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Fungal guild interactions slow decomposition of boreal forest pine litter and humus

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Summary

- Ericaceous understory shrubs and ericoid mycorrhizal fungal communities are ubiquitous in boreal forests, and their interactions with ectomycorrhizal and saprotrophic fungi may determine organic matter dynamics in forest soils.
- We followed decomposition of pine needle litter and mor-layer humus over 3 yr in a factorial shrub removal- and pine root exclusion experiment in an old-growth Scots pine (*Pinus sylvestris*) forest, to evaluate effects of fungal guilds on mass loss.
- Litter mass loss was 23% greater when ectomycorrhizal fungi were excluded suggesting increased saprotrophic activity, independently of ericoid shrub presence. However, this 'Gadgil effect' was only found after 17 months following a summer drought. By contrast, humus mass loss was overall stimulated by ectomycorrhizal fungi, while ericoid mycorrhizal shrubs appeared to counteract this effect, potentially caused by simultaneous addition of recalcitrant organic matter and inhibition of ectomycorrhizal decomposers.
- We conclude that competitive saprotrophic–ectomycorrhizal fungal interactions may slow early-stage litter decomposition, but this effect was small and inconsistent. Furthermore, interactions between ecto- and ericoid mycorrhizal guild members appear to determine the late-stage organic matter balance of boreal forest humus.

Introduction

In coniferous boreal forests, dead plant and microbial remains accumulate as partly decomposed, particulate organic matter, forming a purely organic topsoil, the mor-layer, overlying the mineral soil (Lugato *et al.*, 2018). The carbon in boreal forests constitutes 32% of the global stock (Pan *et al.*, 2011), with 95% of the organic carbon retained in soils (Bradshaw & Warkentin, 2015). High fungal-to-bacterial biomass ratios (Fierer *et al.*, 2009) reflect the pivotal role of fungi in decomposition in coniferous forests, where most biological activity is tightly constrained by low nitrogen availability (Tamm, 1991). Forest ecosystems dominated by ectomycorrhizal symbioses generally accumulate more soil organic matter (Read, 1991) of higher carbon-to-nitrogen ratios (Averill *et al.*, 2014) than forests characterized by arbuscular mycorrhizal symbioses. A proposed explanation is that ectomycorrhizal fungi hinder organic matter decomposition by competing with free-living saprotrophs for nitrogen (Gadgil & Gadgil, 1971; Averill *et al.*, 2014; Steidinger *et al.*, 2019), particularly organic nitrogen forms bound to polyphenolics in lignin-rich litters and less fertile soils (Fernandez & Kennedy, 2016; Smith & Wan, 2019).

The mor-layer in boreal forest undergoes little vertical mixing due to the low pH and lack of burrowing fauna, resulting in a distinct surface litter layer and a more decomposed humus layer beneath. Free-living saprotrophic fungi reside primarily in the litter layer, where they exploit recently shed plant litter for high-quality organic molecules (Lindahl *et al.*, 2007; Barbi *et al.*, 2020). As substrates become depleted in readily hydrolysable organic carbon by early decomposers, oxidation by subsequent decomposers (e.g. white rot fungi) is required to liberate resources locked up in recalcitrant complexes, thereby advancing decomposition (van der Wal *et al.*, 2013; Barbi *et al.*, 2020; Floudas, 2021). In undisturbed humus layers of nutrient-poor boreal forests, ecto- and ericoid mycorrhizal fungi co-dominate, while saprotrophic molds and yeasts make up a smaller community fraction (Lindahl *et al.*, 2007; Clemmensen *et al.*, 2015). Across boreal forests with varying fertility, around three quarters of the carbon in the entire mor-layer originated from roots and associated fungi, rather than from aboveground sources (Kyaschenko *et al.*, 2019), suggesting that processes in the rooting zone are more important than aboveground litter inputs in regulating total soil carbon stocks in the boreal forest (Clemmensen *et al.*, 2013; Kyaschenko *et al.*, 2019). Mycorrhizal fungi depend on

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photo-assimilated organic carbon delivered directly by their plant partners through symbiotic root structures to support mycelial growth (Harley & Smith, 1983). Given this direct supply of simple organic compounds, mycorrhizal fungi may outcompete free-living saprotrophs in organic substrates depleted in hydrolysable carbon (Bödeker *et al.*, 2016), potentially leading to increased accumulation of undecomposed organic matter (Kyaschenko *et al.*, 2017; Frey, 2019; Fanin *et al.*, 2022).

While most ectomycorrhizal fungi lost their abilities to produce hydrolytic and oxidative extracellular enzymes involved in decomposition of plant residues, as they transitioned into the symbiotic lifestyle (Kohler *et al.*, 2015), oxidative capacities have been retained independently in several lineages (Bödeker *et al.*, 2009; Pellitier & Zak, 2018). In particular, extracellular peroxidases and nonenzymatic (Fenton-like) mechanisms may facilitate ectomycorrhizal fungal access to organically bound nitrogen in boreal soils (Lindahl & Tunlid, 2015). Through several lines of evidence, the ectomycorrhizal genus *Cortinarius* has been suggested to be particularly important for decomposition of recalcitrant humus in boreal forests, where saprotrophic basidiomycete decomposers are less abundant in deeper soil layers (Bödeker *et al.*, 2014; Sterkenburg *et al.*, 2018; Lindahl *et al.*, 2021). Ericoid mycorrhizal fungi, associated with the widespread ericaceous understory dwarf shrubs, are another dominant fungal guild in boreal forest humus layers (Lindahl *et al.*, 2007). These fungi can degrade organic matter with laccases and a diverse array of hydrolytic enzymes (Martino *et al.*, 2018), and they assimilate and share organic nitrogen with the plant host (Read *et al.*, 2004); however, ericoid mycorrhizal fungi lack the capacity to produce the most potent oxidative (i.e. white rot peroxidases) enzymes found in many saprotrophic and some ectomycorrhizal Agaricomycetes (Martino *et al.*, 2018; Miyauchi *et al.*, 2020). Soil organic matter stocks have been found to increase along gradients from ectomycorrhizal- to ericoid mycorrhizal dominated ecosystems across subarctic, boreal and temperate areas (Hartley *et al.*, 2012; Clemmensen *et al.*, 2013; Ward *et al.*, 2021). Thus, although ericoid mycorrhizal fungi have retained saprotrophic capacities, their recalcitrant necromass appears to make a significant contribution to the soil organic matter pool (Clemmensen *et al.*, 2015; Adamczyk *et al.*, 2016; Fernandez *et al.*, 2019). Both ericoid- and ectomycorrhizal necromass may be stabilized through chemical complexation with plant-derived tannins (Adamczyk, 2019). Ericoid shrubs and their associated fungi have further been found to inhibit saprotrophic litter decomposition, similar to the ectomycorrhizal 'Gadgil effect' (Fanin *et al.*, 2022), likely because shrubs also act as a sink for soil nitrogen.

To disentangle effects of ectomycorrhizal, ericoid mycorrhizal and saprotrophic fungal guilds, we conducted a series of decomposition experiments with recently shed pine needles and humus incubated in mesh bags in forest plots with or without access by ectomycorrhizal and ericoid mycorrhizal guilds. We used a field experiment in an old-growth boreal pine forest with factorial combinations of tree root exclusion and ericoid dwarf shrub removal, in which we had previously observed largely additive root respiration fluxes from pine roots (40% of soil respiration), ericaceous shrubs (10%) and saprotrophic organisms (50%) over 3 yr (Mielke *et al.*, 2022). Based on litter samples, we first tested the 'Gadgil effect' (Gadgil & Gadgil, 1971) and hypothesized (H1a) that saprotrophic decomposition of pine needle litter would increase via a competitive release, only when both ecto- and ericoid mycorrhizas were eliminated simultaneously, since they both act as sinks for nitrogen and water (Mielke *et al.*, 2022). No partial competitive release of litter saprotrophs following removal of individual guilds was expected. We further expected that any increases in litter decomposition would be related to proliferation of saprotrophic basidiomycetes with white-rot capacity, such as *Mycena* species, rather than ascomycetes. We also hypothesized (H1b) that no 'Gadgil effect', that is, no increase in saprotrophic fungal communities or decomposition, would be evident in well-decomposed and cellulose-depleted humus substrates, due to a lack of colonization by white-rot saprotrophs. Based on humus samples, we examined a 'mycorrhizal guild hypothesis', which predicted that (H2a) the presence of ectomycorrhizal roots and associated fungi would result in faster humus decomposition, while (H2b) presence of ericoid mycorrhizal plants and fungi would counteract decomposition by providing accumulating organic matter (Clemmensen *et al.*, 2015, 2021).

