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Field fungi & their impacts on seedling performance: Examples of the symbiotic spectrum

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By

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ABSTRACT

Field fungi & their impacts on seedling performance: Examples of the symbiotic spectrum

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Ectomycorrhizal association is a prevalent symbiosis that plays a crucial role in supporting temperate and boreal forest ecosystems through the provisioning of fungal foraged nutrients for plant-derived carbon. The stability of this ecological relationship can be viewed as heavily dependent on environmental constraints and resource dynamics. To test how associations with distinct fungal communities impact the performance of *Pinus muricata* seedlings growing under controlled greenhouse conditions, we inoculated seedlings with ectomycorrhizal fungi using field-collected soil from the Jug Handle State Reserve Ecological Staircase in Mendocino, California. We collected field soils from two terraces which have been previously described to have distinct fungal communities, and established four inoculum treatments: one from Terrace 2 (T2), a relatively resource-replete site with tall trees and low carbon-demand fungi, one from Terrace 3 (T3), a comparatively resource-deplete site with dwarfed "pygmy" trees and high carbon-demand fungi, one with both T2 and T3 inoculum, and one control with neither. To track seedling performance under the four inoculum treatments, we performed aboveground measurements on all seedlings for a total of nine months and harvested plants in month 9 (end-of-experiment). Differences between inoculation treatments became more pronounced over time, with uninoculated control seedlings generally maintaining greater height, diameter, and growth rates relative to the inoculated treatments. Seedlings with high-resource (T2) inoculum showed greater representation of *Byssocorticium* and *Rhizopogon occidentalis* in their fungal communities,

whereas under the same environment with low-resource (T3) inoculum, *Wilcokina mikolae* was often dominant. We found that having greater mycorrhization or diversity of fungi did not correlate with increased growth – and having any fungi, regardless of abundance on the root system, led to decreased seedling performance and growth. Our results indicate that reciprocal exchanges of nutrients and carbon are not invariably regulated by mutual benefit in ectomycorrhizal symbioses.

Introduction

Ectomycorrhizal systems, a type of multi-species plant-fungal symbiosis where plants exchange photosynthetically-derived carbon for fungal-acquired nutrients through intercellular root mycorrhization (Smith & Read 1997), are remarkably diverse and dominant in boreal, temperate, and Mediterranean forest ecosystems (Martin and Van Der Heijden 2024). Approximately 6,000 plant species form associations with ectomycorrhizal fungi (Heijden et al. 2015), including economically important timber-producing tree species like *Pseudotsuga menziesii* and range-restricted, threatened species like *Pinus muricata*. They are functionally important for enhancing plant productivity and fundamental for the cycling of nutrients within ecosystems (Pellitier & Donald 2018). Despite their ubiquity and significance in biogeochemical cycles, there is still much to be known about the dynamics of the symbiotic relationship between plants and their ectomycorrhizal fungal symbionts, especially regarding how these interactions shift along the mutualism-parasitism continuum.

These multi-species symbioses are widely considered mutualistic, though the dynamics of resource partitioning can vary considerably under different environmental contexts, rendering the mutualism conditional. Fungal acquisition strategies can considerably enhance plant performance under a variety of stressful conditions (Alrajhei et al. 2022) where the plant's fitness benefit exceeds the carbon investment required to maintain fungal partners. Furthermore, the carbon allocated to fungal partners is a worthwhile investment as nutrient delivery offsets energetic costs to the plant when nutrients that are locked in complex organic forms can only be made accessible from ectomycorrhizal partners, and such is the case in boreal and temperate coniferous forests where these partnerships are dominant (Smith & Read 2008). In nutrient-rich environments, however, where plants can often obtain sufficient

nutrients without assistance from their fungal partners, fungi may not confer much benefit. Since the fungal association still has a carbon cost to the plant, the symbiosis shifts towards parasitism (Johnson et al. 1997).

The exact cost of carbon can depend on fungal identity, with evidence showing that different taxa have different nutrient acquisition strategies, carbon demands, and overall functional traits (Peay et al. 2010). Hyphal development and rhizomorph structure in ectomycorrhizal fungi may be related to their ability to explore different distances, and these differences correlate with modes of mineral nutrient acquisition (Hobbie & Agerer 2010). Long-distance exploration types and rhizomorph structures require more carbon per unit nitrogen due to greater belowground structure building, which may lessen the benefit-to-carbon cost ratio of the symbiosis for the plant. In contrast, short-range exploration types would be an effective strategy in maintaining a smaller carbon expenditure, especially as plant hosts develop and roots become denser (Peay et al. 2011). These varied physiological profiles can alter where the pendulum swings in the mutualism-parasitism continuum, with higher carbon-demanding fungi in environments where plants unable to support that higher cost-to-benefit ratio may collapse/shift into a parasitism.

