypothesis H1b that N addition would reduce standing fungal biomass, we again used linear mixed effects models with block as a random effect. First, we tested the effect of N addition, soil layer, and their interaction on soil ergosterol concentration. We repeated this test using the same alternate model described above for soil fungal necromass because tree root biomass has been positively linked with mycelial biomass (Xie et al., 2024).

To test hypothesis H2 that N addition would increase the lability of fungal necromass, we again used linear mixed effects models with block as a random effect. We then tested the effect of N addition, soil layer, and their interaction on the concentration of condensed tannins in fine roots of mixed species, and repeated this analysis using the concentration of condensed tannins in fine roots of *P. tremuloides*. We then tested the effect of N addition on chitin concentrations in ECM (mixed species)-colonized root tips of *P. tremuloides*, and repeated this analysis using chitin concentrations in *C. geophilum*-colonized root tips of *P. tremuloides*.

## 3 | RESULTS

### 3.1 | Microbial necromass and soil C concentration

Nitrogen addition had no detectable effect on the concentration of fungal necromass, regardless of soil layer ($p = 0.2$, Figure 2a, Table 2a), even when accounting for average annual radial tree growth ($p = 0.2$, Table 2b). Both models showed that fungal necromass concentration was higher in the organic layer than the mineral soil ($p < 0.001$, Figure 2a, Table 2a). Nitrogen addition did not affect soil bacterial necromass ($p = 0.22$, Figure 2b, Table S2a), but soil bacterial necromass was higher in the organic layer compared to mineral soil ($F = 149.6$, $p < 0.001$, Figure 2b, Table S2a). Nitrogen addition did not influence soil C concentration in the organic layer or mineral soil, as measured in 2015 ($p = 0.15$, Figure 2c, Table S2b).

### 3.2 | Chitinase and class II peroxidase potential

Nitrogen addition tended to decrease the relative abundance of chitinase (GH18) gene copies and class II peroxidase (AA2) gene copies of the ECM fungal community, but high variance precluded statistical significance of this trend (Figure 3a,d; Table S3a). There was no relationship between chitinase and class II peroxidase gene abundance and fungal necromass (Figure 3b,e; Table S3b), nor between chitinase and class II peroxidase gene abundance and soil C concentration (Figure 3c,f; Table S3c).