Materials and Methods

Study site and field manipulations

The experiment was conducted at Jädraås IhV in south-central Sweden (60°149 ′N, 16°130 ′E). This is a well-documented 160-yr-old *Pinus sylvestris* L. forest that was naturally regenerated after a fire (Persson, 1983). The forest has a dense understory of ericaceous dwarf shrubs (*Vaccinium vitis-idaea* L., *Calluna vulgaris* L., *Empetrum nigrum* L. and *Vaccinium myrtillus* L.), mosses (*Pleurozium schreberi* Bridel and *Dicranum majus* Turner) and lichens (*Cladonia* spp.). No grasses or herbs, but a few small spruce trees, were present in the site. The soil is a sandy podzol with a 10–20 cm thick organic mor-layer (including litter and humus layers) on a pale (oxidized) eluvial E horizon and a rust-red illuvial B horizon at depth (Lindahl *et al.*, 2007). The mean annual air temperature during the study period (2017–2019) was 4.8°C with a daily maximum of 24.4°C in July and a daily minimum of −20.7°C in January. The mean growing season precipitation was 300 mm, with 335, 227 and 364 mm over the three study years, respectively. During 2018 the site was exposed to an extended summer drought. The approximate duration of snow cover is from late November to late April.

A field manipulation experiment was set up, as presented in detail by Mielke *et al.* (2022). In total, eight replicate blocks ($n = 8$) with five treatment plots in each (1.2 m × 1.2 m, spaced by 5–10 m) were set up, giving a total of 40 plots. The five treatments encompassed control, disturbed control, shrub removal, pine root exclusion and combined pine root exclusion and shrub removal plots. For pine root exclusion, permanent steel barriers were pushed 70 cm through the litter and humus layers down into the mineral soil in November 2016. The shrubs were removed by gently pulling out shoots and attached rhizomes in

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December 2016, and resprouting shoots were removed monthly in the following growing seasons. The disturbed control was constructed by cutting to a depth of 30 cm around the plots with a spade, but without installing permanent barriers, enabling the tracking of the re-establishment of roots and mycelium.

Decomposition experiment

Nylon mesh bags (50 µm-mesh; Sintab Produkt AB, Oxie, Sweden) were filled with oven-dried (40°C) pine needle litter (1.5 g in 3 × 8 cm bags) or humus (3.0 g in 4 × 6 cm bags) collected from the soil surface or the deeper, fully organic layer, respectively, at the same field site. We chose this mesh size to allow ingrowth of fungal mycelia and ericaceous hair roots hosting most of the ericoid mycorrhizal fungal biomass, while excluding pine roots. The humus substrate was homogenized by hand and roots and rhizomes of > 2 mm in diameter were removed. In each plot, two litter bags were placed in the litter layer and two humus bags were placed at 5–10 cm depth into the mor layer at two time points; the first (set 1) in June 2017, and another (set 2) in June 2018. For both sets, one bag was incubated until November the same year (5 months) and one bag was incubated until November the following year (17 months; Supporting Information Fig. S1). At harvest, the bags were cleaned on the outside and kept at −20°C (within 6 h from collection) until freeze-dried, weighed and the content ball-milled to a fine powder. Three extra bags for each of the original substrates were constructed as above and analyzed together with the harvested bags to provide preincubation data. The retrieved sample set consisted of 315 samples (40 plots × 2 substrates × 2 sets × 2 incubations + 6 preincubation samples; except 11 lost samples).

Fungal abundance

DNA was extracted from 50 mg of freeze-dried and milled material from all ingrowth mesh bags using the NucleoSpin Soil kit (Macherey-Nagel, Düren, Germany). The total fungal abundance was estimated by quantitative polymerase chain reaction of the fungal Internal Transcribed Spacer 2 (ITS2) region of the rRNA encoding operon using the fungal-specific primer gITS7 and the reverse primers ITS4 and ITS4arch. Fungal gene copy numbers are not necessarily equivalent to biomass, but they do correlate with alternative biomass-based measurements, such as PLFA and ergosterol (Joergensen *et al.*, 2024). Additionally, quantitative polymerase chain reaction data are compatible with results from community analyses based on the same marker. All samples were run in duplicate plates in reaction volumes of 20 µl using the iQ™ SYBR Green Supermix (Bio-Rad) with 1 ng DNA template, 0.1% bovine serum albumin, gITS7 (0.5 µM), ITS4 (0.3 µM) and ITS4arch (0.1 µM) primers and 0.5 ng μl^{-1} DNA on a CFX Connect Real-Time System (Bio-Rad). Cycling conditions were 5 min at 95°C, and 35 cycles of 30 s at 95°C, 30 s at 56°C and 30 s at 72°C, and a final 7 min at 72°. Standard curves were constructed by serial dilutions of linearized plasmids with cloned fragments of the ITS2 region. The quantification was repeated a third time if the threshold cycle (C_t) of the duplicate runs differed by more than one cycle. PCR inhibition was tested for all samples by comparing amplification of a known amount of the plasmid vector (pGEM-T, Promega) in reactions containing template vs nontemplate controls. No inhibition was detected for any of the samples and amplification efficiencies were 103–110%.

Fungal communities

The ITS2 region was amplified using the same fungal-specific primers as used for quantitative polymerase chain reaction, but with both forward and reverse primers fitted with unique 8 bp sample identification tags. The PCR reactions of 50 µl included 25 ng DNA template, 0.5 µM gITS7 primer, 0.3 µM ITS4 primer, 0.1 µM ITS4arch primer, 0.025 U μl^{-1} polymerase in its buffer (DreamTaq, Thermo Fisher Scientific, Waltham, MA, USA), 200 µM dNTPs and 750 µM $MgCl_2$ (Ihrmark *et al.*, 2012; Clemmensen *et al.*, 2023). Amplification cycles were 5 min at 95°C, and 20–35 cycles of 30 s at 95°C, 30 s at 56°C and 30 s at 72°C, and a final 7 min at 72°C, with PCR cycle numbers optimized to obtain quantitative amplification (Castaño *et al.*, 2020). PCR products were run in duplicates, which were pooled and purified using the AMPure kit (Agencourt, Beckman Coulter, Berverly, MA, USA). Concentrations were determined using a Qubit fluorometer (Thermo Fisher Scientific), and amplicons were pooled in equal amounts into four pools that were further purified using the E.Z.N.A. Cycle Pure kit (Omega Bio-tek, Nocross, GA, USA). An equal amount of a mock community was added to each amplicon pool. The amplicon size distributions were checked on a 2100 Bioanalyzer system (Agilent, Santa Clara, CA, USA). The four pools were sequenced on the Sequel I platform (Pacific Biosciences, Menlo Park, USA) on 1 SMRT (Single Molecule, Real-Time) cell per pool after addition of sequencing adaptors by ligation (SciLifeLab, NGI, Uppsala, Sweden).

A total of 1117 419 sequences were filtered and clustered using the Sequence Clustering and Analysis of Tagged Amplicons (SCATA) pipeline (scata.mykopat.slu.se; Ihrmark *et al.*, 2012). Sequences shorter than 150 bp, with a mean quality score lower than 20, individual bases with a quality score lower than 10, or missing 3′ or 5′ tags were removed. Sequences were screened for primers requiring a minimum match of 90%, and reverse complemented if necessary. This quality filtering removed 33% of the total sequences, and unique genotypes (10% of total sequences) were also removed to reduce the incidence of sequencing errors. To obtain species level clusters, remaining sequences were run through pairwise comparisons with USEARCH (Edgar, 2010) followed by single linkage clustering (equal penalty of gap extensions and mismatches) with the minimum similarity to the closest neighbor set at 98.5% to enter a cluster (Kõljalg *et al.*, 2013). In total, 807 species level clusters (in the following called 'species') were formed from 639 923 sequences passing quality control. Clusters attributed to plants (1.4% of clustered sequences) were almost entirely composed of the four ericaceous shrub species present at our site. Further filtering to focus the data on the community that had grown into the bags during incubations narrowed the community to the 190 fungal species that had at least 1% relative abundance in at least one sample (retaining 98% of

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sequences). Species identifications and guilds were first assigned based on automatic matches with UNITE v.08 (Nilsson *et al.*, 2019) and FUNGALTRAITS (Põlme *et al.*, 2020), and then the 200 most abundant species were further manually annotated to functional groups (ectomycorrhizal fungi, ericoid mycorrhizal fungi, dual ericoid-ectomycorrhizal fungi, other root-associated fungi, opportunistic yeasts and molds, saprotrophic fungi and fungi with unknown ecologies). Yeasts included species in Microbotryomycetes, Tremellomycetes and Saccharomycetales, and molds included species in Mortierellomycota and Mucoromycota and some species in Eurotiales, Hypocreales and Capnodiales. Relative abundances of functional groups were calculated for each sample. Genera with 'white-rot' capacity were specified as the ectomycorrhizal *Cortinarius* and *Hebeloma*, and saprotrophic *Trechispora*, *Mycena*, *Gymnopus*, *Galerina* and *Sistotremastrum*, based on genomic data with multiple genes coding for Mn II peroxidases (Kohler *et al.*, 2015; Miyauchi *et al.*, 2020). As a representation of absolute abundance of each fungal species or group, their relative abundance values were multiplied by total fungal ITS2 copy numbers per unit substrate dry weight (DW).

