



Ectomycorrhizal fungal community response to warming and rainfall reduction differs between co-occurring temperate-boreal ecotonal *Pinus* saplings

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Abstract

Understanding the responses of ectomycorrhizal (ECM) fungi and their tree hosts to warming and reduced soil water availability under realistic future climate scenarios is essential, yet few studies have investigated how combined global change stressors impact ECM fungal community richness and composition as well as host performance. In this study, we leveraged a long-term factorial warming (ambient, +1.7 °C, +3.2 °C) and rainfall reduction (ambient, 30% reduced rainfall) experiment in northern Minnesota, USA to investigate the responses of two congeneric hosts with varying drought tolerances and their associated ECM fungal communities to a gradient of soil moisture induced by a combination of warming and rainfall reduction. Soil drying had host-specific effects; the less drought tolerant *Pinus strobus* had decreased stem growth and lower ECM fungal community richness (fewer ECM fungal Operational Taxonomic Units, OTUs), while the more drought tolerant *Pinus banksiana* experienced no decline in stem growth but had an altered ECM fungal community composition under drier, warmer soils. Taken together, the results of this study suggest that the combined effects of warming and decreased precipitation will largely be additive in terms of their impact on host performance and ECM fungal community richness, but that drier and warmer soil conditions may also differentially impact specific ECM fungal genera independently of host performance.

Keywords Ectomycorrhizal fungi · Diversity · Warming · Rainfall reduction

Introduction

The temperate-boreal ecotone, which represents the transitional region between northern temperate and southern boreal forest, is highly vulnerable to climate change and

already experiencing compositional and functional shifts in plant communities (Frelich et al. 2021, 2024; Ruckstuhl et al. 2008). Rather than responding to a single environmental variable, these forests are being simultaneously affected by both rising temperatures as well as concurrent declines in soil water availability (Gauthier et al. 2015; Liu et al. 2023; Sánchez-Pinillos et al. 2022). It has been demonstrated that warming alone can stimulate photosynthesis and growth of some temperate and boreal tree seedlings (Fisichelli et al. 2014b; Nissinen et al. 2020) and saplings (Reich et al. 2022), but that these positive effects can be negated by concurrent decreases in soil moisture (Fisichelli et al. 2014b; Reich et al. 2018; Stinziano and Way 2014). Further, it has been shown that while moderate warming (+1.6 °C) can increase sapling stem biomass in some species, when combined with reduced rainfall, plot level stem biomass can be decreased by as much as 25% (Reich et al. 2022). Taken together, these results highlight the importance of studying

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multiple climate change stressors concurrently to gain a predictive understanding of the future forest dynamics at the temperate-boreal ecotone.

Despite extensive and growing knowledge of how trees respond to stressors such as warming and reduced rainfall, how these same environmental stressors simultaneously affect their ectomycorrhizal (ECM) fungal symbionts remains less well understood (Bennett and Classen 2020; Cowden et al. 2019; Mohan et al. 2014). Studies suggest that warming results in significant changes to temperate-boreal forest ECM fungal community composition (Fernandez et al. 2017, 2023; Kwatcho Kengdo et al. 2022; Mucha et al. 2018), often due to increases in the relative abundance of ascomycete ECM fungi (Allison and Treseder 2008; Fernandez et al. 2017, 2023). With respect to ECM fungal species richness, evidence is currently mixed, with some studies showing no significant effect of warming (+3.4 °C: Fernandez et al. 2017; Mucha et al. 2018, +3.1 °C: Fernandez et al. 2023, +4 °C: Kwatcho Kengdo et al. 2022) and others showing positive effects (+0.5 °C, Allison and Treseder 2008). Conversely, models of ECM fungal responses to future climate change suggest that warming will reduce ECM fungal species richness in coniferous temperate-boreal forests (Steidinger et al. 2020). Studies of ECM fungal responses to drier soil conditions have shown reduced ECM fungal root tip colonization (Gehring et al. 2020; Kennedy and Peay 2007; Swaty et al. 2004) and decreased ECM hyphal abundance in soil (Castaño et al. 2023; Querejeta et al. 2021), although most of this work is based in seasonally dry climates. While often studied separately, these findings suggest that ECM fungal communities are susceptible to multiple climate stressors and that studying them at the same time (i.e. both warming & reduced soil water availability) is needed to better understand the potential for synergistic effects (Fernandez et al. 2023; Gehring et al. 2020).

The changes in ECM fungal community richness and composition in response to shifting climatic conditions may be the result of either direct effects on fungal growth or indirect effects mediated by their plant host (Kennedy and Peay 2007). While fungi generally have wide temperature tolerances, they tend to perform poorly under water limitation (Coleman et al. 1989). Support for direct effects of drying on fungal performance has been shown in a previous study where declining soil moisture led to decreases in ECM fungal respiration (Heinemeyer et al. 2007). Along with direct fungal responses to altered climatic conditions, ECM fungal communities may also be influenced by the tolerance of their tree hosts for low soil moisture conditions. The ability of plants to cope with water scarcity, commonly referred to as drought tolerance, manifests through several mechanisms that may act simultaneously (Levitt 1980; Moran et al. 2017). Tolerance mechanisms can differ among plant

species but include hydraulic lift (to redistribute water from lower soil layers), altered belowground carbon (C) allocation to roots (to increase surface area available for water absorption), and/or stomatal regulation (to prevent water loss), all of which have important implications for resource exchange (C and H₂O) between tree hosts and mycorrhizal fungal partners (Brunner et al. 2015; Horton and Hart 1998; Lehto and Zwiazek 2011; McDowell et al. 2008).

Co-occurring temperate-boreal conifers, which are likely to change abundances in response to altered climatic conditions (Fisichelli et al. 2012; Fisichelli et al. 2014a; Reich et al. 2022), can differ in their ability to withstand warmer, drier conditions. For instance, long-term experimental warming (+3.1 °C) and reduced rainfall has been shown to reduce the survival and growth of temperate *Pinus strobus* saplings, but not boreal *Pinus banksiana* saplings (Reich et al. 2022). Warming (when soil moisture was sufficient) also was found to increase photosynthesis rates for *P. banksiana*, and other temperate-boreal species, but not for *P. strobus* (Reich et al. 2022). These varying responses are reflective of the higher drought tolerance of *P. banksiana* compared to *P. strobus* (drought tolerance indices = 4 and 2.29, respectively, on a scale where ‘5’ represents the highest tolerance of drought conditions including low soil water potentials, low precipitation and high evapotranspiration, while ‘1’ represents the lowest drought tolerance, Niinemets and Valdares 2006). Varying responses of temperate-boreal tree hosts to gradients of warming and soil drying may have significant implications for ECM fungal communities given that reduced tree host photosynthesis may indicate less carbon available for ECM fungi (Fernandez et al. 2017, 2023; Pickles et al. 2012).