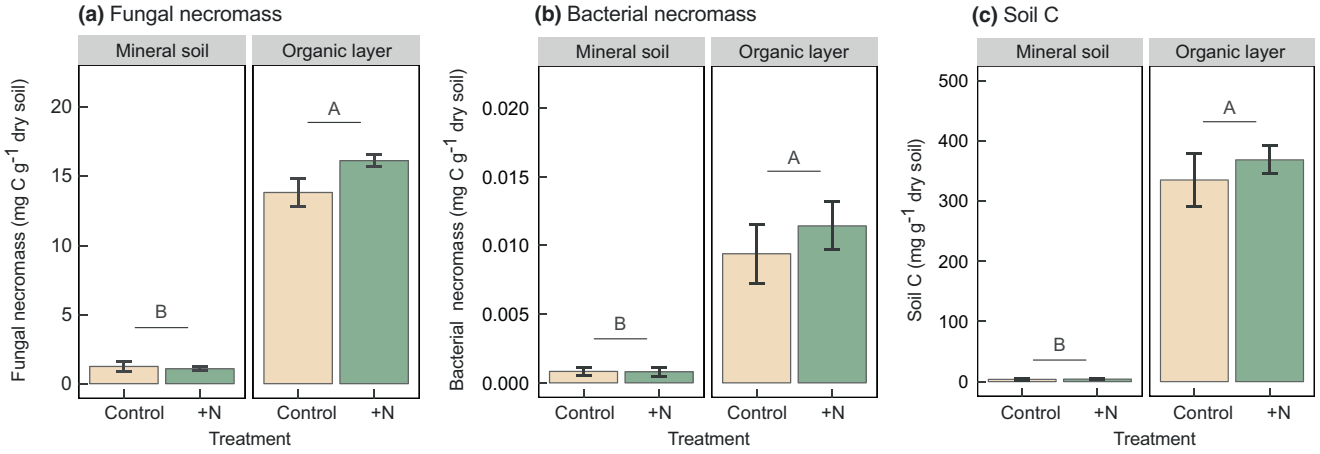


FIGURE 2 Mean (±SE) (a) soil fungal necromass (mg C g$^{-1}$ dry soil); (b) soil bacterial necromass (mg C g$^{-1}$ dry soil), and (c) soil C (mg g$^{-1}$ dry soil) in mineral and organic soils with and without added nitrogen (30 kg N ha$^{-1}$ year$^{-1}$ for 13 years) in boreal *Populus tremuloides* forests in Alberta, Canada ($n = 4$ per N × soil layer treatment combination (necromass) or 3 per N × soil layer treatment combination (soil C)). Uppercase letters indicate significant differences between soil layers.

**TABLE 2** (a) Initial model of the effects of nitrogen (N) addition and soil layer on soil fungal necromass concentration (mg C $g^{-1}$ dry soil, $n = 4$ per N × soil layer treatment combination), according to linear mixed effects models. Block was included as a random effect in all models. $DF_{num}$, degrees of freedom (numerator); $DF_{den}$, degrees of freedom (denominator). (b) Alternate model controlling for average annual tree stem radial growth.

| | √(soil fungal necromass (mg C $g^{-1}$ dry soil)) | | | |
|---|---|---|---|---|
| | $DF_{num}$ | $DF_{den}$ | *F* value | *p* value |
| **(a) Initial model** | | | | |
| N addition | 1 | 9 | 1.7 | 0.222 |
| Soil layer | 1 | 9 | 792.2 | **<0.001** |
| N addition × soil layer | 1 | 9 | 3.0 | 0.118 |
| **(b) Alternate model** | | | | |
| N addition | 1 | 8 | 1.6 | 0.247 |
| Soil layer | 1 | 8 | 715.7 | **<0.001** |
| Average annual tree stem radial growth | 1 | 8 | <0.1 | 0.895 |
| N addition × soil layer | 1 | 8 | 2.7 | 0.139 |

*Note:* Bold values denote statistical significance ($p < 0.05$).

### 3.3 | Standing fungal biomass, condensed tannins, and chitin

We could not detect effects of N addition on soil ergosterol concentrations in organic or mineral soils ($p = 0.94$, Figure 4a, Table S4a), indicating that standing fungal biomass was insensitive to N addition. The results were similar when controlling for average annual radial tree growth. When examining mixed species roots, tannin-chitin complex formation did not appear disrupted by N addition; we could not detect differences in the concentration of condensed tannins ($p = 0.27$, Figure 4b, Table S5a) or chitin ($p = 0.23$, Figure 4c, Table S5b).

Similar to mixed species roots, we could not detect changes in the concentration of condensed tannins or chitin in species-specific root samples. Nitrogen addition did not affect condensed tannins in fine roots of *P. tremuloides* ($p = 0.73$; Table S6a), nor did it affect chitin concentrations in fine roots colonized by *C. geophilum* ($p = 0.95$, Table S6b).

## 4 | DISCUSSION

Our hypotheses were unsupported. We found that inorganic N addition had no detectable effect on soil fungal necromass, even when controlling for average annual radial tree growth. Soil C concentrations were unaffected by inorganic N addition. We also did not detect inorganic N addition effects on chitinase or class II peroxidase potential, standing soil fungal biomass concentration (as measured by ergosterol concentration), condensed tannin concentration, or chitin concentration of ECM-colonized root tips. These results are based on eight *P. tremuloides* stands (four fertilized stands and four control stands); broader inferences should consider the relatively modest sample size in the current study, which is in line with that of other N addition field studies (Karst et al., 2021).