This difference in cost-benefit relationship among fungal taxa may explain their varied distribution on the landscape scale, with high-cost, high-foraging fungi found more frequently in low-resource environments (Bui et al. 2020). For example, ectomycorrhizal communities have been observed to shift towards more stress-tolerant functional traits (melanization, rhizomorph formation, long-distance hyphae) in arid environments, which require high carbon allocation from the plant for substantial water uptake and nutrient acquisition (Bui et al. 2020). Under these conditions, the benefit that the fungi provide may

outweigh the carbon costs, which allows these fungi to remain largely mutualistic because they improve host survival and performance in stressful environments. The observed selection for or against specific fungal traits and strategies is seen along environmental gradients, where mycorrhizal community composition and foraging strategies are known to vary as nutrient and water availability changes (Moeller et al. 2014). Particularly, trees under more stressful conditions along landscape gradients host fungi with more carbon-intensive foraging strategies, like rhizomorph formation, long-distance foraging, and hydrophobic hyphae. By understanding how the abiotic and biotic factors play into the distribution of fungi across a heterogeneous landscape for a single host, we can provide insight into the relationships between resource availability, plant community function, and fungal identity and their associated carbon costs.

Here, we tested whether fungal symbionts from high-resource and low-resource environments vary in their carbon demands on seedlings by inoculating Bishop pine *(Pinus muricata)* with fungi from different field environments at the Ecological Staircase Jug Handle State Reserve (JHSR) in Mendocino California. This region is a landscape comprised of a series of marine-derived terraces with soil ages ranging from 100,000-500,000 years, where the most fertile and diverse forests exist on the younger terraces, and the most nutrient poor and pygmy forests occur on older terraces (see Westman and Whittaker, 1975, for a detailed site description). As nutrients leach throughout time, the aged soils form terraces with a water-impermeable iron-hardpan, which results in the dwarfing of endemic pine species, including *P. muricata*. Despite drastic edaphic conditions, *P. muricata* grows on each of the five terraces, yet the mycorrhizal communities differ. The edaphic variation along this striking Northern California coast chronosequence allows us to target these bigger picture

questions using a single tree species, *P. muricata*, and their associated mycorrhizal communities. We conducted a 9-month greenhouse experiment that sought to identify changes in *P. muricata*-ectomycorrhizal seedling performance and mycorrhization patterns when grown with inoculum representing high-resource, low-resource, and dual fungal communities at JHSR. By examining these plant-fungal symbiosis dynamics, we aimed to link variation in fungal traits and carbon demands on seedlings with the distribution of fungi across the chronosequence. We tested whether seedling performance is greater in high-resource inoculum using metrics like seedling height, biomass, greenness, diameter, mycorrhization rates, and foliar nutrient content. We predicted that fungal communities from high-resource soils should have a lower carbon cost and therefore have greater potential for mutualistic interactions with *P. muricata* seedlings. We uncovered how the non-fixed nature of this dynamic symbiosis (resource exchange regulation) underpins the symbiosis as a form of conditional mutualism.

Materials and Methods

Experimental Design

To test how associations with distinct fungal communities impact the performance of *Pinus muricata* seedlings, we inoculated seedlings with ectomycorrhizal fungi using field-collected soil. We collected soil from the Jug Handle Ecological Staircase in Mendocino County, California, USA (39°22'N, 123°47'W, Figure 1), where the fungal community is known to vary based on soil condition (Moeller et al. 2014). To generate two distinct fungal communities, we collected soils from Terrace 2 ("high-resource" hereafter; a relatively resource-replete site with tall trees and low carbon-demand fungi, Moeller et al. 2014) and

Terrace 3 ("low-resource" hereafter; a comparatively resource-deplete site with dwarfed "pygmy" trees and high carbon-demand fungi, Moeller et al. 2014). On each terrace, we identified three locations that were surrounded by *P. muricata* trees, and collected 2.5 liters of soil from each location by scraping away the litter layer and digging out soil to a maximum depth of 25 cm. Collected soils were stored in ziplock bags at 4 °C for four weeks, at which point they were passed through a 4 mm sieve and pooled by terrace.

We used these field soils to create three inoculation treatments: high-resource (T2 inoculum), low-resource (T3 inoculum), and dual inoculation fungal communities. The single-terrace inoculations contained 10% field soil, and the dual inoculation contained 20% field soil (10% from high-resource, 10% from low-resource). The remainder of soil was an autoclaved greenhouse mix made from vermiculite, peat, grow mulch/compost, perlite, and horticultural sand (in a 2:1:1:1:0.5 ratio). A fourth control treatment contained 100% autoclaved greenhouse mix.

Meanwhile, we germinated *P. muricata* seedlings from seed (Sheffield's Seed Company, Locke, NY) to serve as hosts for fungal communities. Seeds were soaked overnight in water and then sown in twice-autoclaved potting mixture comprised of 6 parts peat : 3 parts pumice : 1 part sand. The potting mixture was allowed to cool overnight between autoclave sessions. The seeds were then cold-stratified for one month at 4 °C before germination at room temperature in the University of California, Santa Barbara (UCSB) greenhouse. Germinated seeds were then planted into bleach-sterilized pots containing one of the four inoculum treatments. All seedlings were checked for moisture three times weekly, and when dry they were watered with deionized water to saturation.

To track seedling performance, we performed aboveground measurements on all seedlings once a month for a total of nine months. At each timepoint, seedlings were measured for height, basal stem diameter, branch count and length, and greenness. Greenness refers to a foliar color scale ranging from 1-5, where 1 indicates yellow or chlorotic needles representing light or nutrient stress, and 5 indicates dark green needles, or otherwise apparently healthy seedlings.