Here we leveraged the ‘Boreal Forest Warming at an Ecotone in Danger’ (B4WarmED) experiment to investigate the responses of ECM fungal communities on two congeneric hosts with varying drought tolerances to a gradient of soil moisture induced by a combination of warming and rainfall reduction.

We hypothesized that ECM fungal richness and community composition would respond linearly to declining soil moisture, demonstrated by declines in ECM fungal OTU (Operational Taxonomic Unit) richness and linear shifts in the relative abundances of individual ECM fungal genera in response to declining soil moisture. With respect to the role of tree host drought tolerance, we further hypothesized that ECM fungal communities on the more drought tolerant *P. banksiana* would be less responsive to declining soil moisture than communities on *P. strobus*.

Methods

Study site and experimental design

This study was conducted at the southern site of the B4WarmED experiment (Rich et al. 2015), which is a long-term chamber-free experimental warming and rainfall reduction study located at the University of Minnesota Cloquet Forestry Center (CFC), Cloquet, MN, USA (46° 40' 46" N, 92° 31' 12" W, 382 m a.s.l., 4.5 °C mean annual air temperature (MAT), 807 mm mean annual precipitation). The experimental site was cleared between 2007 and 2008 and included mixed *Populus* stands with coarse upland soils (Rich et al. 2015). At CFC, there are 3-meter diameter circular plots located within three 'block' replicates with factorial above and belowground experimental warming (ambient, +1.7 °C, +3.2 °C, infrared lamps for aboveground and soil heating cables for belowground) and reduced rainfall (ambient, 30% reduced rainfall, rain removed using rainout shelters, (Reich et al. 2022; Rich et al. 2015; Stefanski et al. 2020). The warming treatments were initiated in 2009, and the rainout shelters were installed in 2012 at randomly selected plots of each temperature treatment type (Reich et al. 2022; Rich et al. 2015; Stefanski et al. 2020). Warming and reduced rainfall treatments are performed yearly between early spring and late fall (for further details see: Rich et al. 2015; Stefanski et al. 2020). The plots contain a mix of randomly assigned ~1-4-year-old saplings of multiple co-occurring native Minnesota tree species (*Abies balsamea* L., *Acer rubrum* L., *A. saccharum* Marshall., *Betula papyrifera*, *Picea glauca* (Moench) Voss., *Pinus banksiana* Lamb., *Pinus strobus* L., *Populus tremuloides* Michx., *Quercus macrocarpa* Michx., *Quercus rubra* L.), as well as several non-native invasive species (*Rhamnus cathartica* L., *Frangula alnus* Mill., *Lonicera morrowii* A. Gray, *Lonicera tatarica* L.). Saplings were planted into randomly assigned locations in a grid with 20 by 20 cm spacing (Reich et al. 2022; Rich et al. 2015).

The *Pinus* saplings included in this study were planted in spring 2017 (~2 years old at planting) and harvested in April-May 2021.

Sapling growth measurements

At the end of each growing season, we measured the diameter of each *Pinus banksiana* and *P. strobus* sapling at 5 cm above the ground surface, as well as the total height of the saplings following the leader (central stem) in the fall of each experimental year (September-October). Stem biomass of each sapling was estimated from height and diameter, using an allometric equation developed based on a subset of

saplings that were destructively sampled in 2011 (Reich et al. 2015; see Supplementary Information for details).

Soil and climate measurements

Soil volumetric water content (VWC, 0–20 cm depth) in each plot was continuously measured (hourly) using a Campbell Scientific CS-616 probe inserted into the ground at 45° (Rich et al. 2015). Aboveground temperature was measured hourly at canopy level height using surface tissue-imitating acrylic blocks with embedded thermocouple sensors, and belowground temperature was measured hourly using sealed thermocouples at 10 cm soil depth (Rich et al. 2015; Stefanski et al. 2020). The hourly soil VWC, aboveground temperature, and belowground temperature data were trimmed to include only data between April 1st and October 31st, which represents the approximate growing season period. Additionally, only years when both the rainfall and temperature treatments were turned on were included (2018–2020). Hourly data was averaged across all growing seasons per each individual plot.

Ectomycorrhizal root sampling

In Spring 2021 (April-May), 1–3 saplings per tree species were harvested from each of the 18 plots (3 plots per treatment type per block), sampling one experimental block at a time (3 blocks total). The number of saplings collected depended on the survival rate within each treatment combination. To keep root systems intact, saplings were excavated with trowels, taking care to avoid damaging the fine roots. Following excavation, root systems were separated from the stem and adhering soil was gently removed by submerging roots in water and rinsing off any visual soil or organic matter. Each root system was then quartered, and one quarter was randomly selected for ECM fungal analysis. The selected portion was rinsed thoroughly to remove any remaining soil. The selected roots from each sapling were oven-dried at 38 °C for 48 h and stored at -20 °C prior to processing. To prepare the sampled roots for DNA extraction, we used methods previously implemented by other studies in this experimental system (see Fernandez et al. 2017; Fernandez et al. 2023). Dried root systems were gently crushed in a clean piece of printer paper to detach the ECM fungal colonized fine roots from the larger higher order roots (Kong and Ma 2014). To homogenize the detached fine root material, 20 mg was ground to a fine powder using a bead beater for 30 s (Mini-Beadbeater-96, BioSpec Products, Bartlesville, OK, USA).

Molecular methods

Total genomic DNA was extracted from all root samples using a standard chloroform method (see methods detailed in Kennedy et al. 2003). The ITS2 rDNA subunit was PCR amplified using a barcoded fungal-specific ITS5.8SFun-ITS4-Fun primer set, 35 total cycles, and cycling conditions detailed in Taylor et al. (2016). Both negative and positive (Palmer et al. 2018) controls were also included. All positively amplified samples were cleaned using the Charm Just-A-Plate kit (Charm, San Diego, CA, USA) following manufacturer's instructions. Samples were quantified on a Qubit fluorometer (Thermo Scientific, Waltham, MA, USA), mixed at approximately equimolar concentration into a single library, and sequenced using Illumina MiSeq 2×3000 bp v3 chemistry at the University of Minnesota Genomics Center.