### 4.1 | Mechanisms that favour soil fungal necromass accumulation

We hypothesized (H1a) that inorganic N addition would decrease chitinase and class II peroxidase potential of the ECM fungal community, which would likely favour soil fungal necromass accumulation. However, we found little supporting evidence. Class II peroxidases are powerful enzymes secreted by some agaricomycetous ECM fungi to degrade complex organic matter and liberate nutrients locked within (Kohler et al., 2015). The abundance and expression of the chitinase gene GH18 differs among ECM taxa and, recent work has suggested that there is a direct link between chitinase gene abundance and N uptake from chitin (Maillard et al., 2023). Though our hypothesis was not supported, the marginally lower class II peroxidase potential under long-term inorganic N addition that we found is consistent with other studies. For example, Forsmark et al. (2024) found that 20 years of inorganic N addition decreased manganese peroxidase (a type of class II peroxidase) activity in pine forests of boreal Sweden. Other work in boreal peatlands of Minnesota, USA, found that the potential class II peroxidase gene abundance of the ECM fungal community of spruce seedlings declined with increasing site fertility (Hupperts & Lilleskov, 2022). The production of enzymes such as chitinases and class II peroxidases is an ostensibly costly trait (Zheng et al., 2020), and long-term inorganic N addition would presumably favour an ECM fungal community that has fewer gene copies of these enzymes, but we did not find strong support for this hypothesis. Earlier work from the same experiment found shifts in the abundances of some ECM taxa (Karst et al., 2021), but our work suggests that there was little change in community-level genetic potential to produce chitinases or class II peroxidases.

We also hypothesized (H1b) that inorganic N addition would increase standing fungal biomass, consequently increasing fungal necromass and soil C concentration. Despite a higher relative abundance of distance exploration types under N addition (Karst et al., 2021), and therefore presumably more exploratory mycelia in the soil, we found no treatment effect on soil ergosterol concentrations or soil C concentrations. A recent study in Swedish pine forests found that N additions of 50 kg $ha^{-1}$ $year^{-1}$ increased soil ergosterol concentration (Forsmark et al., 2024), and we expected to find similar results in our present study. However, Forsmark et al. (2024) simultaneously found that the same treatment decreased the concentration of the fungal PLFA marker 18:2ω6, which is another indicator of standing fungal biomass. The authors concluded that the higher stability of ergosterol relative to PLFA markers may unintentionally capture noise from fungal necromass, thus blurring the results when using ergosterol as an indicator of standing fungal biomass. Alternatively, a recent study found that exploration type assignments were poor