Harvest

Nine months after the start of the experiment, seedlings from each treatment were destructively harvested. Here, fungal mycorrhization rates, biomass, fungal communities, and soil nutrient data were measured.

Fungal identification

To measure fungal mycorrhization rates, seedling root systems were rinsed with tap water and placed under a dissecting microscope, and uncolonized and colonized root tips were counted using a multi-digit clicker device. Root tips with fungi were first categorized into distinct morphologies, and then used to guide the sampling approach for characterizing fungal communities with Sanger sequencing. To identify the ectomycorrhizae present, we observed 5 distinct morphotypes across all seedlings and sampled them on random seedlings from each treatment. We did this by dissecting small (sizes anywhere between a 2-4mm) portions of root tips and placing them in 10 uL of Sigma-Aldrich Extraction Solution (Sigma-Aldrich Co. LLC, St. Louis, MO; Product no. E7526) in a 96-well plate. Samples were then subject to an extraction process following manufacturer's instructions at 65℃ for

10 minutes and 95℃ for 10 minutes before being given 10uL of Sigma-Aldrich Neutralization Solution B (Sigma-Aldrich Co. LLC, St. Louis, MO; Product no. N3910). Extracted DNA samples were then stored at -20 °C until amplification and sequencing.

To characterize the fungal communities, we targeted the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA genes on the root tips. Extracted samples were diluted with nuclease-free water (1 part DNA extract : 10 parts water) and amplified using a mixture of Promega GoTaq Green Mastermix (Promega Corp., Madison, WI), ITS1-F (Gardes & Bruns 1993) and ITS4 (White et al. 1990) primers. Samples were then sequenced at Molecular Cloning Laboratories (South San Francisco, CA USA). All subsequent sequence data analyses were done using Geneious (Biomatters Inc., Auckland NZ), a bioinformatics software program. Returned sequencing products were trimmed and clustered into contigs, where the consensus sequences determined from the contig assembly were used to assign taxonomic groups of fungi. For samples that did not cluster, we manually trimmed the sequences and assigned taxonomy using the Basic Local Alignment And Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov).

Using the UNITE database (Nilsson et al. 2019), taxonomic assignments were generated through the assignTaxonomy() function from the *dada2* package in R and the ectomycorrhizal ecological guild was parsed through FUNGuild assignments (Nguyen et al. 2016). For tips that did not successfully amplify or returned as poor quality sequences, additional rounds of PCR and amplicons were prepared for sequencing. These new attempts targeted the morphotypes corresponding to unsuccessfully amplified root tips, using samples from the same or different seedlings within the relevant treatment group. For unsequenced tips, we assigned taxonomy through parsimonious analysis of sequenced tips and raw total

mycorrhization counts at the individual seedling level. To do this, we took known sequencing data on morphotypes, assessed quality and proportion of sequences, and projected sequenced taxa to each tip within a given morphotype.

Foliar and soil nutrient analysis

Following root tip morphotyping and sample collection, above-ground foliar (needle), above-ground woody (stem), and below-ground root tissue were subsequently packed into coin envelopes and dried at 60℃ for 72 hours before weighing to obtain biomass. Soil samples were prepared by pooling two randomly selected seedlings from each treatment group until there were three sets per treatment, each with a minimum of 0.5 lbs, yielding 24 total samples (3 sets x 2 pooled seedlings x 8 treatments). All 24 soil samples were placed in labelled ziplock bags and sent to Brookside Laboratories (New Bremen, OH USA; catalog no. AG11, S001, and S202) for their sample preparation, standard soil, and Carbon:Nitrogen ratio nutrient analyses.

In addition to these measurements, foliar nutrient analyses were performed. For each inoculum treatment, seven randomly-selected seedlings were dried for a second time at 60℃ for 24 hours, and funneled into plastic scintillation vials that were prepared with small metal rods inside. Each vial was placed inside large plastic cylindrical containers and compressed with paper towels such that the potential for movement was minimized once inside. We then placed the cylindrical containers on a roller mill for 48 hours to achieve full pulverization of foliar tissue. After 48 hours, the samples were fully reduced to a powder and the metal rods were removed. The vials containing the powdered needles were packaged and sent for

carbon, hydrogen, and nitrogen analysis using an elemental analyzer at the Marine Science Institute Analytical Lab (Santa Barbara, CA USA).

Statistical Analysis

All statistical analyses were performed in R (v.4.4.2) in RStudio (RStudio Team 2021). Any p-values that were at or below 0.05 were considered to be significant across all tests. Plots were produced with *ggplot2* (Wickham, 2016). Differences among inoculation treatments were assessed using an analysis of variance (ANOVA). After ensuring that the assumptions for normality and equal variances were met, post-hoc pairwise comparisons were taken using Tukey's honestly significant differences (Tukey-HSD). In the event that the data violated one or both assumptions, a Kruskal-Wallis test was performed followed by a Dunn's pairwise comparison test, which was used to either validate or replace ANOVA results represented as significance letters on plots.