Bioinformatic processing

The raw demultiplexed .fastq files were processed using the 'amptk' pipeline (v1.5.4, Palmer et al. 2018). Briefly, primers were removed, and sequences trimmed to 300 bp. Based on initial quality control assessments of the 'synmock' community, it was determined that including both forward and reverse reads resulted in both fewer reads per OTU as well as greater OTU inflation (likely due to the poorer quality of the reverse reads). As such, forward-only sequences were denoised using DADA2 algorithm (Callahan et al. 2016) and clustered at 97% similarity. Read counts in the OTU x sample matrix were adjusted to account for index bleed using 1% as the filter percentage. Taxonomy was assigned using a hybrid algorithm that integrates results from a USEARCH global alignment against the UNITE database (v8, Nilsson et al. 2019) and both UTAX and SINTAX classifiers. After samples were sequenced and processed, functional guilds were assigned using 'FUNGuild' (Nguyen et al. 2016). Raw sequences and associated metadata were deposited in the NCBI Short Read Archive under Bioproject ID #: PRJNA1079312. Prior to statistical analyses, we assessed the negative control sample for the presence of contamination, and then subtracted any OTU sequence reads present in the negative control from all study samples. Additionally, sequence reads less than or equal to 31 per sample were removed from the OTU table based on OTU read counts for the 'synmock' (Palmer et al. 2018) positive control sample (15 OTUs had reads > 100, and 3 OTUs had low read counts of 31 or fewer). This approach is consistent with previous research suggestions for high throughput sequencing quality control (Lindahl et al. 2013). The final OTU table was then filtered to include only ECM fungal taxa, and 6 low-read (< 1000 reads) samples were removed

from the dataset (as they were below the 90th percentile of ~2000 ECM fungal reads).

Statistical analyses

All analyses were carried out using R version 4.3.1 (R Core Team 2023). Differences in soil VWC amongst the experimental treatment types were assessed with a two-way, type III mixed-effects ANOVA using the 'lme' function in the 'nlme' package (Pinheiro et al. 2024), and then the 'Anova' function in the 'car' package (Fox et al. 2023). The model included treatment as a fixed effect, and experimental block as a random effect to account for any variation in soil VWC by block. Tukey HSD post hoc tests were performed using the 'emmeans' function in the 'emmeans' package (Lenth et al. 2024). To assess tree host growth responses to the experimental treatments, the relative increase in stem biomass during the last experimental year for each sapling was calculated ((final stem biomass in 2020 - stem biomass in 2019)/initial stem biomass at start of study). Preliminary analyses showed this response was similar when earlier experimental years were also included, but only the final year was used here since it was the time period closest to when the ECM fungal communities were characterized. Relative stem growth data were square root transformed to achieve normality before performing statistical tests. The relationships between relative stem growth and soil VWC, aboveground temperature, and belowground temperature were assessed with separate linear mixed effects models using the 'lme' function in the 'nlme' package (Pinheiro et al. 2024). For each model, experimental block was included as a random effect to account for unexplained block variation. Models were run on the full dataset (including tree host species as a fixed effect in addition to soil VWC, aboveground temperature, or belowground temperature), as well as for each individual tree species. Adjusted R squared values for linear mixed effects models were calculated using the 'rsq.lmm' function in the 'rsq' package (Zhang 2023).

Prior to statistical analysis of ECM fungal richness and community composition, the ECM fungal OTU table was normalized using a relative abundance transformation, where the abundance of each OTU per sample was divided by the total number of reads for that sample (thus all values were between 0 and 1). This approach accounts for potential variation in sequencing depth across samples (McKnight et al. 2019) and aligns with previous approaches used in this study system (Fernandez et al. 2017). ECM fungal OTU richness (the number of unique OTUs) was also square root transformed to achieve normality before performing statistical tests. To assess the effects of soil VWC, aboveground temperature, belowground temperature, and sapling relative stem growth on ECM fungal OTU richness, separate

linear mixed effects models were performed using the ‘nlme’ package (Pinheiro et al. 2024). All models included experimental block as a random effect. Similar to relative stem growth models, richness models were run on the full dataset including both tree host species, as well as for each individual tree species, and Adjusted R squared values were calculated using the ‘rsq.lmm’ function in the ‘rsq’ package (Zhang 2023). For results of all linear mixed effects models in this study, we report both the conditional Adjusted R squared (Adj. $R^2_{(c)}$) which includes both fixed and random effects, as well as the marginal Adjusted R squared values (Adj. $R^2_{(m)}$) which only includes variance from fixed effects.

To assess the effects of tree host species and experimental treatment on ECM fungal community composition, a permutational multivariate analysis of variance (PERMANOVA) test was run on a Bray-Curtis dissimilarity matrix of the transformed OTU table using PRIMER V7 (with PERMANOVA+) (Clarke and Gorley 2015). The model included tree host species and treatment as fixed effects, block as a random effect, and their interactions. Additionally, PERMANOVA models were run for each individual tree host species, and these models included treatment as a fixed effect, block as a random effect, and their interaction. All PERMANOVA models were run with 999 permutations. Beta dispersion tests were run using the ‘betadisper’ function in the ‘vegan’ package to assess the effects of each predictor variable on ECM fungal community dispersion (Oksanen et al. 2022). To assess how much of the variability in ECM fungal community composition could be explained by tree host species, soil VWC and temperature, a distance-based redundancy analysis was performed on the same Bray-Curtis dissimilarity matrix using the ‘dbrda’ function in the ‘vegan’ package (Oksanen et al. 2022). The full model included tree host species, soil VWC, aboveground temperature, and belowground temperature. Forward and reverse selection was then used to determine the best dbrda model. Distance-based redundancy analyses were also performed for each individual tree host species. These models included soil VWC, aboveground, and belowground temperature. Finally, to assess the effects of warming and decreasing soil VWC on the relative abundances of the most abundant ECM fungal genera, we calculated mean relative abundances across all samples from the same tree host species and treatment type. Then the mean relative abundance of each genus was assessed with linear regression models using soil VWC, aboveground temperature, or belowground temperature as separate predictor variables. These models were run separately for each of the two tree host species. Mean relative abundance data for samples from *P. banksiana* were square root transformed to achieve normality.

Results

Temperature and soil volumetric water content

Between 2018 and 2020, the B4WarmED experimental treatments warmed plots by an average of +1.7 °C and +3.2 °C for the two warming treatment types (see Supplementary Table S1). Soil VWC from 2018 to 2020 was significantly different between the experimental treatments and the control treatment (ambient temperature, ambient rainfall), with mean soil VWC for each of the five experimental treatments being significantly lower than the control treatment ($p < 0.01$, see Supplementary Table S2). Soil VWC decreased with increasing warming and/or rainfall reduction, from highest in the ambient temperature-ambient rainfall (control) to lowest in the +3.2-reduced rainfall treatment (which for convenience we call ‘highest stress’, see Supplementary Figure S1). There was no significant variation in soil VWC by experimental block.

Sapling host growth

Responses of sapling relative stem growth varied by tree host species (Fig. 1), and thus we present the results of the linear mixed effects models separated by individual tree host. For *P. strobus*, sapling relative stem growth did not respond significantly to aboveground temperature and belowground temperature ($p = 0.15$ and $p = 0.18$) but decreased significantly with declining soil VWC ($p = 0.04$, Adj. $R^2_{(c)} = 0.19$ and Adj. $R^2_{(m)} = 0.13$, Fig. 1b). In contrast, the relative stem growth of *P. banksiana* saplings did not respond to aboveground temperature, belowground temperature, or soil VWC ($p = 0.60$, 0.56 , and 0.81 , respectively).