Statistical analyses of pine and shrub effects

We used a permutational multivariate analysis of variance (PERMANOVA) with the adonis2 function in the VEGAN package (Oksanen *et al.*, 2018) in R (v.4.1.3) run at 999 permutations to assess the effect of pine and shrub presence, substrate type, bag set and incubation duration on fungal community composition. We present sequential *P* values; changing the order of tested terms did not affect overall conclusions. Community data were Hellinger transformed and converted into Bray–Curtis dissimilarity matrices for this analysis. Humus and litter samples were first analyzed together, then individually. For the humus substrates, two outliers were removed to allow the analysis to converge. The betadisper function in 'vegan' was employed to assess the assumptions of similar dispersion within the treatments, sets and incubation durations.

The effects of pine and shrub presence on mass loss and on total and guild-wise fungal abundance (ITS2 copies g DW^{-1}) were tested using linear mixed effects models in the NLME package for litter and humus substrates separately (Pinheiro *et al.*, 2019). Pine and shrub presence (binary), bag set and incubation duration were specified as fixed effects, and all interactions were included. Block and plot were set as random effects to account for the spatial dependency of the randomized block design and multiple incubations. All fungal community variables were square root or log transformed to maintain homoscedasticity. However, since pine exclusion largely eliminated the ectomycorrhizal guild (preventing homogenous variances), shrub and pine effects on ectomycorrhizal abundance were examined by individual *t*-tests, by testing the shrub effect only in treatments with pine present and testing the difference between plots with or without pine root ingrowth. When outputs indicated strong interactions with incubation duration and/or bag set, individual statistical tests were rerun for each duration and/or set. The disturbed control treatment was not included in statistical tests but was displayed in figures for reference.

Bayesian decomposition models

We implemented Bayesian hierarchical models to investigate potential fungal community drivers of pine needle litter and humus decomposition using the package RJAGS (v 4-13) in R (Plummer, 2003, 2015). We focused these models on the 17-month incubations where fungal abundance had increased the most and where potential effects of the initial disturbance on for example leaching and opportunistic colonizers had declined. For litter we used both bag sets after 17 months, while for humus we only used the second bag set after 17 months because the drought during 2018 limited fungal ingrowth and mass loss of the first set. Thus, three data sets were tested for relationships between fungi and decomposition, each utilizing all variation across the 40 treatment plots. The explanatory variables (total fungal and guild abundances (ITS2 copies g DW^{-1})) were *Z*-score normalized to facilitate result interpretation and to increase the performance of the Just Another Gibbs Sampler (JAGS) algorithm. We used vague Normal priors for all intercepts (α) and slopes (β) and Half-cauchy priors for all sigmas (σ) (Gelman *et al.*, 2008). Two different sets of initial values were defined for all estimated parameters and latent variables and ran the models until convergence and mixing of three Markov chain Monte Carlo (MCMC) chains (Gelman & Rubin, 1992). We extracted parameter estimates at every 10th step from a total of 15 000 samples from each MCMC chain, that is each coefficient of guild abundances was based on 4500 samples. The Gelman–Rubin criterion was used to diagnose chain convergence and posterior predictive checks on each model to compare the predicted mean, coefficient(s) of variation and residuals with the original data (Gelman & Rubin, 1992; Hooten *et al.*, 2015). This approach bases inferences on combined uncertainty in the response estimates (*k* and mass loss) and model residuals and thus decreases the risk of Type I errors.

Litter decomposition model Pine needle litter was expected to decompose following an asymptotic decay equation (Eqn 1a). In short, this function behaves like a simple negative exponential function but has a much longer tail (Eqn 1a):

$$M(t) = M(0)(1 + kt)^{-\gamma} \quad \text{Eqn 1a}$$

$M(0)$ is the initial mass, $M(t)$ is mass remaining, (t) is the incubation length in days, k is a latent, specialized decomposition parameter (d^{-1}), and γ is a median decomposition constant (-1.19) adjusted for climate conditions in the boreal forest (Bosatta & Ågren, 2003). We fitted this decay function to the data using a Gamma distribution with σ denoting variance and μ denoting mean of the mass (m) (Eqn 1b):

$$Mt_i \sim \text{Gamma}\left(\frac{\mu m_i^2}{\sigma m^2}, \frac{\mu m_i}{\sigma m^2}\right) \quad \text{Eqn 1b}$$
$$\mu m_i = M0_i(1 + k_i t)^{-\gamma}$$

We then fitted a linear mixed model using a Gamma distribution to k_i based on total fungal biomass (βBm) as the explanatory variable (Eqn 1c). In a separate model, we split the total fungal biomass into white rot saprotrophs (βW) and nonwhite rot

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saprotrophs (βN) as the explanatory variables (Eqn 1d) (biomass of biotrophs was negligible in the litter). A grouping parameter accounted for the nested design of plots (e_{block_i}) in blocks 1–8:

$$k_i \sim \text{Gamma}\left(\frac{\mu k_i^2}{\sigma k^2}, \frac{\mu k_i}{\sigma k^2}\right)$$
$$\log(\mu k_i) = \alpha + \beta\text{Bm} \times \text{Bm}_i + e_{\text{block}_i} \quad \text{Eqn 1c}$$
$$e_{\text{block}_i} \sim \text{Normal}(0, \sigma\text{block})$$

$$k_i \sim \text{Gamma}\left(\frac{\mu k_i^2}{\sigma k^2}, \frac{\mu k_i}{\sigma k^2}\right)$$
$$\log(\mu k_i) = \alpha + \beta N \times N_i + \beta W \times W_i + e_{\text{block}_i} \quad \text{Eqn 1d}$$
$$e_{\text{block}_i} \sim \text{Normal}(0, \sigma\text{block})$$

Humus decomposition model We expected humus mass (Mt) to accumulate or decline following a Gaussian linear model either with the total abundance of fungi (Eqn 2a) or in another model (Eqn 2b) with abundances of ericoid mycorrhizal fungi (β_{ericoid}), saprotrophs (β_{sap}), molds and yeasts (β_{mold}) and ectomycorrhizal fungi (β_{ecto}) as explanatory variables. Archaeorhizomycetes were excluded in the final model because they co-varied with ectomycorrhizal fungi as a clearly pine-associated guild. Ectomycorrhizal and saprotrophic guilds could not be further sub-divided according to their decomposition strategies (e.g. white rot capacity) due to limited and variable species-wise occurrences across replicates.

$$Mt_i \sim \text{Normal}(\mu m_i, \sigma m)$$
$$\mu m_i = \alpha + \beta\text{Bm} \times \text{Bm}_i + e_{\text{block}_i} \quad \text{Eqn 2a}$$
$$e_{\text{block}_i} \sim \text{Normal}(0, \sigma\text{block})$$

$$Mt_i \sim \text{Normal}(\mu m_i, \sigma m)$$
$$\mu m_i = \alpha + \beta_{\text{ericoid}} \times \text{ericoid}_i + \beta_{\text{ecto}} \times \text{ecto}_i + \beta_{\text{sap}} \times \text{sap}_i + \beta_{\text{mold}} \times \text{mold}_i + e_{\text{block}_i} e_{\text{block}_i} \sim \text{Normal}(0, \sigma\text{block}) \quad \text{Eqn 2b}$$

Results

Fungal abundance and community composition

Total fungal abundance (ITS2 copies gDW^{-1}) in the litter clearly increased over the duration of incubation, particularly from 5 to 17 months, and fungal ingrowth was more evident in the second bag set initiated during the second year (Figs 1a, S1). There were no significant effects of pine root exclusion or shrub removal on total fungal ingrowth in either of the litter sets (Table S1). The species-level fungal community composition differed markedly between the pine needle litter and humus substrates (Fig. S2). In both litter sets, community composition changed with incubation length ($P = 0.001$) with a convergence after 17 months (Fig. 2a; Table 1a). Fungal community composition in litter was affected by the presence of shrubs, but not pine, and the shrub effect depended on incubation length (Table 1a). The estimated abundance of fungal guilds in the litter was largely unaffected by the treatments, except for ericoid mycorrhizal fungi (0–2.5% of total fungi) which were more abundant when shrubs were present in the second set (Fig. 1a; Table S1). The white rot saprotrophic genus *Mycena* (mainly *Mycena clavicularis*) was the dominant fungus in the pine needles after 17 months; however, shrub and pine root presence did not significantly affect the abundance of white rot saprotrophs in the litter (Fig. 3a; Table S1).