To visualize fungal community differences, a non-metric multidimensional scaling (NMDS) ordination was used with a PERMANOVA statistical analysis in the package *vegan* (Oksanen et al. 2008). To assess correlations, we used generalized linear models (GLMs) chosen from the lowest Akaike's Information Criterion (AIC) which represented the best supported models. For these models, the predictor variable was inoculation and mycorrhized tips, proportion root tips mycorrhized, and fungal richness with the response variables as total seedling biomass, aboveground biomass, foliar biomass, percent nitrogen in foliage, and total nitrogen in foliage.

Results

Aboveground seedling performance

Seedling growth increased steadily from the beginning through the middle of the experiment, plateauing around July (approx. five months from the start of the experiment, Fig. S1). Differences between inoculation treatments were minimal early in the experiment and became more pronounced over time, with control seedlings generally maintaining greater height, diameter, and growth rates relative to the inoculated treatments. Furthermore, dual inoculation treatments displayed the lowest values across most metrics, including terminal height, total aboveground stem length (stem + branches), and growth rates (Fig. S1).

Seedling growth rates differed between the inoculation treatments ($p < 0.05$, ANOVA, Fig. 2). Particularly, height growth rates of seedlings were significantly lower in high-resource inoculum and dual-inoculated treatments than that of the control treatment (Fig. 2, $p < 0.05$ and $p < 0.001$, respectively). Dual-inoculation treatment had a lower height growth rate than the low-resource treatment, while the low-resource treatment did not significantly differ from the control. Total aboveground growth rates (stem + branches) showed a similar pattern, where both high-resource ($p < 0.001$) and dual ($p < 0.001$) treatments had substantially reduced growth relative to the control and low-resource treatments, which were both not significantly different from one another.

Diameter growth rate and terminal diameter were significantly reduced in all inoculated treatments relative to the control, with the dual-treatment showing the greatest reduction (Fig. 2, S2). Seedlings, regardless of treatment, had no significant differences in greenness both over time and at the terminal timepoint (Fig. 2, S2). Terminal seedling height and total aboveground stem length differed among treatments, with control seedlings consistently having the longest length and dual-inoculated seedlings the shortest (Fig. S2).

Destructive harvest & Fungal interactions

There were pronounced treatment effects on seedling biomass; namely, aboveground (sum of stem and foliar) biomass was highest in the control seedlings and lowest in dual-inoculated seedlings, with single-inoculation treatments being intermediate (Fig. 3). Foliar biomass showed similar trends. Root biomass and total biomass showed a distinct pattern with control seedlings exceeding all inoculated treatments, whereas no one inoculated treatment showed any significant difference from the other.

Fungal mycorrhization did not vary between single and dual-inoculated treatments (Fig. 4), and only two seedlings from the control treatment (24 replicates) had two instances of fungal presence on the root system throughout the entire experiment, with the rest successfully having no fungi. Fungal species richness differed among inoculation treatments, with lowest values generally in the low-resource inoculum treatment, intermediate values in the high-resource inoculum treatment, and highest within the dual inoculum treatment group. However, pairwise differences were modest and partially overlapping.

When looking at seedling-level community composition, there was variability in relative abundance among seedlings, with communities typically dominated by one or two taxa. High-resource treatment seedlings showed greater representation of *Byssocorticium* and *Rhizopogon occidentalis,* whereas in low-resource treatment seedlings, *Wilcokina mikolae* was often dominant (Fig. S3). Seedlings from the dual-inoculated treatment group exhibited more mixed communities relative to those in the single-inoculated treatments, with representative fungal species from both single-inoculum treatments.

Community composition differed strongly among inoculation treatments ($F = 5.62$, $p < 0.001$, PERMANOVA). An NMDS ordination revealed separation among low-resource and

high-resource treatments for the most part, with overlap in both for the dual treatment (Fig. 5). Species vectors indicated that the differences in community composition were driven primarily by variation in the relative abundance of *W. mikolae* for low-resource treatments, *Byssocorticium* and *R. occidentalis* for high-resource treatments.

The low-resource inoculum showed the lowest percent nitrogen in foliar tissue, where every other treatment group showed no statistical differences from each other (Fig. 6). Total foliar nitrogen content varied significantly, with control and high-resource treatments containing more nitrogen overall than low-resource and dual treatments (Fig. 6). This pattern reflects combined effects of both biomass production and tissue nitrogen content.

There was no strong evidence of interaction effects of mycorrhization and inoculum, meaning that the effect of inoculation did not depend on mycorrhization. None of the metrics for mycorrhization (number of mycorrhized root tips and proportion mycorrhized) or fungal richness were significant predictors of seedling biomass across all metrics (total plant, aboveground, and foliar) in generalized linear models (Fig. S4).

Additionally, inoculation treatment was the main driver of proportional and total nitrogen content, whereas mycorrhizal metrics were largely non-significant and had a slight tendency towards negative relationships. All inoculated treatments significantly reduced total foliar nitrogen with respect to the control treatment (Fig. S5).