Ectomycorrhizal fungal community richness

2,184,260 fungal sequence reads passed quality control, with 909,136 of these reads belonging to ECM fungi. After removing the 6 samples with low read sums, the final dataset included 907,106 ECM fungal reads, with a mean of 15,640 reads per sample (range: 2,089–69,960, see Supplementary Table S3). Responses of ECM fungal OTU richness varied by tree host (Fig. 2); thus, we present the results of the linear mixed effects models separated by individual tree host. For *P. banksiana*, there were no significant responses of ECM fungal OTU richness to soil VWC, aboveground, or belowground temperature ($p = 0.24$, $p = 0.62$ and $p = 0.69$, respectively). ECM fungal OTU richness of *P. strobus* samples, however, decreased significantly in response to declining soil VWC ($p < 0.01$, Adj. $R^2_{(c)} = 0.35$ and Adj. $R^2_{(m)} = 0.22$, Fig. 2b), increasing mean aboveground temperature ($p < 0.01$, Adj. $R^2_{(c)} = 0.39$ and Adj. $R^2_{(m)} = 0.23$,

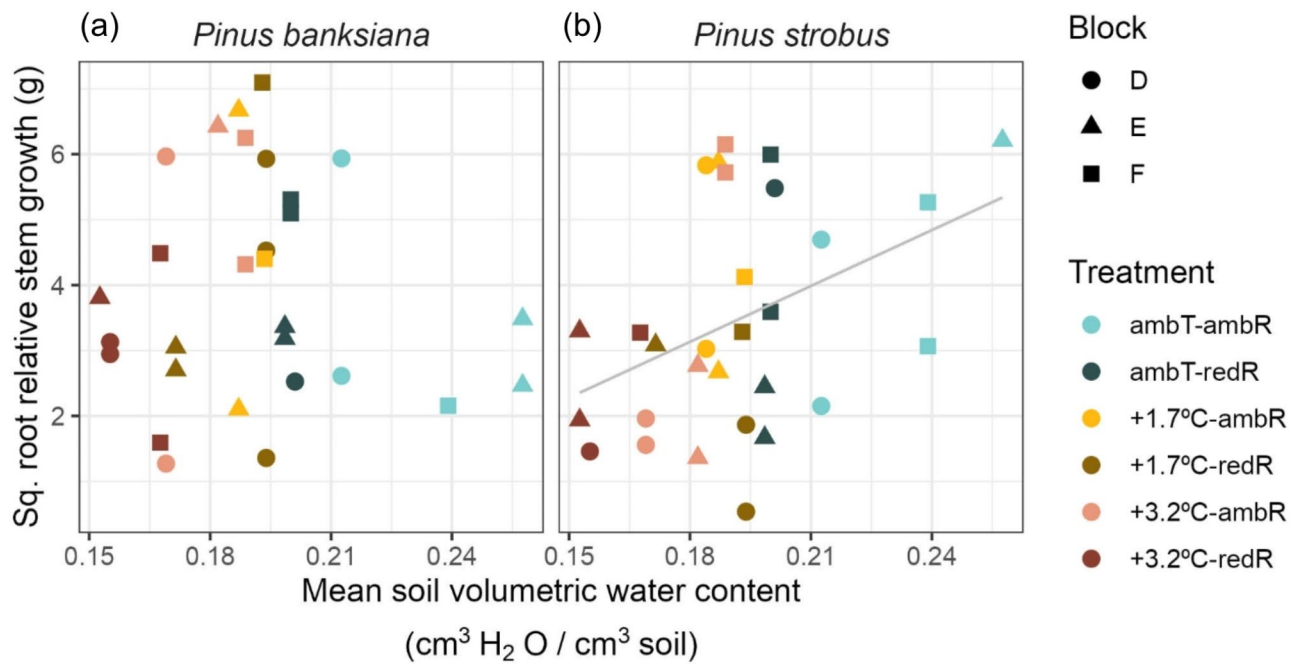


Fig. 1 Responses of square root transformed sapling relative stem growth (final 2020 stem biomass- stem biomass in 2019)/initial stem biomass at start of study) to mean plot soil volumetric water content (VWC) by tree host species (*Pinus banksiana* (a), *Pinus strobus* (b)). Point colors correspond to combined warming and rainfall treatment

type. Point shapes correspond to experimental block. The gray trend line going through points for *P. strobus* (b) depicts the fixed effects model of $\sqrt{\text{relative stem growth}} \sim \text{mean soil VWC}$ ($p=0.03$, Adjusted R-squared=0.13), and does not account for the random effect of block

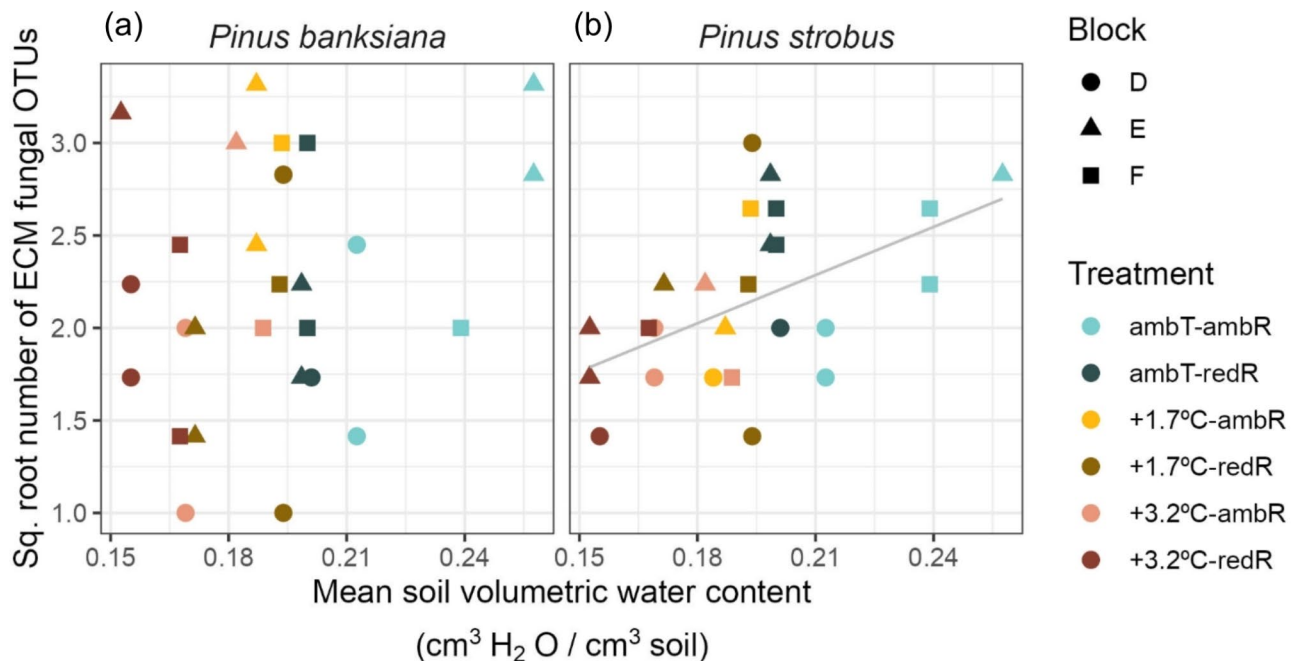


Fig. 2 Responses of the square root transformed number of unique ECM fungal OTUs (ECM fungal OTU richness) to mean plot soil volumetric water content (VWC) by tree host species. Point colors correspond to combined warming and rainfall treatment type. Point shapes correspond to experimental block. The gray trend line going

through points for *P. strobus* (b) depicts the fixed effects model of $\sqrt{\text{number of ECM fungal OTUs}} \sim \text{mean soil VWC}$ ($p<0.01$, Adjusted R-squared=0.22), and does not account for the random effect of block