Fungal ingrowth in humus increased over the duration of the incubations, and as for litter, a higher abundance of fungi was found in the second (start in 2018) than in the first (start in 2017) set (Fig. 1b). Pine roots increased the total fungal abundance in the second set after 17 months (Fig. 1b; Table S1). In both humus sets, *Penicillium spinulosum* coll. was the most abundant species, but total guild abundances of molds and yeasts, and saprotrophs were unaffected by shrub or pine presence (Fig. 1b). In the second set, shrub presence tended to promote the abundance of ericoid mycorrhizal fungi, such as *Serendipita* spp. (Fig. 3d), while pine roots promoted the abundance of nonwhite rot ectomycorrhizal fungi, such as *Piloderma sphaerosporum* (Fig. 3b). The abundance of undefined root-associated fungi, such as *Archaeorhizomyces* spp., was stimulated by the presence of pine roots in the second set and tended to have higher abundance in the presence of pine roots in the first set of incubations (Fig. 3f; Table S1). The fungal species composition in humus also shifted significantly over the course of the incubation, but the shift over time depended on both the set and pine root exclusion, with a greater shift in the second set and with pine roots present (Fig. 2b; Table 1b). Both pine roots and shrubs, and their interaction, had a strong overall effect on community composition in the humus (Table 1b). The amounts of fungal template DNA in pine needle litter and humus samples before incubation were several orders of magnitude lower than in the ingrowth bags.

Litter and humus mass loss

Mass loss of both substrates varied across sets and incubation times (Fig. 4; Table S2). Litter had lost *c.* 25% of the original dry mass after 5 months and 50–65% after 17 months (Fig. 4a,b). In the first set after 17 months, litter mass loss was *c.* 23% lower in the presence of pine roots, irrespective of shrub presence (Fig. 4a; Table S2). In the second litter set, shrubs had a positive effect on mass loss across both incubations, irrespective of pine root presence (Fig. 4b; Table S2). After 17 months, total fungal abundance in the pine needle litter had no clear link to the decomposition parameter k for the first and second set, respectively (cumulative posterior probability: $P(\beta\text{Bm} < 0) = 0.851$ and 0.426, respectively; Fig. S3a). White rot saprotrophic fungal abundance had a clear positive effect on the decomposition parameter k for both litter sets ($P(\beta\text{W} > 0) = 0.904$ and 0.889), while nonwhite rot saprotrophic fungi had a negative effect in both sets ($P(\beta\text{N} < 0) = 0.981$ and 0.986; Fig. 5a,b).

Over the first 5 months, 5–15% of the initial humus mass was lost in both sets (Fig. 4c,d). After 17 months, humus mass loss increased in the second set (by an additional 24–30% of the initial mass), but not in the first set (Table S3). Pine presence, overall, increased humus mass loss in both sets (Table S3). In the

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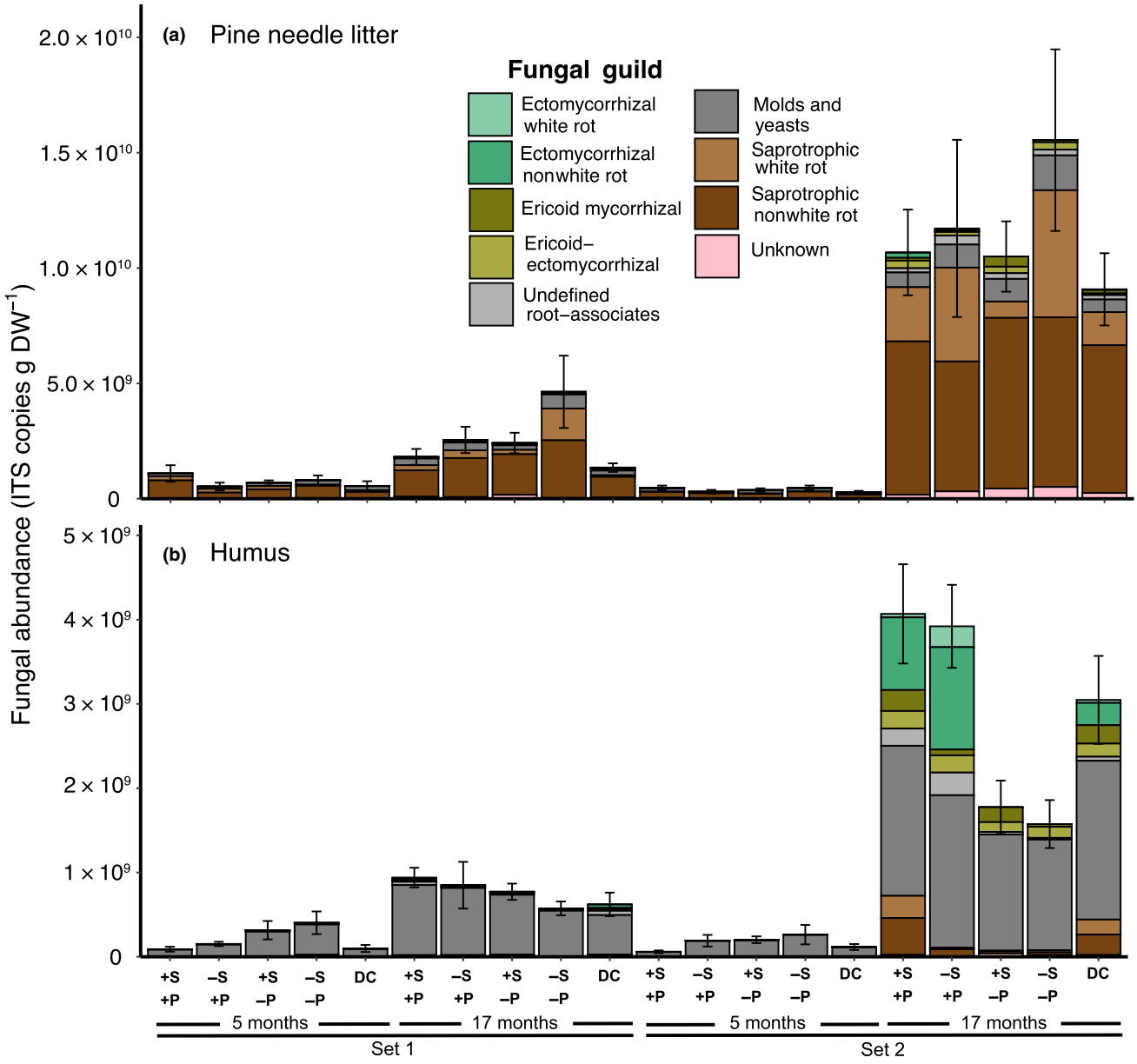

Fig. 1 Fungal ingrowth in *Pinus sylvestris* litter and humus substrates. Total fungal (entire bars) and fungal guild (stacked bars) abundance Internal Transcribed Spacer 2 (ITS2) copies g^{-1} dry weight (DW) substrate) in (a) decomposing pine needle litter and (b) humus incubated over 5 and 17 months in decomposition bag sets initiated in June 2017 and June 2018 (see Supporting Information Fig. S1). The bags were incubated in a factorial field experiment involving plots with or without shrubs (+S/−S) and with or without access by pine roots (+P/−P). Total fungal abundance was estimated as fungal ITS2 copies (quantitative polymerase chain reaction) and guild abundances were estimated by multiplying total fungal ITS2 copies with relative abundances of each guild derived from DNA sequencing of the same ITS2 marker. Error bars are SE of the means of total fungal biomass ($n = 8$). See Table S1 for statistical tests. The disturbed control (DC) is displayed for comparison but was not statistically tested.

second set, however, the positive effect of pine was conditional on shrubs and incubation time (Table S3); after 17 months, pine roots increased mass loss only in the absence of shrubs, and shrubs slowed mass loss (Fig. 4d). After 17 months in the second set, total fungal abundance was weakly negatively related to percent mass loss in the humus (Eqn 2a; $P(\beta \mathrm{Bm} > 0) = 0.367$; Fig. S3b). In the more elaborate model (Eqn 2b), the abundance of ectomycorrhizal fungi was positively associated with mass loss ($P(\beta_{\mathrm{ecto}} > 0) = 0.718$; Fig. 6a), molds and yeasts were negatively associated with mass loss ($P(\beta_{\mathrm{mold}} < 0) = 0.836$; Fig. 6b), while the abundance of ericoid mycorrhizal fungi ($P(\beta_{\mathrm{Ericoid}} < 0 = 0.514$; Fig. 6c) and saprotrophs ($P(\beta_{\mathrm{Saprotroph}} < 0) = 0.665$; Fig. 6d)) had an indeterminant effect.