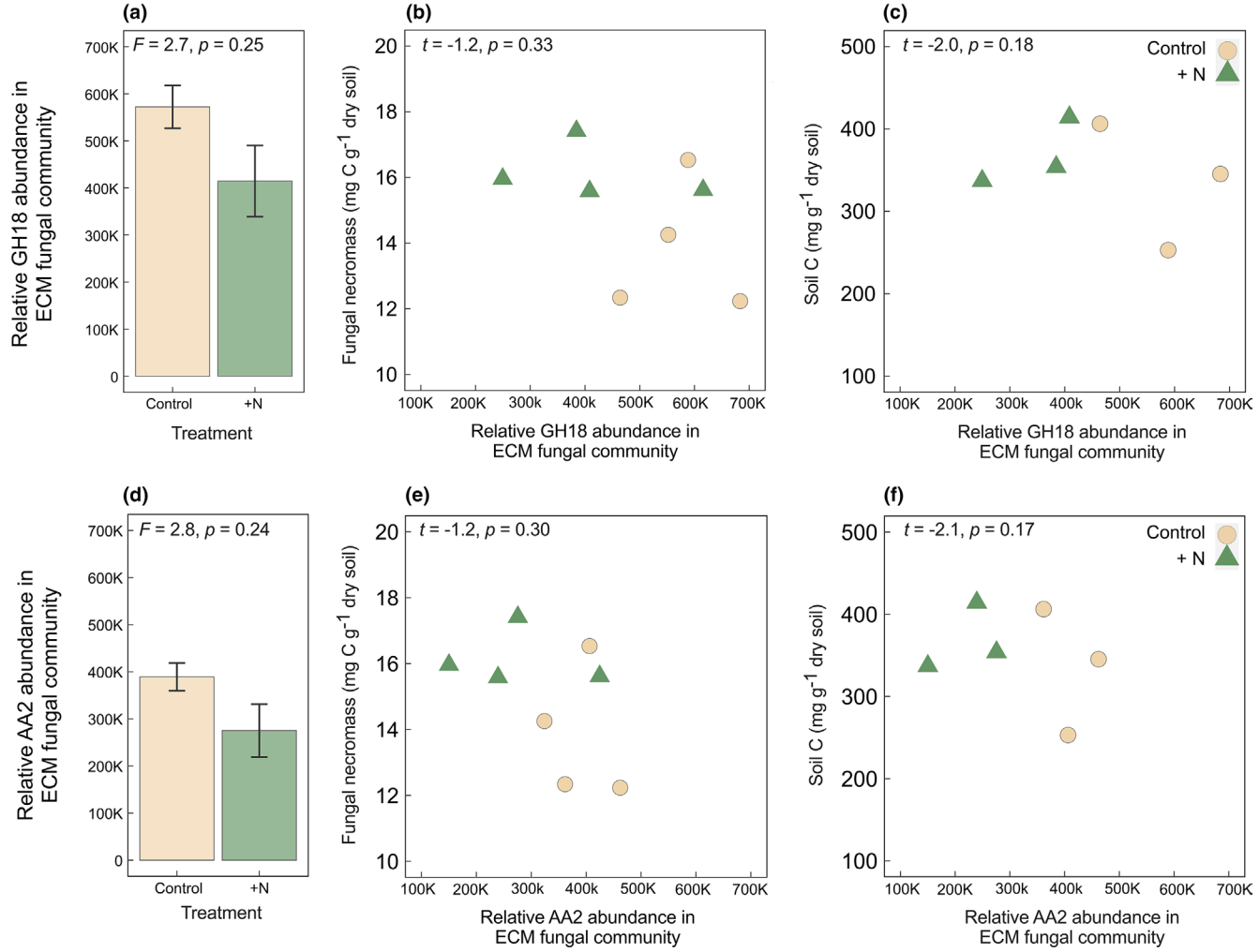


**FIGURE 3** (a) Mean (±SE) relative abundance of chitinase gene (GH18) copies in the ECM fungal community with and without added inorganic nitrogen (30 kg N $ha^{-1}$ $year^{-1}$ for 13 years) in boreal *Populus tremuloides* forests in Alberta, Canada ($n = 4$ per N treatment); (b) relationship between fungal necromass (mg C $g^{-1}$ dry soil) and the relative abundance of chitinase gene (GH18) copies in the ECM fungal community; (c) relationship between soil C (mg $g^{-1}$ dry soil) in the organic layer and the relative abundance of chitinase gene (GH18) copies in the ECM fungal community. Panels (d–f) show relative class II peroxidase (AA2) gene copies.

predictors of the relative abundance of DNA from exploration types in soil (Jörgensen et al., 2022). Regardless, the higher relative abundance of distance exploration types found by Karst et al. (2021) in the same *P. tremuloides* stands that were used for the present study suggest higher biomass of ECM fungi. However, ECM fungal biomass has been estimated to comprise approximately half of total soil fungal biomass in deciduous broadleaf forests (Awad et al., 2019), indicating that ECM fungal traits may not be the best predictors of total soil fungal biomass or necromass. It may also be possible that standing fungal biomass remains unchanged but has higher turnover, resulting in more necromass inputs despite no change in soil fungal biomass.