Supplemental Figure S2), and increasing belowground temperature ($p < 0.01$, Adj. $R^2_{(c)} = 0.39$ and Adj. $R^2_{(m)} = 0.22$, Supplemental Figure S3). However, there was no significant relationship between relative sapling stem growth and ECM fungal OTU richness for either host species.

Ectomycorrhizal fungal community composition

Of the 76 total ECM fungal OTUs, 34 were shared across the two hosts, 16 were shared across the three warming treatments, 33 OTUs were shared across the two rainfall treatments, and 5 OTUs were shared across all 5 combined warming/rainfall reduction treatments. ECM fungal community composition varied significantly by treatment ($F_{5,23} = 1.37$, $p = 0.02$), block ($F_{2,23} = 2.03$, $p < 0.01$), and a block by treatment interaction ($F_{10,23} = 1.54$, $p < 0.01$), but not by tree host species ($p = 0.08$). When the ECM fungal community on each host was analyzed separately, a marginally significant effect of treatment ($F_{5,12} = 1.39$, $p = 0.055$), but not block ($p = 0.24$) was present for *P. banksiana*. For *P. strobus*, there was no significant effect of treatment ($p = 0.41$), but there was a significant effect of block ($F_{2,11} = 1.74$, $p = 0.04$) and a significant block by treatment interaction ($F_{10,11} = 1.56$, $p < 0.01$) effect on ECM fungal community composition. Beta dispersion tests indicated homogeneity of dispersion amongst tree species, treatments, and blocks ($p > 0.05$). A distance-based redundancy analysis including

tree host, soil VWC, aboveground temperature, and belowground temperature indicated significant effects of tree host species ($F_{1,53} = 2.01$, $p < 0.01$), soil VWC ($F_{1,53} = 2.16$, $p < 0.01$), and aboveground temperature ($F_{1,53} = 2.31$, $p < 0.01$) on ECM fungal community composition (Model Adj. $R^2 = 0.06$, $p < 0.01$). Forward and reverse model selection indicated that the best model included tree species ($F_{1,55} = 1.96$, $p < 0.01$), and soil VWC ($F_{1,55} = 2.11$, $p < 0.01$), explaining 6.88% of the variation in ECM fungal community composition (Adj. $R^2 = 0.03$, $p < 0.01$, Supplementary Figure S4). Distance-based redundancy analyses by individual tree host species indicated significant effects of soil VWC ($F_{1,25} = 2.31$, $p < 0.01$) and aboveground temperature ($F_{1,25} = 1.98$, $p < 0.01$) on ECM fungal community composition of *P. banksiana* (Adj. $R^2 = 0.08$, $p < 0.01$, see Fig. 3a). Forward and reverse model selection indicated that a model with soil VWC alone ($F_{1,27} = 2.23$, $p < 0.01$) was the best predictor of *P. banksiana* ECM fungal community composition, explaining 7.62% of the variation (Adj. $R^2 = 0.04$, $p < 0.01$). The distance-based redundancy analysis model for *P. strobus* was not significant (Fig. 3b).

Of the 16 ECM fungal families present in the dataset, the top 10 (each comprising 1% or greater relative abundance) were: Inocybaceae (21.48%), Russulaceae (15.83%), Sebacinaceae (15.71%), Pyrenomataceae (12.71%), Thelephoraceae (10.90%), Gloniaceae (8.84%), Tylosporaceae (6.74%), Tuberaceae (4.63%), Hydangiaceae (1.40%),

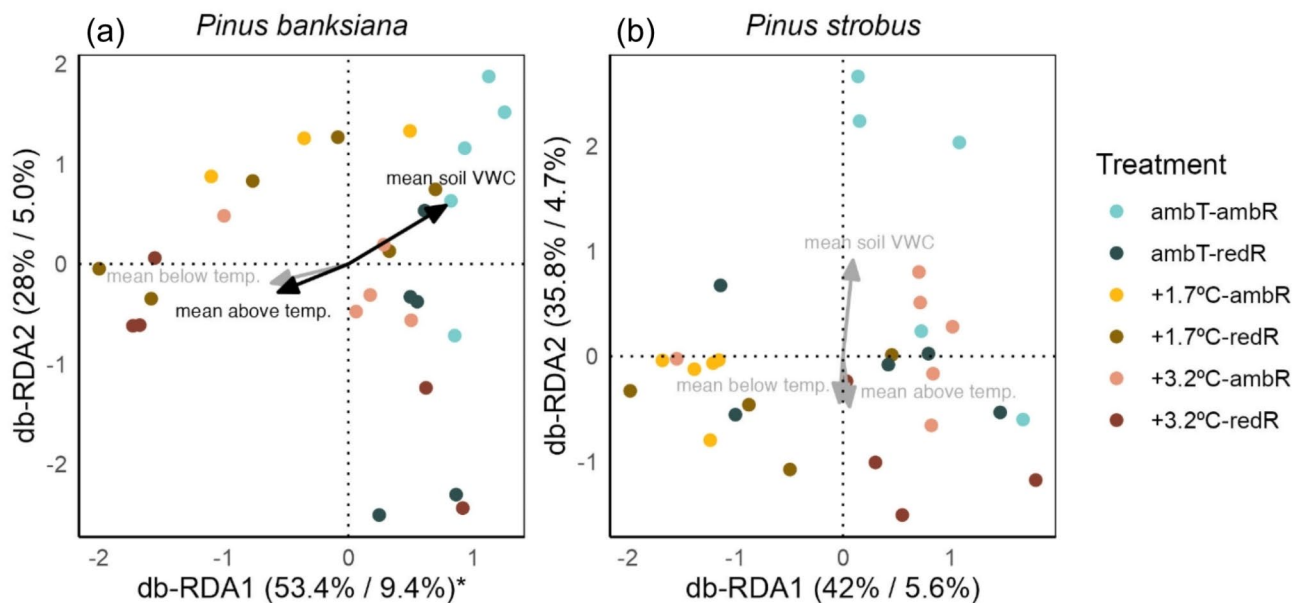


Fig. 3 Distance based redundancy analysis biplots by tree species based on the normalized Bray-Curtis dissimilarity matrix of the ECM fungal community data, constrained to mean plot soil volumetric water content (mean soil VWC), mean aboveground temperature (mean above temp.), and mean belowground temperature (mean below temp.). Point colors correspond to combined warming and rainfall treatment type. Axis titles include the relative contribution of each axis to the total