Discussion

Pine needle decomposition

We hypothesized (H1a) that only the combined removal of the ericoid and ectomycorrhizal guilds would cause a Gadgil effect by

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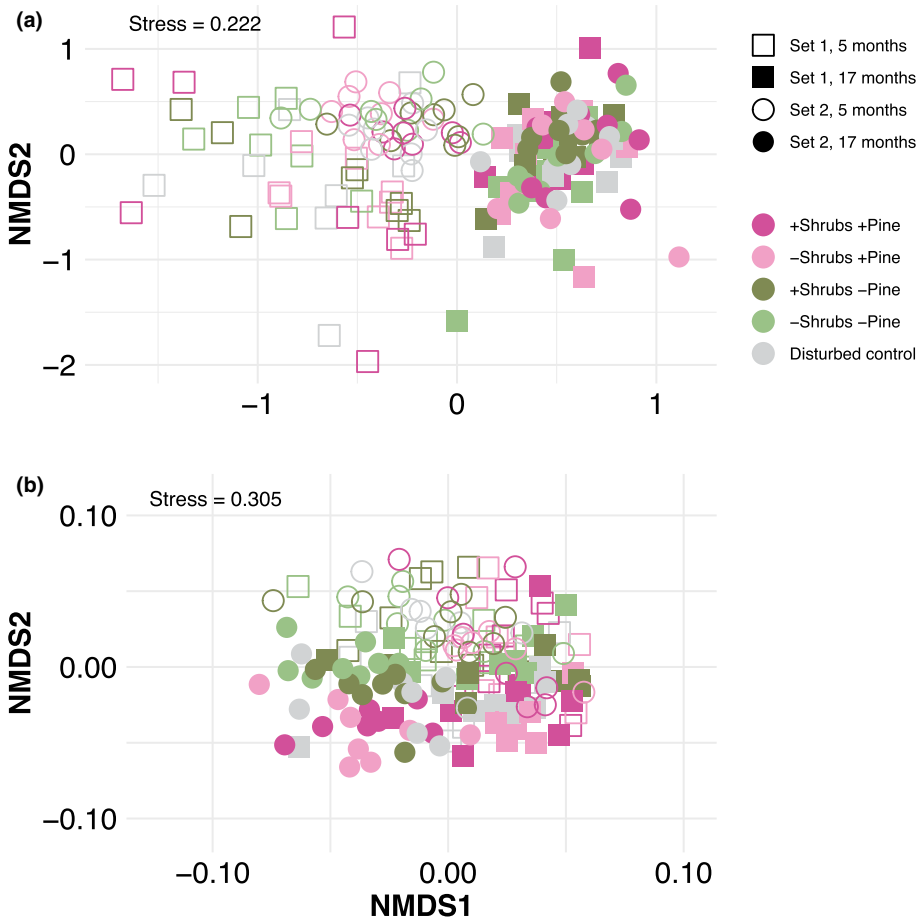

Fig. 2 Fungal community composition in decomposing *Pinus sylvestris* needle litter and humus as analyzed by nonmetric multidimensional scaling (NMDS). Symbols indicate community similarity for individual decomposition bags of litter (a) and humus (b) after incubation for 5 months (open symbols) or 17 months (filled symbols) in set 1 (squares; initiated June of the first year) and set 2 (circles; initiated June of the following year). The decomposition bags were incubated in treatment plots with the presence or absence of shrubs and pine roots (displayed in color). Fungal communities were based on DNA sequencing of the fungal Internal Transcribed Spacer 2 (ITS2) marker.

Table 1 Effects of *Pinus sylvestris* root and ericaceous shrub presence, bag set (first or second year) and incubation duration (5 or 17 months) on total fungal community composition in decomposing litter (a) and humus (b).

	R^2	F	P
(a) Litter			
Shrub	0.010	2.06	0.029*
Set	0.038	7.94	0.001***
Incubation	0.179	37.3	0.001***
Shrub × incubation	0.009	1.81	0.033*
Set × incubation	0.030	6.28	0.001***
(b) Humus			
Pine	0.021	3.49	0.001***
Shrub	0.012	2.05	0.001***
Set	0.025	4.17	0.001***
Incubation	0.032	5.41	0.001***
Pine × shrub	0.009	1.57	0.007**
Pine × incubation	0.009	1.47	0.011**
Set × incubation	0.023	3.93	0.001***

Pinus sylvestris roots and ericaceous shrubs were encoded as present (1) or absent (0) in a PERMANOVA including all interaction terms. Statistically significant terms are indicated for each substrate (***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$).

allowing saprotrophs to access more nitrogen and increase decomposition of pine needle litter when released from competition from the mycorrhizal guilds (Gadgil & Gadgil, 1971). After 17 months of incubation in the first set, a 23% increase in decomposition of pine needle litter was indeed found in response to pine root exclusion, indicative of an ectomycorrhiza-mediated Gadgil effect, similar to that originally described (Gadgil & Gadgil, 1971) and previous studies at similar sites (Berg & Lindberg, 1980; Sterkenburg *et al.*, 2018). However, this effect was not observed in the second litter set, incubated 1 yr later. Ericoid shrubs have previously been found to inhibit decomposition of shrub leaf litter in boreal forests (Fanin *et al.*, 2022), in a similar manner as in the original ectomycorrhiza-mediated Gadgil effect. Contrary to our hypothesis, the hampered litter decomposition in the first set was independent of whether shrubs were present, and in the second set, ericoid shrubs even promoted early litter decomposition, irrespective of pine root presence (Fig. 7).

The fungal communities in the pine litter underwent clear successional trajectories from a dominance by nonwhite rot saprotrophs, encompassing a particularly large abundance and diversity of ascomycetes such as *Calycellina* spp., *Venturiaceae* spp., and *Desmazierella acicula* at early stages, towards an increasing proportion of white rot saprotrophs, primarily *Mycena* spp., at later stages. The fungal community in the decomposing litter was not dramatically affected by root exclusion or shrub removal, although shrub presence promoted the abundance of ericoid mycorrhizal fungi in the second litter set. Thus, we found little evidence of whether the observed Gadgil effect (first set) was underpinned by