Our finding that soil C concentrations were unchanged under N addition is consistent with previous work in evergreen-dominated boreal forests (Maaroufi et al., 2015). However, work in other ecosystems has found that N fertilization increased topsoil C concentrations (Huang et al., 2011; Tipping et al., 2017), and we anticipated similar treatment effects in the present study. Upscaling soil C concentrations to soil C stocks (kg $ha^{-1}$) may reveal treatment effects, as shown by studies in boreal forests where inorganic N addition increased soil C stocks (Forsmark et al., 2020; Maaroufi et al., 2015). We did not quantify soil C stocks in the present study, which requires additional measurements of organic layer depth and bulk density.

The results shown here, and those of Karst et al. (2021), might suggest a weak link between soil fungal necromass and the relative abundance of distance ECM exploration types. Earlier work has found that distance exploration types contribute more fungal necromass to forest soils (Weigt et al., 2012). One of the most common ECM fungal species that produces emanating hyphae, *Cenococcum geophilum*, is known for having highly melanized cells that are resistant to decay (Certano et al., 2018; Fernandez et al., 2019). However, the abundance of *C. geophilum* (measured by ITS reads) was unaffected by N addition (Karst et al., 2021). Other traits that may confer necromass stability include hydrophobicity and small hyphal diameter (Ekblad et al., 2013; McCormack et al., 2010; Rillig et al., 2007). The genus *Piloderma* has varied exploration types (Agerer, 2001; Hagenbo et al., 2018), produces hydrophobic mycelia (Agerer, 2001; Lilleskov