constrained proportion, and the relative contribution of each axis to the total inertia, respectively. Asterisks indicate axis significance ($p < 0.05$). Black arrows with black text indicate significant variables ($p < 0.05$) while gray arrows with gray text indicate variables that were not significant in each model ($p > 0.05$). The model for *P. banksiana* (a) was statistically significant ($p < 0.05$) and the model for *P. strobus* (b) was not significant ($p > 0.05$)

and Clavulinaceae (1.12%) for *P. banksiana*, and Suillaceae (26.78%), Thelephoraceae (16.22%), Tylosporaceae (14.25%), Russulaceae (10.60%), Sebacinaceae (9.00%), Inocybaceae (8.08%), Tuberaceae (4.23%), Hydnangiaceae (3.01%), Pyronemataceae (2.73%), and Clavulinaceae (2.10%) for *P. strobus* (see Fig. 4). Of the 22 ECM fungal genera present, the top 10 (each comprising 1% or greater relative abundance) were: *Inocybe* (20.96%), *Sebacina* (14.19%), *Wilcoxina* (12.71%), *Tomentella* (10.90%), *Lactarius* (9.29%), *Cenococcum* (8.84%), *Amphinema* (6.74%), *Russula* (6.53%), *Tuber* (4.63%), and *Helvellosebacina* (1.51%) for *P. banksiana*, and *Suillus* (26.78%), *Tomentella* (16.22%), *Amphinema* (14.25%), *Sebacina* (8.40%), *Inocybe* (8.03%), *Russula* (7.00%), *Tuber* (4.23%), *Lactarius*

(3.60%), *Laccaria* (3.01%), and *Wilcoxina* (2.72%) for *P. strobus*. Two ECM fungal genera, *Inocybe* and *Tomentella*, had significant trends across treatments on *P. banksiana* saplings. The mean relative abundance of *Inocybe* on *P. banksiana* significantly increased with declining soil VWC ($p=0.02$, Adj. $R^2=0.71$, Fig. 5a), but did not significantly respond to aboveground or belowground temperature ($p=0.12$ and 0.13 , respectively). Conversely, the mean relative abundance of *Tomentella* on *P. banksiana* significantly decreased with declining soil VWC ($p<0.01$, Adj. $R^2=0.95$, Fig. 5b), increasing aboveground temperature ($p=0.01$, Adj. $R^2=0.79$, Supplementary Figure S5), and increasing belowground temperature ($p=0.02$, Adj.

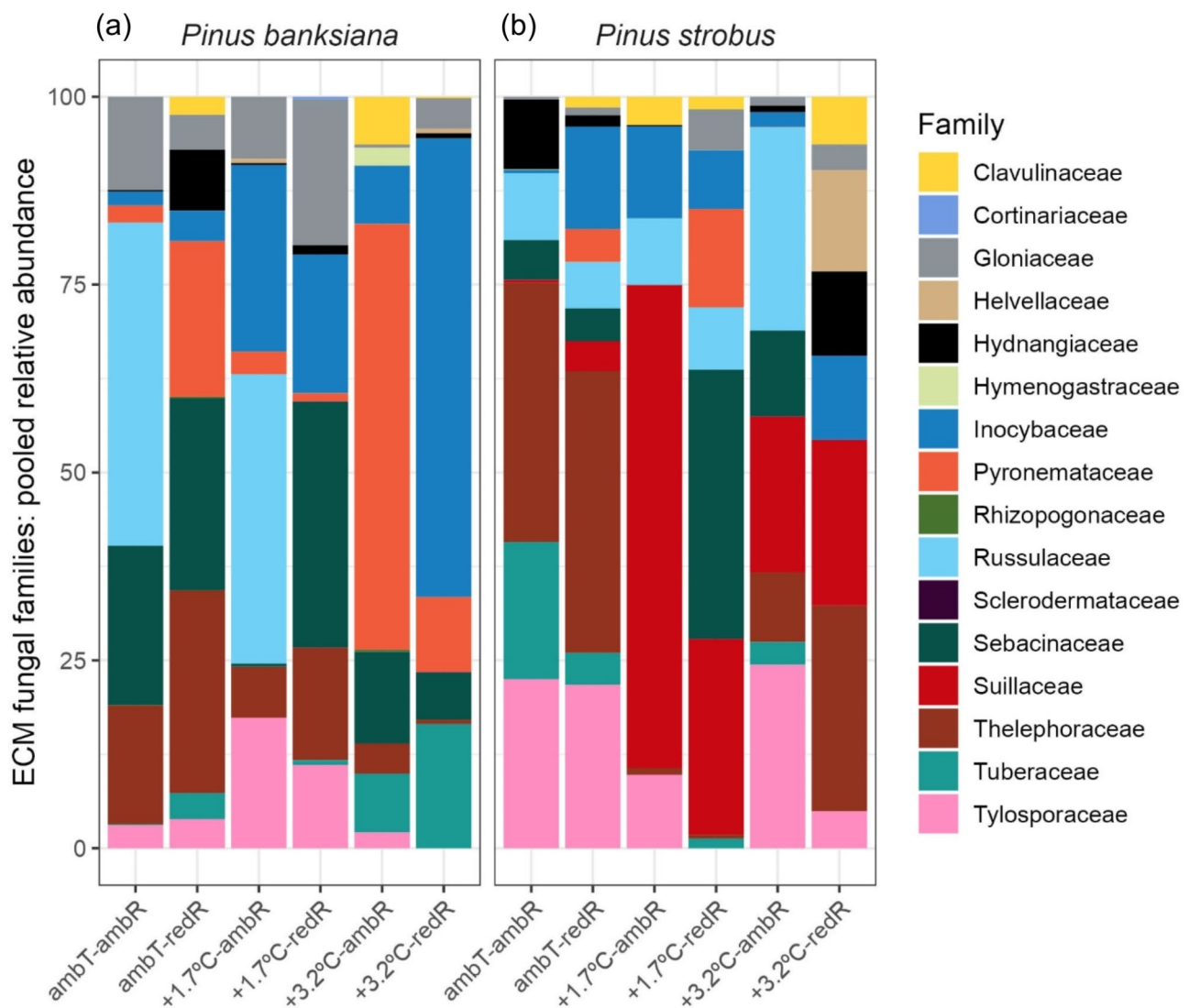


Fig. 4 Stacked bar plot of the relative abundance of each ECM fungal family pooled by tree host species and treatment type. Colors correspond to ECM fungal families