Discussion

Our study focuses on understanding how fungal traits might ultimately impact their distribution across a landscape through a 9-month controlled greenhouse experiment for a single host species, *P. muricata*, with four inoculation treatments: single source (either

high-resource or low-resource environments), both environments, or neither as a control. There were consistent effects on seedling growth across multiple metrics where control seedlings outperformed all inoculated treatments. Although plant performance did not markedly differ among inoculation treatments for the first 3 months, over time, interactions between seedlings and their belowground fungal communities increasingly influenced non-destructive measures of growth (measurements in Fig. 2). Specifically, inoculated seedlings in our study experienced distinct effects of reduced growth rates and biomass compared to control seedlings. Negative effects were particularly pronounced in the dual-inoculation treatment, which contained double the amount of soil inoculum, suggesting that combining fungal inoculum sources may have imposed greater carbon costs to the host. This could be due to increased competition when there are multiple symbionts competing for host-derived carbon, potentially creating imbalances in resource exchanges.

This pattern was consistent across height, total aboveground length, diameter, and total biomass. All growth outcomes may have been, to some degree, the product of relatively high nutrient study conditions (Fig. S6), which likely artificially rendered fungal communities unable to assist the seedlings. Work from Näsholm et al. (2013) shows similar outcomes: ectomycorrhizal fungi can exacerbate nitrogen limitation by retaining nutrients within their biomass rather than transferring it to their host trees. In these circumstances, the fungi become a net carbon drain while the plant host still supplies photosynthates to maintain the symbiosis. Fungi shifting towards the parasitic end of the symbiosis spectrum, although not the intended aim of the study, was thus unsurprising given the resource availability and ecologically plastic nature of mycorrhizae (Johnson et al. 1997).

In our experiment, there were slight reductions in plant performance for dual-inoculated seedlings relative to single-inoculation treatments, particularly in diameter growth rate and aboveground biomass. Multi-partner frameworks show that symbionts may face trade-offs between competitive ability and mutualistic quality, implying that a dominant taxon doesn't necessarily mean a beneficial taxon (Moeller and Peay 2016). This dynamic could be responsible for the slight reduction in plant performance we see in dual-inoculum treatment seedlings, where increased competition among fungal partners may have further exacerbated carbon limitation, though we did not see any pronounced differences in fungal richness in this experiment.

Besides treatment effects on plant growth, we asked whether fungal mycorrhization would be greatest in high-resource treatments compared to the other inoculated treatments due to prior work that demonstrates fungi from high-resource (T2) environments generally having lower carbon costs on their host plants (Moeller et al. 2014). We found that fungal mycorrhization did not significantly differ among inoculated treatments and variation in seedling growth could not be explained by the number of root tips colonized, the proportion of mycorrhization, or fungal richness. Essentially, having more or a greater diversity of fungi did not translate into increased growth – and having any fungi, regardless of abundance on the root system, decreased seedling performance and growth. Alternative explanations include possible soil environment changes that were detrimental to seedlings over the course of the experiment (Fig. S6), though other soil variables that might have impacted seedling performance are difficult to disentangle. Another possibility would be that pathogens may have led to the lower seedling performance; although, in a separate experiment, we

inoculated seedlings with field soil and did not see negative effects on seedlings in high soil nutrient environments (data not shown).

Our findings align with the framework proposed by Johnson et al. (1997), in which mycorrhizal symbiosis operates along a mutualism-parasitism continuum depending on environmental context and partner traits. Consistent with this interpretation, other work demonstrates that when hosts have access to alternative resource acquisition strategies via their own roots, or when the cost of maintaining symbionts outweigh the benefits, there is a mutualism breakdown (Werner et al. 2018). Under these nutrient-replete environments, plants have the opportunity to fulfil their own needs without the help from a fungal symbiont, though the fungus may still draw carbon from the plant and result in parasitism. This is relevant to our findings given that there was a lack of a strong nutrient limitation that might have warranted immediate beneficial interactions between the fungi and seedlings.

Although mycorrhization rate did not predict growth, fungal community composition differed strongly among inoculation treatments, which may explain some of the differences observed in plant performance. When observing fungal communities across inoculation treatments, there was relatively clear compositional separation, indicating that inoculum source strongly structured fungal assemblages. These compositional differences were driven by variation in the dominance of key taxa. High-resource inoculum treatments were dominated by *R. occidentalis* and *Byssocorticium,* which have long-distance and short-distance exploration type hyphae, respectively (DEEMY database: http://www.deemy.de/). Along with a lack of rhizomorph formation, *Byssocorticium* produces a short distance body plan, consistent with existing data on fungal body plans in high resource environments at Jug Handle State Reserve (DEEMY database:

http://www.deemy.de/, Moeller et al. 2014). Low-resource environments showed a greater representation of *W. mikolae,* which lack rhizomorphs. The prevalence of certain taxa over others within individual seedlings highlights dominance patterns that may have important functional consequences for host trees, as different ectomycorrhizal fungi may exhibit contrasting strategies in nutrient acquisition and exploration type, thus impacting carbon demand (Peay et al. 2011). Dual-inoculum seedlings exhibited communities that resembled fungi from both single-inoculum treatments, though this coexistence may not have persisted under longer-term experiments due to potential for competitive exclusion or host-mediated selection.