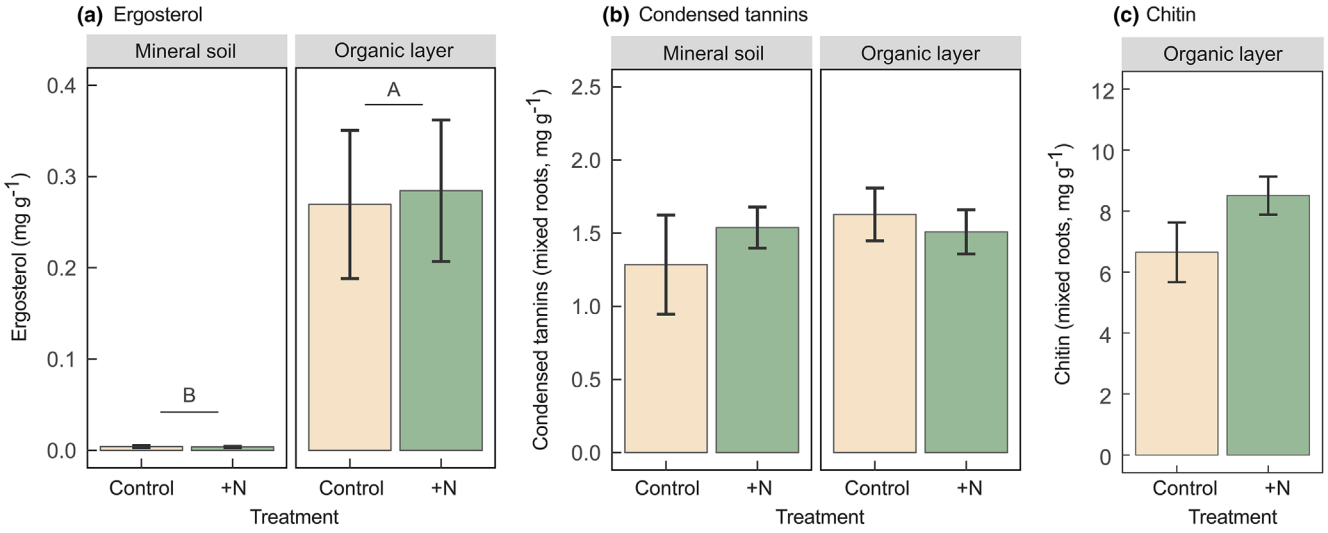


**FIGURE 4** (a) Mean (±SE) ergosterol concentration (mg g$^{-1}$ dry soil) with and without added inorganic nitrogen (30 kg N ha$^{-1}$ year$^{-1}$ for 13 years) in boreal *Populus tremuloides* forests in Alberta, Canada ($n = 4$ per N × soil layer treatment combination); (b) Mean (±SE) condensed tannin concentration in mixed roots (mg g$^{-1}$ dry root) with and without added inorganic nitrogen (30 kg N ha$^{-1}$ year$^{-1}$ for 13 years) in boreal *Populus tremuloides* forests in Alberta, Canada ($n = 4$ per N × soil layer treatment combination); (c) Mean (±SE) chitin concentration in mixed roots (mg g$^{-1}$ dry root) with and without added inorganic nitrogen (30 kg N ha$^{-1}$ year$^{-1}$ for 13 years) in boreal *Populus tremuloides* forests in Alberta, Canada ($n = 4$ per N × soil layer treatment combination). Uppercase letters in panel (a) indicate significant differences between soil layers.

et al., 2011) and was found to increase under N addition in these forests (Karst et al., 2021), therefore being potentially responsible for the non-significantly higher concentration of fungal necromass in soils under N addition in the current study. More work is needed to identify traits of ECM fungi that affect necromass stability.

Soil fungal necromass concentrations may also create soil conditions where distance explorations types are favoured, resulting in a positive feedback loop. For example, studies have shown that lower fertility soil is associated with a higher relative abundance of long- or medium-distance ECM exploration types (Khalfallah et al., 2024). In the present experiment, inorganic N addition may have partially relieved N limitations but caused other nutrient limitations (e.g. phosphorus) that would select for the ostensibly wider foraging capacity of distance exploration types. Further, the trending decline in chitinase and class II peroxidase gene abundance of the ECM community under N addition in the present study, but simultaneous increase of distance ECM exploration types in Karst et al. (2021), suggest a decoupling of organic matter-degrading enzyme potential and mycelial biomass production. This decoupling warrants further research to investigate which nutrient limitations favour the ability to produce costly enzymes and which limitations favour different ECM exploration types, as our study suggests that community-level enzyme production potential and exploration types may be driven by different factors.

## 4.2 | Mechanisms that constrain soil fungal necromass accumulation

Our competing hypothesis, H2, was that inorganic N addition would lower condensed tannin concentrations and increase chitin concentrations of fine roots, resulting in more labile fungal necromass and therefore lower soil fungal necromass concentrations. Our findings did not support this hypothesis; N addition did not appear to disrupt potential tannin-chitin complex formation. Specifically, condensed tannin concentrations of *P. tremuloides* roots did not change, suggesting the availability of tannins did not limit the formation of stable complexes. Within *P. tremuloides*, the effect of N addition on condensed tannins in roots appears inconsistent; whereas we and Kosola et al. (2006) found no change with added N, Stevens et al. (2014) found 47% higher concentrations under low than high N conditions. Similar to condensed tannin concentrations, N addition did not influence the chitin concentration of *C. geophilum* colonized root tips or of the aggregate community of ECM root tips, suggesting that this trait is relatively conserved or that ECM fungi did not access the added N. Previous work found large differences in ECM chitin concentrations across multiple ECM fungal taxa (Wallander et al., 1997), suggesting that shifts in ECM community composition may affect soil chitin concentrations. However, Karst et al. (2021) reported no change in ECM fungal community composition, which could partly explain our findings. In any case, inorganic N addition unlikely disrupted the formation of stable chitin-tannin complexes. Our study does not rule out the importance of biochemical interactions between roots and ECM fungi on stabilizing fungal necromass (Adamczyk, 2021); however, in this *P. tremuloides* forest, these interactions appear resistant to inorganic N addition. This apparent absence of a treatment effect on tannin-chitin complexes, and therefore no increase in the lability of fungal necromass, may partially contribute toward relatively unchanged soil fungal necromass concentrations under N addition.