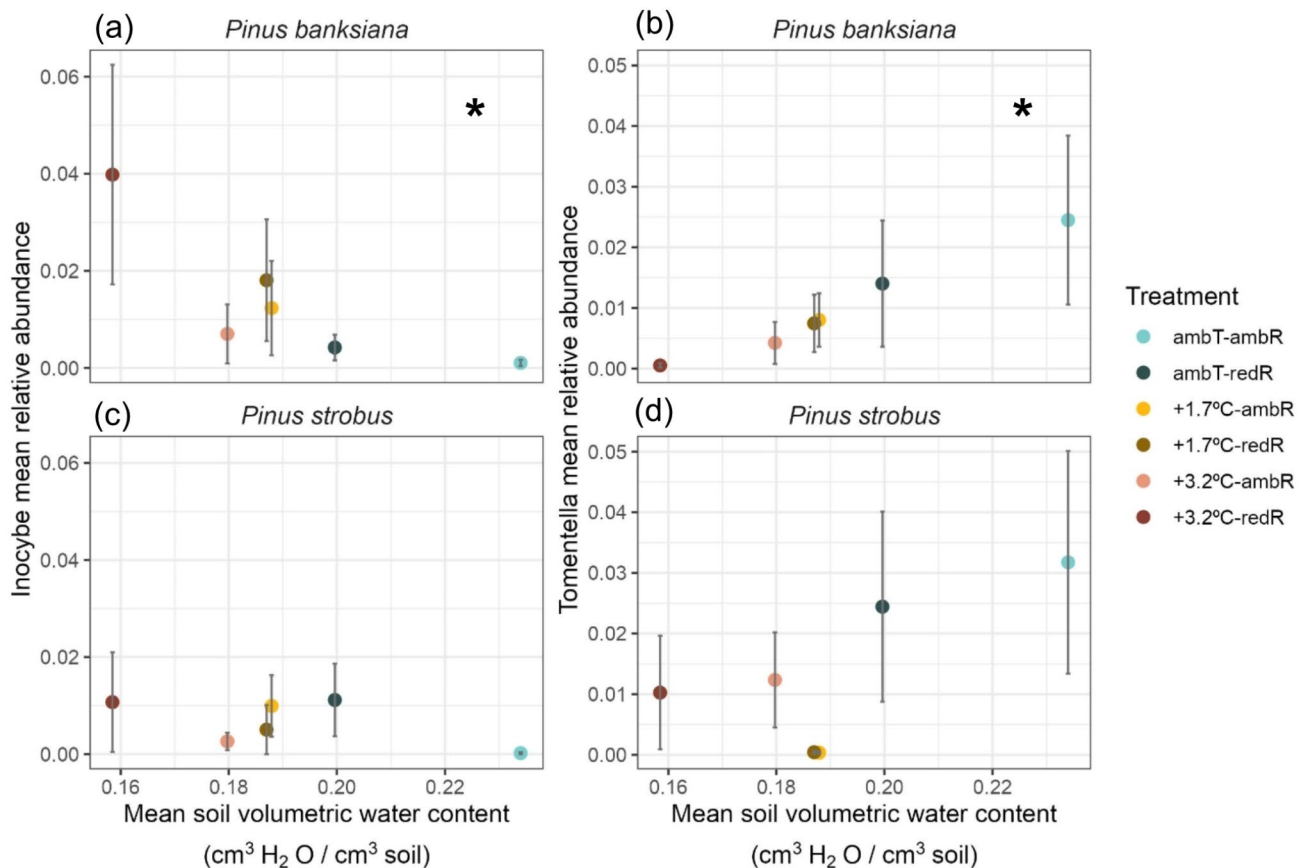


Fig. 5 Responses of the mean relative abundance of *Inocybe* (left) and *Tomentella* (right) on *P. banksiana* and *P. strobus* to mean soil volumetric water content. Point colors correspond to combined warming and rainfall treatment type. Standard error bars are displayed in dark gray. Asterisks indicate significant relationships for *Inocybe* on

P. banksiana (a, $p=0.02$, Adjusted R-squared=0.71 for square root transformed relative abundance) and *Tomentella* on *P. banksiana* (b, $p<0.01$, Adjusted R-squared=0.95 for square root transformed relative abundance)

$R^2=0.75$, Supplementary Figure S6). No significant genus level trends were observed for *P. strobus*.

Discussion

ECM fungal communities in boreal forests are amongst the most diverse globally (Tedersoo et al. 2012), yet increasingly exposed to rapidly changing climates (Ruckstuhl et al. 2008). Understanding fungal responses to warming and reduced soil water availability under realistic future climate scenarios is thus essential, yet few studies have investigated how combined global change stressors impact ECM fungal community richness and composition. Our study demonstrates that combined warming and soil water deficit can significantly affect ECM fungal community structure, but that responses vary by tree host. In support of our hypothesis, we observed a positive relationship between ECM fungal richness (number of unique OTUs) and soil VWC for the less drought tolerant *P. strobus*, indicating fewer OTUs

under increasingly drier and warmer conditions. In contrast to our expectation about community composition, however, we observed that the ECM fungal community composition on the more drought tolerant *P. banksiana* varied significantly in response to decreasing soil VWC.

The observed negative effect of soil drying (due to experimental warming and rainfall reduction) on ECM fungal richness of the less drought tolerant *P. strobus* was consistent with previous studies documenting declines in ECM fungal richness in response to long-term warming in Alaskan tundra (Geml et al. 2015) and combined long-term warming and drought in the southwestern U.S. (Gehring et al. 2020), as well as model predictions of decreasing ECM fungal diversity with future warming at the temperate-boreal ecotone (Steidinger et al. 2020). At the same time, the lack of a similar negative response for *P. banksiana* matches other studies that have documented no significant reduction in ECM fungal richness due to either warming (Fernandez et al. 2017; Mucha et al. 2018), rainfall reduction (Richard et al. 2011), or warming and rainfall reduction combined

(Fernandez et al. 2023). Taken together, these mixed results suggest that predicting ECM fungal richness responses to shifting climatic conditions will be challenging, and likely dependent on both ECM fungal community composition as well as host performance.

Interestingly, in the case of *P. strobus*, the lack of shift in ECM fungal community composition with declining soil VWC suggests that the decline in ECM fungal richness reflects a general loss of ECM fungal species rather than a loss from any one specific taxonomic group. While Gehring et al. (2020) observed a positive association between shoot growth and ECM fungal Shannon diversity, we did not observe a clear link between ECM fungal richness and *P. strobus* host stem growth. This lack of relationship may be in part due to a temporal mismatch in the measurements (i.e. growth measured in the fall and the ECM fungal community measured the following spring), but this explanation could be impacted by ECM fungal community shifts from fall to spring, which we did not measure (see Štursová et al. 2020; Koide et al. 2007 for differences in soil fungal communities by season). Further, shoot growth is likely more indicative of photosynthate availability for ECM fungal symbionts than stem growth, as it includes leaf biomass. Looking forward, we recommend investigating whether tree host drought tolerance metrics such as stomatal conductance or hydraulic safety margin (Anderegg et al. 2016; McDowell et al. 2008; Meinzer et al. 2009) are predictive of ECM fungal richness to potentially better connect host and fungal responses to shifting environmental conditions. For example, stricter regulation of stomatal conductance by drought-tolerant trees might decrease carbon available for their ECM fungal partners, potentially reducing the relative abundance of ECM fungal taxa that are considered to be of higher carbon demand (Fernandez et al. 2017, 2023; McDowell et al. 2008; Pickles et al. 2012).