Increased nitrogen uptake did not translate into increased growth, suggesting a distinct imbalance between nutrient acquisition and carbon costs. It's possible that there may have been an imbalance between nutrient acquisition and carbon disbursement, where any possible benefit of the symbiosis was offset by the costs of initiating and supporting the symbionts. However, given that nitrogen was not strongly limiting in our system (Fig. S6), any potential for positive seedling performance was weakened by the short timeframe of the experiment. Work by Horning et al. (2023) on soil resource conditions constraining reciprocal exchange further emphasizes that these interactions are not so tightly regulated by mutual benefit. Further, strategies that low-resource fungi utilize in the field, which make them successful symbionts to plant hosts in those nutrient environments, may not be as beneficial in a small, controlled setting with nutrient homogeneity relative to natural settings. The temporally and spatially fixed organization of this study's greenhouse experiment may also not have been sufficiently long or contained a complex enough soil matrix to warrant the benefits of costly physiological traits like rhizomorph formation or hydrophobicity.

Our results did not support our hypothesis that high-resource inoculum treatment seedlings would have greater potential for mutualistic interactions, that is: high-resource inoculum treatment seedlings would show greater seedling performance and mycorrhization rates compared to the other treatments. In our system, inoculation treatments altered fungal community assemblages and, in general, reduced plant growth and performance compared to control treatments with no mycorrhizae. The fungi in this experiment acted as additional carbon sinks, next to host roots, and displayed signs of parasitism rather than mutualism. This shift emphasizes the inherent duality of symbiotic systems that operate variably under changing ecological conditions. These findings underscore the importance of considering environmental context, fungal identity, and community composition when investigating the nuance of plant-ectomycorrhizal interactions. Future work testing a factorial treatment for soil resource environments and inoculation treatments could explore the mechanisms behind the distribution of functionally different fungi along the Mendocino landscape.

Figures and figure captions

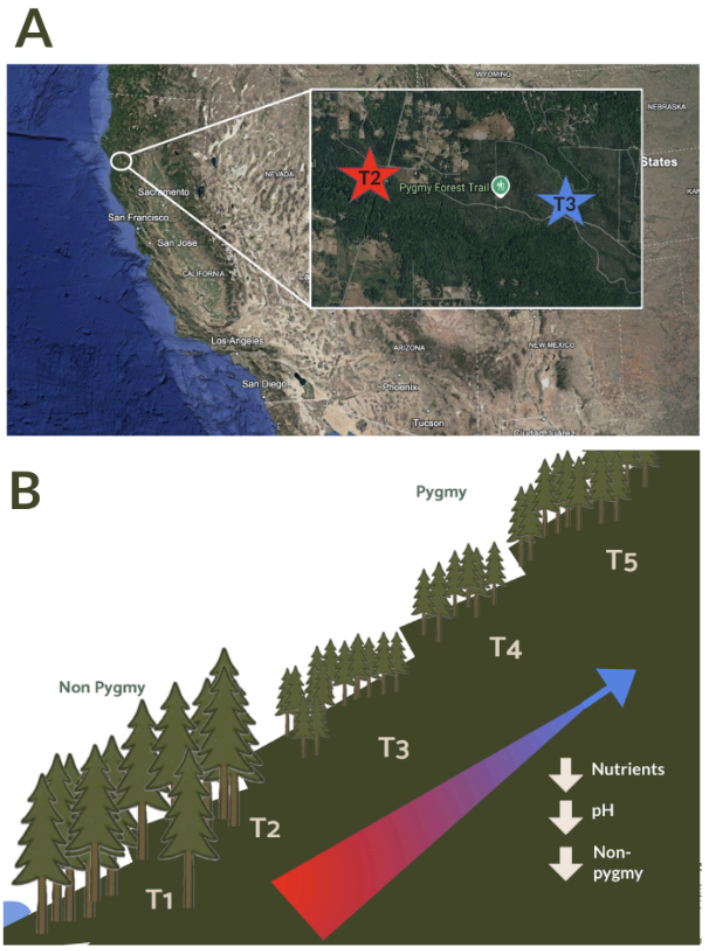

Figure 1: Field site location. Jug Handle State Reserve Ecological Staircase is located on the coastline of Northern California (panel A) and includes five marine-derived terraces that increase in age from the coastline towards inland (panel B). We collected soils from Terrace 2 (high-resource) and Terrace 3 (low-resource), which are marked with stars on the site map and indicated as “T2” and “T3” respectively.