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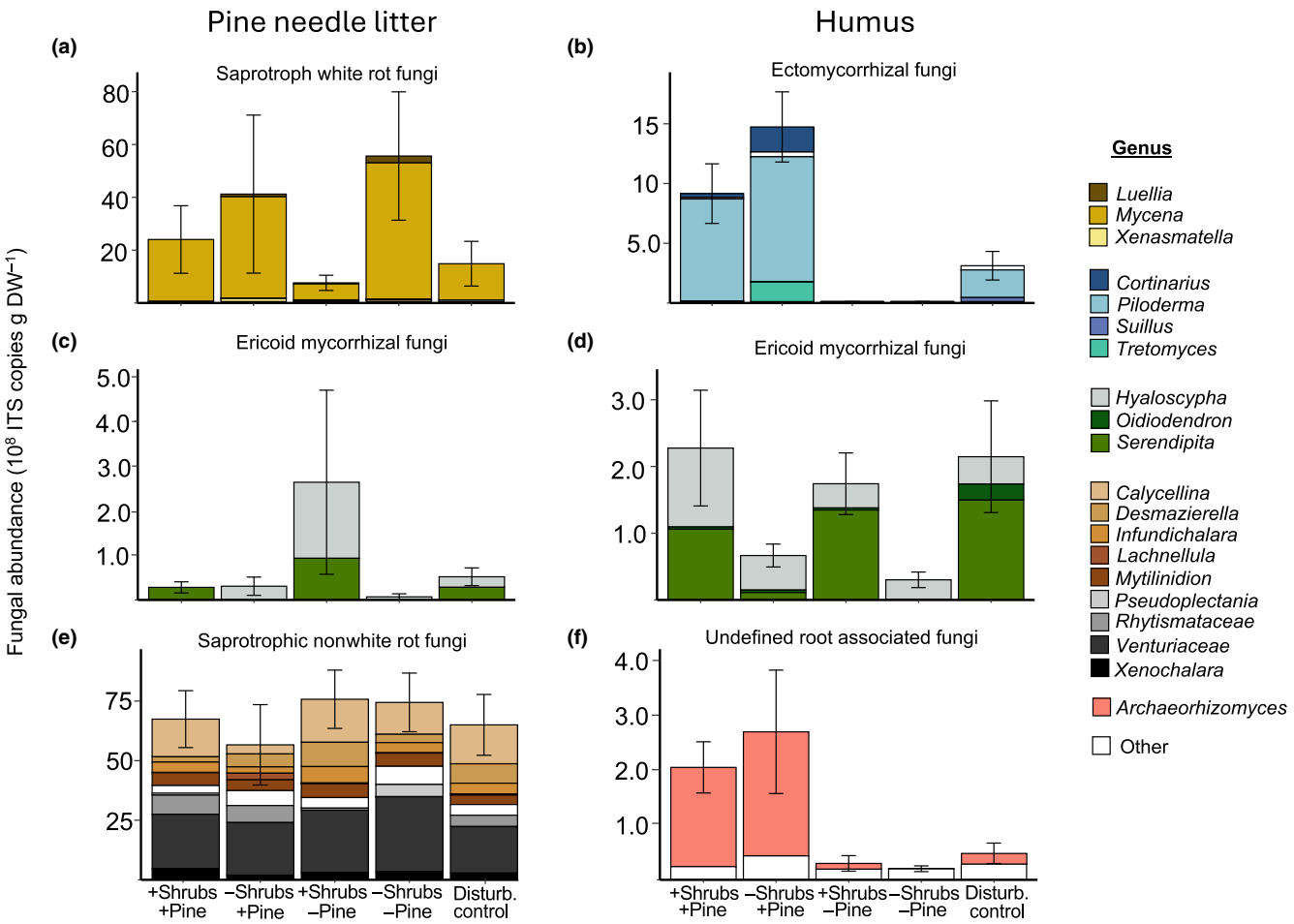

Fig. 3 Abundance of fungal guilds and individual genera. *Pinus sylvestris* needle litter (a, c, e) and humus (b, d, f) substrates incubated for 17 months (set 2) in a factorial field experiment involving plots with or without shrubs (+/−Shrubs) and with or without access by pine roots (+/−Pine). Fungal abundances (entire bars) were estimated as copies of the fungal Internal Transcribed Spacer 2 (ITS2) region (10^8 g^{-1} dry substrate mass (DW)) by multiplying total fungal ITS copies by the relative abundances of each guild and genus as determined by sequencing of the ITS2 marker. Fungal genera with low abundance are grouped in the 'Other' category. Error bars are SE of the means of total fungal abundances ($n = 8$). The disturbed control (DC) is displayed for comparison but was not included in statistical tests.

direct competitive interactions between saprotrophic and ectomycorrhizal fungi in the decomposing pine litter. In a temperate, continental pine plantation in North America, proliferation of tomentelloid fungi into the litter layer was linked to slowed decomposition (Fernandez *et al.*, 2020). In agreement with our expectation, a high abundance of white rot saprotrophs was related to faster decomposition of pine needle litter in both sets, while nonwhite rot saprotrophs were associated with slower decomposition rates. These relationships are, however, inferred from correlations between fungal communities analyzed at one time point and mass loss integrated over a longer period of time. Variation in timing of the succession from nonwhite rot (mainly ascomycetes) to white rot saprotrophs over the course of the decomposition process may, thus, have overruled the relatively minor variation in mass loss linked to the fungal guild exclusions, limiting our capacity to detect the community change related to the Gadgil effect.

Lower soil water availability has been highlighted as a potential mechanism behind ectomycorrhizal suppression of saprotrophic activities, since the uptake of water from the soil by ectomycorrhizal fungi and roots limits the amount of water available for saprotrophic fungi, particularly during periods of drought (Brownlee *et al.*, 1983; Koide & Wu, 2003). In our field experiment, mycorrhizal fungi to a larger extent, reduced soil inorganic nitrogen and water availability during the second growing season (2018) after a summer drought (Mielke *et al.*, 2022). This suggests that a greater increase in available nitrogen after pine root removal may have supported increased saprotrophic fungal nitrogen import growth and decomposition. Decomposition of high carbon-to-nitrogen ratio pine needle litter by white rot saprotrophs has been found to be stimulated by import of nitrogen from deeper layers (Boberg *et al.*, 2014), and relaxed competition for nitrogen outside of the decomposition bags may have caused the observed Gadgil effect, even though no ectomycorrhizal fungi were observed in the litter bags. Furthermore, indirect mechanisms, such as competition for nitrogen and water, are likely to be coupled (Fernandez & Kennedy, 2016), and it seems plausible

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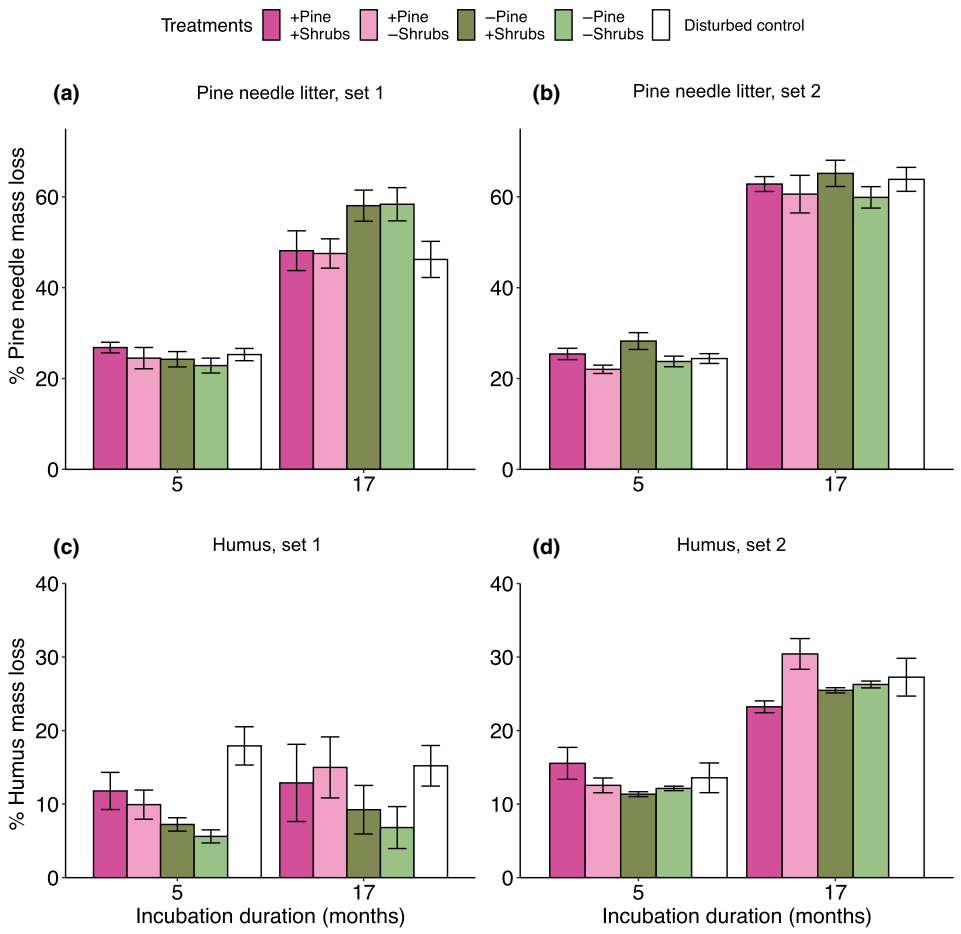

Fig. 4 Mass loss in *Pinus sylvestris* needle litter and humus substrates over two consecutive incubation periods. Mass loss (% of initial mass) of two sets of litter (a and b) and two sets of mor-layer humus (c and d) decomposition bags, with each set consisting of 50 µm-mesh bags incubated for either 5 or 17 months in each of the treatment plots (Supporting Information Fig. S1). Treatments, indicated in color, involved factorial exclusion of *P. sylvestris* roots and removal of shrubs. In disturbed control plots (white bars) roots were initially severed but were allowed to re-establish over the full duration of the experiment. Error bars are SE of the means ($n = 8$). See Tables S2 and S3 for statistical tests.