The shift in composition of the ECM fungal communities associated with *P. banksiana* due to soil drying was consistent with previous studies that indicate significant effects of warming and rainfall reduction on ECM fungal community composition of roots at the B4WarmED experiment (Fernandez et al. 2017, 2023; Mucha et al. 2018) as well as other locations (Gehring et al. 2020; Kwatcho Kengdo et al. 2022). Previous studies have shown that ECM ascomycete fungi are typically favored under drier conditions (Allison and Treseder 2008), including at the B4WarmED experiment (Fernandez et al. 2017, 2023), but here we found no significant increase in the abundance of *Cenococcum*, *Tuber*, or *Wilcoxina*, which were among the dominant ECM fungal genera on *P. strobus* and *P. banksiana*. The variation in *P. banksiana* ECM fungal composition was significantly explained by mean plot-level soil VWC and aboveground temperature, which affected different ECM fungal

genera differently. Specifically, we observed that *Inocybe* relative abundance significantly increased with decreasing soil VWC (Fig. 5a) while *Tomentella* relative abundance declined significantly with decreasing soil VWC (Fig. 5b) and increasing above and belowground temperature for *P. banksiana*. Previous studies at B4WarmED have observed community shifts toward higher relative abundance of *Inocybe* under +3.4 °C warming (Fernandez et al. 2017) as well as +3.1 warming combined with reduced rainfall (Fernandez et al. 2023). Conversely, Mediterranean studies indicate a decrease in *Inocybe* taxa abundance in *Pinus pinaster* soils under drought conditions (Castaño et al. 2023), as well as lower abundance of *Inocybe hirtella* on *Quercus Ilex* root tips under rainfall reduction (Richard et al. 2011). With respect to sporocarp production, evidence suggests a positive relationship between sporocarp abundance of several species of *Inocybe* and accumulated rainfall from August–November in Mediterranean *P. pinaster* forests (Gassibe et al. 2015). Though observations from these drier/seasonally dry ecosystems are opposite from our findings for *Inocybe* on *Pinus banksiana*, they demonstrate the potential for the abundance of this ECM fungal genus to vary with soil moisture. The observed decrease in *Tomentella* abundance under drier soil conditions is consistent with the observed absence of *Tomentella* on *P. edulis* roots by Gehring et al. (2020) under combined warming and drought, compared to the control, warming-only, and drought-only treatments in their New Mexico, U.S. study.

The absence of similar compositional shifts in the ECM fungal communities associated with *P. strobus* under drier conditions was surprising given the strong relationship between soil drying and ECM fungal richness on that host. We speculate this may be the result of the greater carbon limitation under warmer and drier conditions experienced by *P. strobus* (as evidenced by the decline in stem growth), which would likely minimize the ability of this host to differentially invest in members of its ECM fungal community that may provide benefit under drier conditions (Shi et al. 2002). Closer analysis of the significant treatment by block interaction for *P. strobus* ECM fungal community composition revealed that blocks D and E appeared to differ in community composition by treatment, but not block F (though treatment sample sizes after data quality control also differed slightly by block, see Supplementary Figure S7). Further investigation would be needed to determine whether factors such as neighboring plant community composition or differences in edaphic conditions may explain this block variation. Given the different responses across hosts in this study as well as variation across studies, further research is needed to understand how differences in ecosystem type (e.g. semi-arid or seasonally dry versus mesic) as well as the magnitude of warming and water stress may influence

ECM fungal community compositional responses to climate manipulations.

We acknowledge there are multiple limitations to our study. The first is that the ECM fungal richness patterns observed may have been influenced by potential differences in mycorrhizal colonization. Root samples were observed under a dissecting microscope to confirm evidence of ECM colonization, but not specifically scored for percent colonization. Previous studies have found lower ECM fungal colonization with drought, so the decline in ECM fungal richness we observed on *P. strobus* at lower soil moisture levels may result from lower total colonization (Gehring et al. 2020; Kennedy and Peay 2007; Swaty et al. 2004). Additionally, the two *Pinus* species included in this study differ in their geographic ranges, with *P. strobus* having a temperate range, and *P. banksiana* having a boreal range (Burns and Honkala 1990a, b; Reich et al. 2015). It is possible that the poor host performance and observed decline in ECM fungal richness on *P. strobus* in drier soils may in part be due to inadequate soil conditions present at the northern/boreal limit of this host's range, as observed in previous research studies (Carteron et al. 2020; Solarik et al. 2020). Finally, we recognize that we only have two hosts that differ in their drought tolerance, and testing other hosts with greater differences in tolerance will be important in better linking host drought strategy with ECM fungal community responses to changing climatic conditions. Despite these limitations, we believe this study provides important new insight about tree-host specific ECM fungal responses to warming and declining soil water availability at the temperate-boreal ecotone.

Conclusions

The present study found that ECM fungal richness on *Pinus strobus* roots declines with warming and decreasing soil VWC, indicating potential negative effects of even moderate warming scenarios on ECM fungal richness. Additionally, ECM fungal community composition on *Pinus banksiana* significantly shifted in response to declining soil VWC, with overall increases and decreases in the relative abundances of particular ECM fungal genera. Given that the ECM fungal communities of these two co-occurring hosts differed in their climate responses even at the scale of 3-meter diameter plots, the projected changes in forest tree species performance and composition at the temperate-boreal ecotone due to climate change (Frelich et al. 2021, 2024; Ruckstuhl et al. 2008) will likely have cascading effects belowground on ECM fungal diversity.

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Author contributions D.J.N., P.B.R., and P.G.K. designed the research study. D.J.N., A.S. and R.B. performed data collection. A.S. and R.B. harvested saplings and collected climate and sapling growth data. D.J.N. processed sapling roots in the lab and performed DNA extractions, PCR, and DNA library preparation for all samples. D.J.N. performed data analysis with guidance from K.V.B. and P.G.K. D.J.N. wrote the first draft of the manuscript. All authors commented on previous manuscript drafts, and approved the final submitted version.

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Data availability Raw sequences and associated metadata were deposited in the National Center for Biotechnology Information (NCBI) Short Read Archive under Bioproject ID #: PRJNA1079312. The OTU table analyzed in this manuscript, along with the corresponding temperature, soil volumetric water content, and sapling growth data are provided as supplementary material.

Declarations

Competing interests The authors declare no competing interests.

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