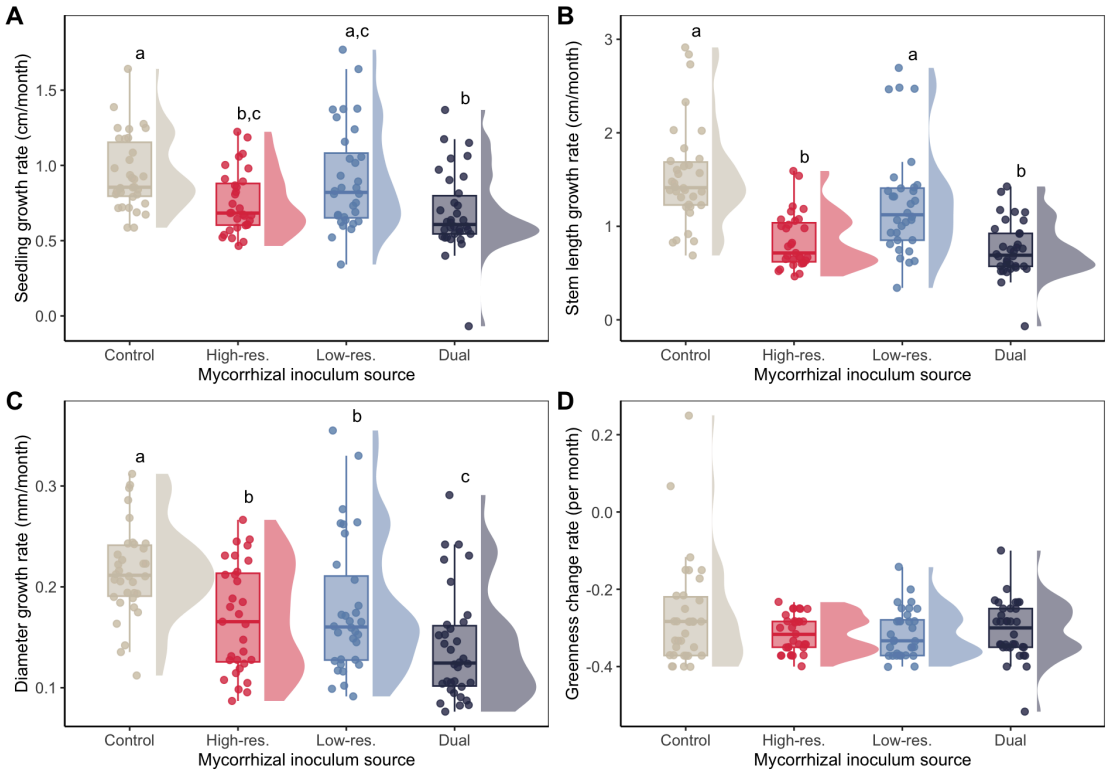

Figure 2: Inoculation with ectomycorrhizal fungi decreased growth in seedlings. Generally, control seedlings grew faster than fungal-inoculated seedlings when growth was measured as (A) aboveground height, (B) aboveground stem length, and (C) diameter at the soil surface. Seedlings that received high-resource inoculum (either alone, or in combination with low-resource inoculum) grew the least. All seedlings exhibited similar decreases in greenness (D) over the course of the experiment. Boxes are interquartile ranges with the horizontal line representing the median, and letters represent statistically significant differences at the $p < 0.05$ level. A two-way ANOVA was performed, followed by a Tukey-HSD test for pairwise differences between groups.