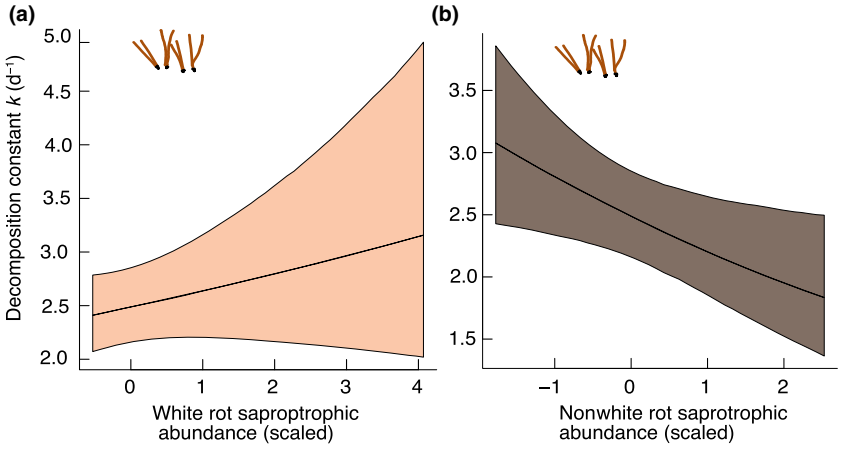

Fig. 5 Decomposition rates in relation to the abundance of saprotrophic fungi in *Pinus sylvestris* litter. Predicted decomposition rate (k, d^{-1}) of litter in relation to abundance of (a) white rot saprotrophs and (b) nonwhite rot saprotrophic fungi as modelled in Eqn 1b in both litter sets across all experimental plots. The decomposition constant k is presented at magnitude 10^{-3}. Credible intervals (95%) surround the median. Note, biomass is scaled and is not comparable between the functional groups.

that released competition for both water and nitrogen after drought was particularly important, restricting the Gadgil effect to the drought-prone year.

Contrary to expectations, shrub presence, linked to a higher abundance of ericoid mycorrhizal fungi in the litter, overall promoted mass loss in the second litter set. While pine litter inputs to the soil surface were unmanipulated in all plots, shrub removal also involved eliminating leaf litter inputs from ericaceous shrubs. It is possible that a different community of particularly the nonwhite rot saprotrophs was associated with ericaceous litter and that this, together with other synergistic effects of litter mixing (Gartner & Cardon, 2004), promoted increased decomposition of the pine litter. However, ericoid mycorrhizal fungi may also have contributed to increased litter decomposition when shrubs were present. In contrast to ectomycorrhizal fungi, which mostly lost their cellulolytic capacities (and with that, their potential for a free-living saprotrophic lifestyle) when evolving a mutualistic lifestyle (Kohler *et al.*, 2015), genome studies suggest

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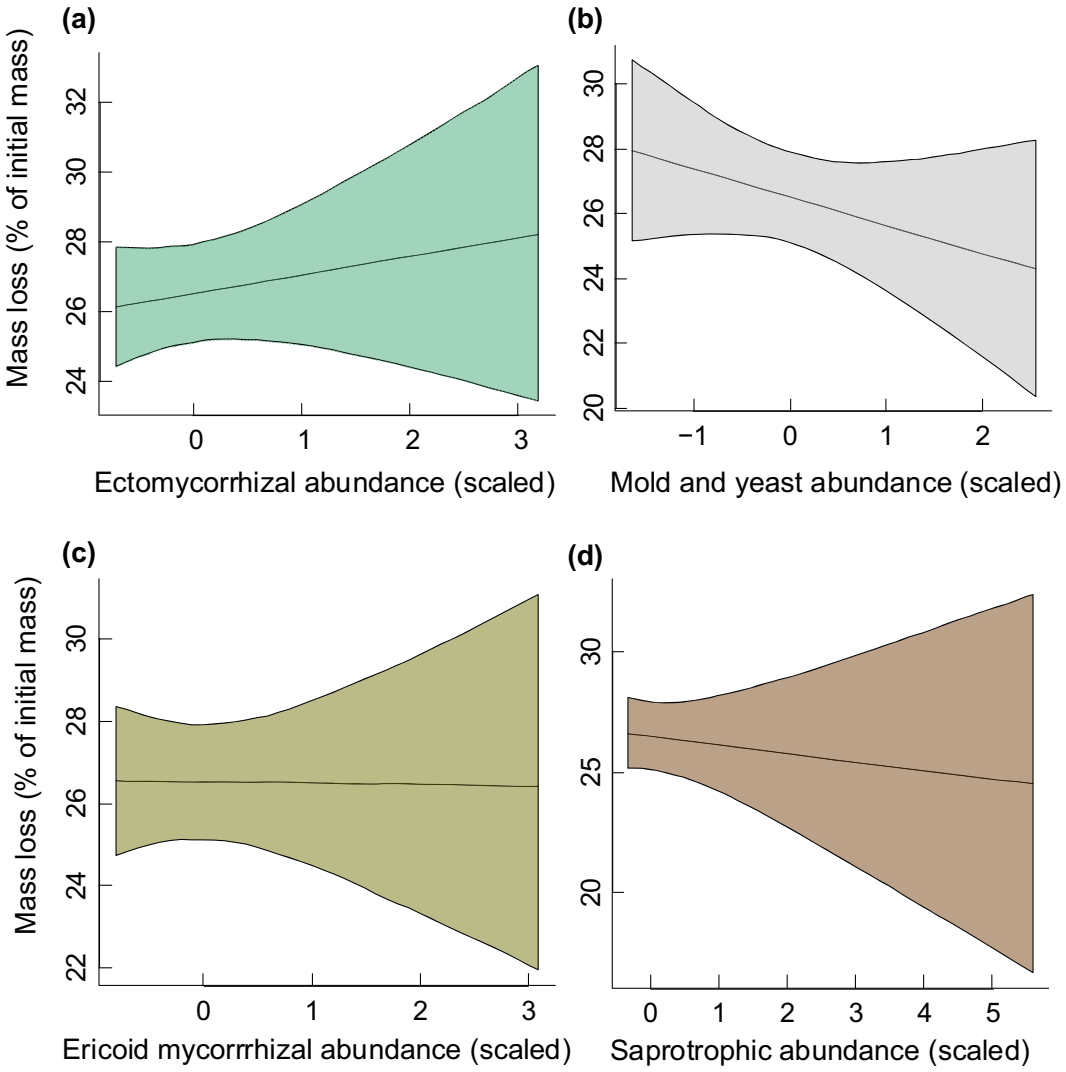

Fig. 6 Decomposition rates in relation to the abundance of fungal guilds in boreal *Pinus sylvestris* forest humus. Predicted humus mass loss (%) of humus in relation to abundance of (a) ectomycorrhizal fungi, (b) molds and yeasts, (c) ericoid mycorrhizal fungi, and (d) saprotrophic fungi as modelled in Eqn 2b from the second set of 17-month incubations across all experimental plots. Credible intervals (95%) surround the median. Note that biomass is scaled and is not comparable between the functional groups.

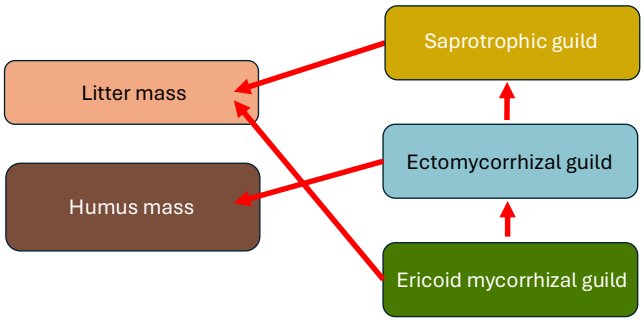

Fig. 7 A simplified conceptual diagram illustrating the possible direct and indirect impacts of fungi associating with trees and ericaceous shrubs on the decomposition of *Pinus sylvestris* needle litter and humus. A red arrow between guild and substrate represents a negative effect of that guild on substrate mass (i.e. increased decomposition) while a red arrow between guilds represents a counteracting effect on decomposition.

that ericoid mycorrhizal fungi have a versatile ecology and have retained extracellular enzymatic capacity for decomposition with enzymes hydrolyzing carbohydrates in parallel to their development of a symbiotic lifestyle (Martino *et al.*, 2018). A potential explanation for our observation is that ericoid mycorrhizal fungi were able to colonize and stimulate decomposition of recently shed litter, and that this shift towards a saprotrophic ecology may have been initiated during the drought period (in 2018) of presumed poor supply of carbon via the symbiotic root associations (Mielke *et al.*, 2022), and then partly sustained into the 17-month incubations through priority effects.