### 4.3 | Summary

Thirteen years of inorganic N addition had no detectable effect on soil fungal necromass, and potential underlying mechanisms were similarly unresponsive. There was no treatment effect on soil fungal biomass concentrations and no disruption to potential tannin-chitin complex formation, suggesting that fungal necromass inputs and the lability of fungal necromass were unaffected by inorganic N addition. Furthermore, the relative abundances of chitinase and class II peroxidase gene copies in the ECM fungal community were seemingly unaffected by N addition despite downward trends with N addition. Taken together with Karst et al. (2021), our findings suggest a decoupling of key physiological traits (organic matter-degrading enzyme potential) and morphological traits (exploration type) for acquiring nutrients from organic matter. Ultimately, our present study suggests that soil fungal necromass and biomass, and their drivers, are largely insensitive to over a decade of inorganic N addition in deciduous broadleaf-dominated boreal forests.

## AUTHOR CONTRIBUTIONS

Justine Karst conceived the study. Scott X. Chang designed and maintained the field experiment. Justine Karst, Jean C. Rodriguez-Ramos, Guncha Ishangulyyeva, Josh Wasyliw, Joseph D. Birch, James Franklin, Jin-Hyeob Kwak, and Nadir Erbilgin collected data. Stefan F. Hupperts and Joseph D. Birch analysed data. Stefan F. Hupperts wrote the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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## CONFLICT OF INTEREST STATEMENT

None.

## DATA AVAILABILITY STATEMENT

Data is archived with University of Alberta Dataverse: https://doi.org/10.5683/SP3/P1AMSK (Karst, 2024).

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**Table S1.** Summary of chitinase (GH18) and class II peroxidase (AA2) gene copies among fungal genera in this study, retrieved from the MycoCosm database.

**Table S2.** Effect of nitrogen (N) addition and soil layer on (a) soil bacterial necromass concentration (mg C $g^{-1}$ dry soil, $n = 4$ per N × soil layer treatment combination), and (b) soil C concentration (mg $g^{-1}$ dry soil, $n = 3$ per N × soil layer treatment combination), according to linear mixed effects models.

**Table S3.** (a) Effect of nitrogen (N) addition on the relative abundance of chitinase (GH18) and class II peroxidase (AA2) gene copies in the ECM fungal community, according to linear mixed effects models ($n = 4$). (b) Relationship between the relative abundance of chitinase (GH18) gene copies in the ECM fungal community and soil fungal necromass, and class II peroxidase (AA2) gene copies in the ECM

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fungal community and soil fungal necromass, according to linear mixed effects models ($n = 4$).(c) Relationship between the relative abundance of chitinase (GH18) gene copies in the ECM fungal community and soil C concentration, and class II peroxidase (AA2) gene copies in the ECM fungal community and soil C concentration, according to linear mixed effects models ($n = 3$).

**Table S4.** Effect of nitrogen (N) addition and soil layer on (a) soil ergosterol concentrations (mg $g^{-1}$ dry soil) according to linear mixed effects model ($n = 4$ per N × soil layer treatment), (b) soil ergosterol concentrations (mg $g^{-1}$ dry soil) when controlling for average annual tree radial stem growth, according to linear mixed effects model ($n = 4$ per N × soil layer treatment).

**Table S5.** Effect of nitrogen (N) addition on (a) condensed tannin concentrations (mg $g^{-1}$ dry root) in mixed species fine roots, according to linear mixed effects model ($n = 4$ per N × soil layer treatment combination); (b) chitin concentrations (mg $g^{-1}$ dry root) in mixed ectomycorrhizal root tips, according to linear mixed effects model ($n = 4$ per N treatment).

**Table S6.** Effect of nitrogen (N) addition on (a) condensed tannin concentrations (mg $g^{-1}$ dry root) in *Populus tremuloides* fine roots in the organic-mineral soil interface, according to linear mixed effects model ($n = 3–4$ per N treatment), and (b) chitin concentrations (mg $g^{-1}$ dry root) in *Populus tremuloides* fine roots colonized by *Cenococcum geophilum*, according to linear mixed effects model ($n = 4$ per N treatment).

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