The root trenching treatment *per se* could also have stimulated the Gadgil effect through stimulated saprotrophic decomposition of severed roots in the surrounding plot (i.e. through a priming effect) during the first two growing seasons (Mielke *et al.*, 2022). However, litter decomposition patterns in our disturbance

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controls, which were trenched at the onset of the experiment but then left with no permanent barrier, reflected patterns in untrenched control plots, suggesting that trenching side effects were not the main cause of the observed Gadgil effect. Overall, the lack of a large and consistent inhibitory effect of mycorrhizal guilds on litter decomposition suggests that a Gadgil effect is at most modest and transient, even in our old-growth, nutrient-poor boreal forest. The extent to which competitive release from mycorrhizal guilds leads to enhanced saprotrophic decomposition may depend on multiple factors, such as litter quality, soil fertility, water availability, weather conditions, and stage of decomposition (Fernandez & Kennedy, 2016; Smith & Wan, 2019; Choreño-Parra & Treseder, 2024).

Humus decomposition

Previous studies on local (Gadgil & Gadgil, 1971; Averill & Hawkes, 2016; Kyaschenko *et al.*, 2017) and global (Averill *et al.*, 2014; Steidinger *et al.*, 2019) scales have suggested that ectomycorrhizal fungi increase overall soil organic matter stocks by competing with free-living saprotrophs and thereby restrict organic matter decomposition. That is, the Gadgil hypothesis has been extrapolated and assumed to be valid beyond the early litter decomposition phase and to affect older soil substrates, promoting accumulation of organic stocks. Here we hypothesized (H1b) that saprotrophic decomposition and the related Gadgil effect would not be important in well-decomposed humus, and (H2a) that divergent ecologies of ecto- and ericoid mycorrhizal fungi instead would have counter-balancing effects on mor-layer humus decomposition. Overall, the presence of pine roots did indeed stimulate humus mass loss, and we also found support for the idea that ericaceous shrubs counteracted the humus decomposition mediated by pine roots. We, thus, confirm that it is not valid to extrapolate the Gadgil hypothesis to explain decomposition dynamics of humus in boreal forest, where a major fraction of soil organic matter is not directly derived from aboveground litter inputs, and where decomposition dynamics appear to differ between recently shed litter and older organic substrates in the rooting zone (Clemmensen *et al.*, 2013; Kyaschenko *et al.*, 2017).

Pine root exclusion eliminated ectomycorrhizal fungal ingrowth in all humus bags, which led to an overall decline in fungal abundance. Presence of pine roots stimulated decomposition in both humus sets, but in the second set, pine only stimulated decomposition in the absence of ericoid shrubs. Furthermore, the presence of shrubs hampered humus mass loss, but only in the presence of pine roots. Shrubs also marginally increased the abundance of the ericoid mycorrhizal fungi in humus, but this guild continued to make up part of the fungal community even after the host removal. These results support our hypotheses, that (H2a) the ectomycorrhizal guild overall promoted humus mass loss, while (H2b) the ericoid mycorrhizal guild counteracted mass loss. However, the significant interactions indicate that effects of the two mycorrhizal guilds on mass loss were not simply additive but potentially depended on within-guild community variation across the treatments.

The 10–15% humus mass loss during the first months of both humus sets was likely caused by a combination of leaching and assimilation of recently dead organic residues by molds and yeasts without the capacity to contribute to long-term humus decomposition. Molds and yeasts are normally encountered at very low abundances in undisturbed boreal forest soils, but may proliferate after disturbances releasing easily available substrate (Lindahl *et al.*, 2010; Bödeker *et al.*, 2016). Pine roots promoted longer-term colonization of humus by ectomycorrhizal fungi and root-associates with undefined ecology (mainly *Archaeorhizomycetes* spp.). Fungal ingrowth, correspondingly, was more variable among plots with pine present, likely linked to stochastic substrate colonization and variable extramatrical mycelial growth of different ectomycorrhizal fungi across treatments and individual bags (Jörgensen *et al.*, 2023). *Cortinarius* species have previously been found to be stimulated by long-term shrub removal in northern boreal forest (Fanin *et al.*, 2022). In our case, the presence of shrubs also appeared to reduce colonization by white rot ectomycorrhizal fungi, but species-level colonization was variable among replicates, and treatments were not significantly different for the finer fungal groupings. Our decomposition model supported the idea that higher abundance of the ectomycorrhizal fungal guild as a whole was related to larger humus mass loss at the 17-month time point. Whether ectomycorrhizal fungi with oxidative decomposition capacity (e.g. *Cortinarius*) or other ectomycorrhizal decomposers (e.g. *Piloderma*) using alternative laccase-like reaction mechanisms (Heinonsalo *et al.*, 2015) were responsible for decomposition was not evident, due to the lack of sufficient replication to capture the inherent stochasticity in ectomycorrhizal mycelial colonization. Additionally, high abundance of molds and yeasts was found to be related to slower decomposition, likely because this guild was more abundant in replicates with less competition and ongoing decomposition by ectomycorrhizal fungi.

The decomposition model did not clearly link ericoid mycorrhizal fungal abundance to slower decomposition of organic matter, potentially because this fungal guild persisted in the humus despite elimination of their hosts. It was thus not possible to evaluate whether direct organic matter inputs via shrub roots and ericoid mycorrhizal mycelia led to increasing humus mass, or whether they primarily restricted colonization and decomposition by the ectomycorrhizal fungi. This distinction was hindered because the finer time-scale organic matter dynamics in the humus were masked by the 10–15% initial disturbance-mediated decrease in humus mass coinciding with mold colonization. However, it is noticeable that the least mass loss was observed in control plots with presence of both pine roots and shrubs. This may indicate that co-existence of the ecto- and ericoid mycorrhizal guilds, and a potential shrub-mediated alteration of the ectomycorrhizal fungal community (i.e. fewer white rot ectomycorrhizal decomposers), facilitates organic matter accumulation in the boreal forest. Another mechanism that may stabilize organic matter disproportionately when the two guilds co-exist is chemical complexation of exudates and residues derived from pine and shrub roots and mycorrhizal necromass (Adamczyk, 2019; Fanin *et al.*, 2022).

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In conclusion, we found support for a 23% increase in recent litter decomposition upon ectomycorrhizal exclusion, that is, a Gadgil effect, but the importance of this effect for organic matter inputs into the long-term stabilized organic pool (such as humus) is judged to be minimal, transient, and context-dependent (Fig. 7). We found no support for an ericoid-mediated Gadgil effect, but rather somewhat increased litter decomposition in the presence of the ericoid guild, potentially related to a shift from mutualistic to a saprotrophic lifestyle of the ericoid fungi after a drought or to synergistic effects of litter mixing. The litter decomposition model supported the idea that white rot saprotrophs, primarily *Mycena* spp., are related to litter decomposition in this old-growth boreal pine forest. Pine roots and associated ectomycorrhizal fungi were linked to larger mass loss of humus; however, this stimulation of decomposition by ectomycorrhizal fungi appeared to be hampered by the presence of shrubs. It was not possible to unravel whether the ingrowth of ericoid plant roots and associated fungi elicited an increase in mass towards later stages of humus decomposition or whether they primarily hindered the decomposer activities of ectomycorrhizal fungi. Interactions between root-associated guilds or individual species, thus, appear to be more important mechanisms underlying humus accumulation in boreal forests than the Gadgil effect (Clemmensen *et al.*, 2015), at least in the time perspective of this study. Future research priorities should include examination of decomposition and decomposer dynamics in a more mechanistic context, for example, by transcriptomics or proteomics, over greater temporal and spatial scales, to better understand whether increased saprotrophic activities could cause major losses of mor-layer humus stocks in situations where mycorrhizal guilds are hampered, for example through tree felling, soil disturbance, or pathogen outbreaks.

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Competing interests

None declared.

Author contributions

The field experiment was initiated and designed by KEC, BDL, RDF and AE and the specific study was conceived by all authors. The field and laboratory work was performed by LAM and KEC. Data were analyzed by LAM. The Bayesian models were developed by LAM and JK. The manuscript was written by LAM and all authors contributed to the data interpretation and revisions.

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Data availability

Raw sequence data can be found under BioProject accession number PRJNA834027 in the Sequence Read Archive. All scripts and data are available on GitHub (https://github.com/mielk132/jadraas_needles_humus).

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Supporting Information

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

Fig. S1 Timeline for humus and needle litter incubation experiment.

Fig. S2 NMDS of fungal community composition in decomposing substrates.

Fig. S3 Association of total fungal abundance with decomposition parameters.

Table S1 Linear mixed models of fungal abundances.

Table S2 Linear mixed models of pine litter mass loss.

Table S3 Linear mixed models of humus mass loss.

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