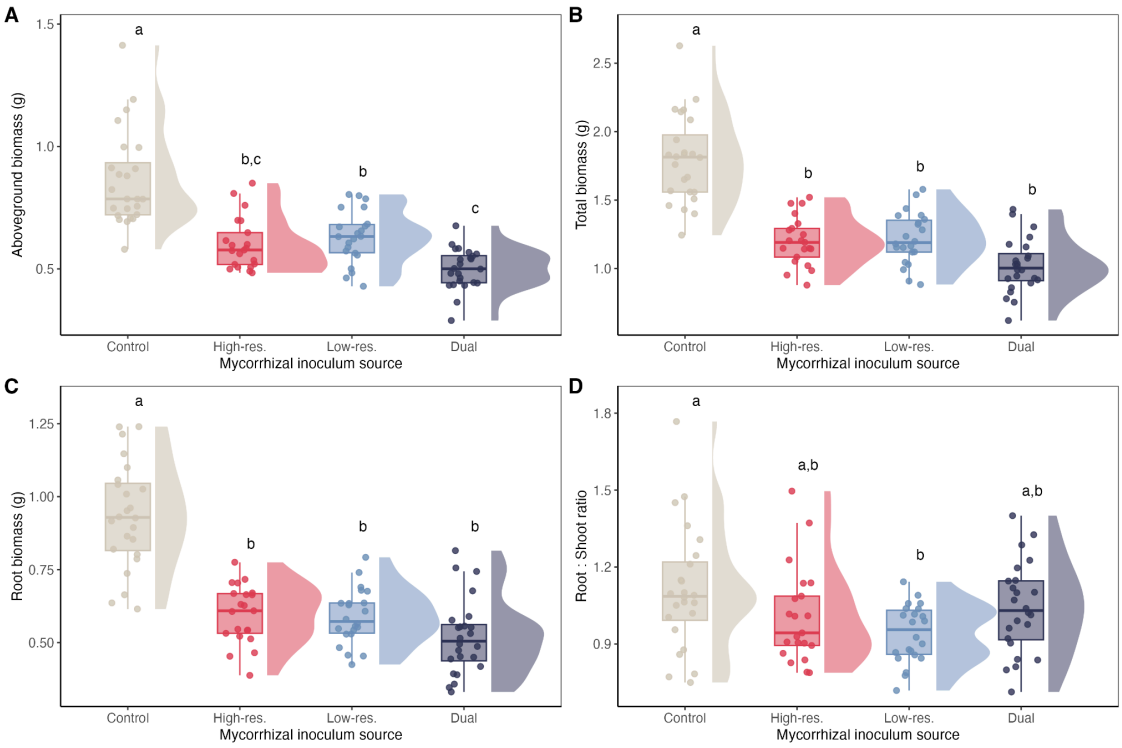

Figure 3. Mixed outcomes for terminal foliar, aboveground, belowground, and root:shoot biomass at harvest. Echoing growth rate data, seedlings in control treatments generally grew faster than fungal-inoculated seedlings. Average (A) aboveground biomass, the sum of woody stem and foliar tissue, (B) total biomass, the sum of woody stem, foliar, and root tissues, (C) root tissue biomass, and (D) root to shoot ratios are represented. Boxes are interquartile ranges with the horizontal line representing the median, and letters represent statistically significant differences at the $p < 0.05$ level. A two-way ANOVA was performed for data in panels (A) (B) and (D) followed by a Tukey-HSD test for pairwise differences between groups. Assumptions of equal variances were violated for (C) root to shoot ratios, leading to a non-parametric Kruskal-Wallis test followed by reported results of Dunn's post-hoc analyses to identify differences in groups.

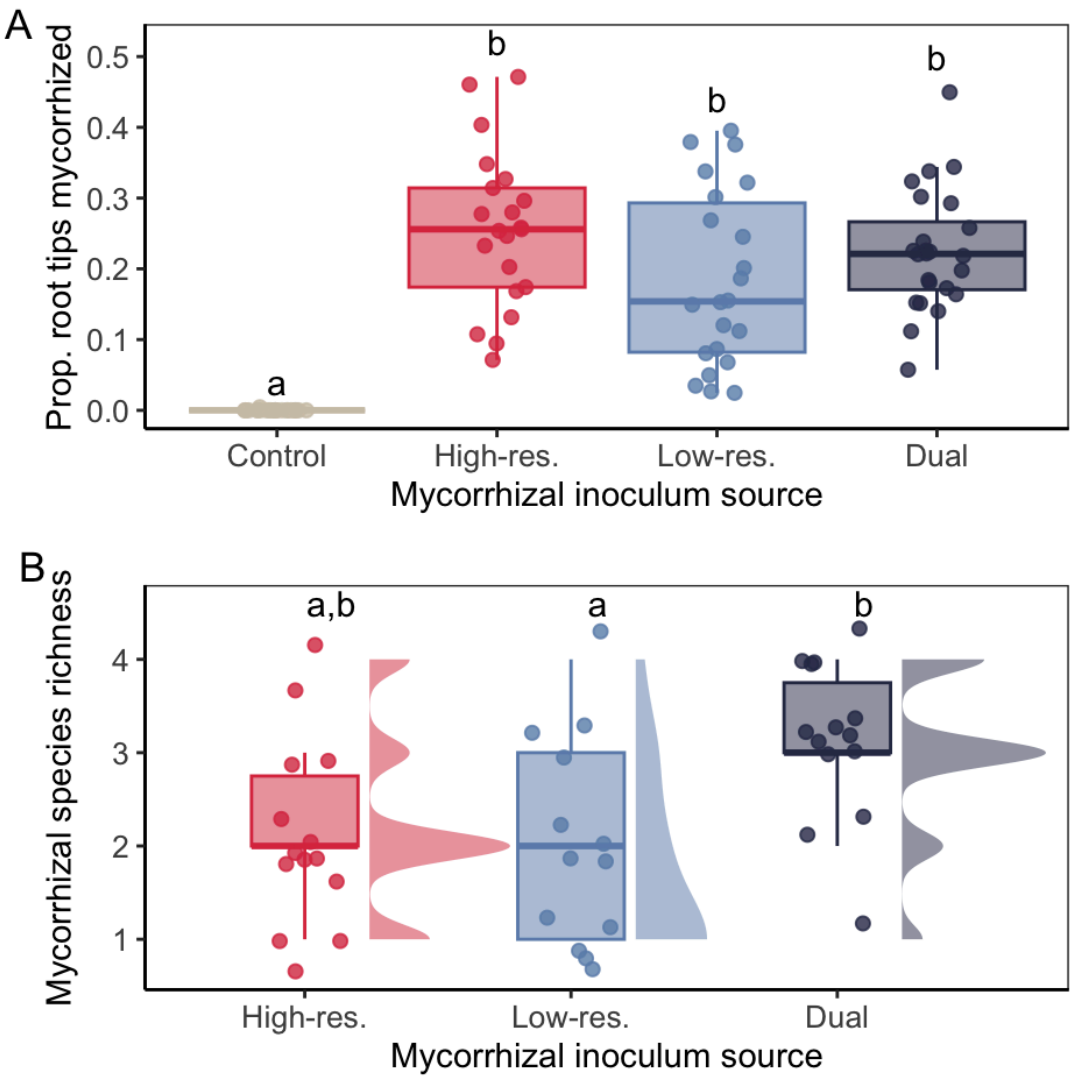

Figure 4: Fungal mycorrhization and species richness generally showed no differences across inoculum treatments. Both (A) proportion of root tips with fungi and (B) fungal richness violated assumptions of normality and equal variances, thus Kruskal-wallis tests and post-hoc Dunn's analyses were performed and significant letters are presented. Boxes are interquartile ranges with the horizontal line representing the median, and letters represent statistically significant differences at the $p < 0.05$ level.

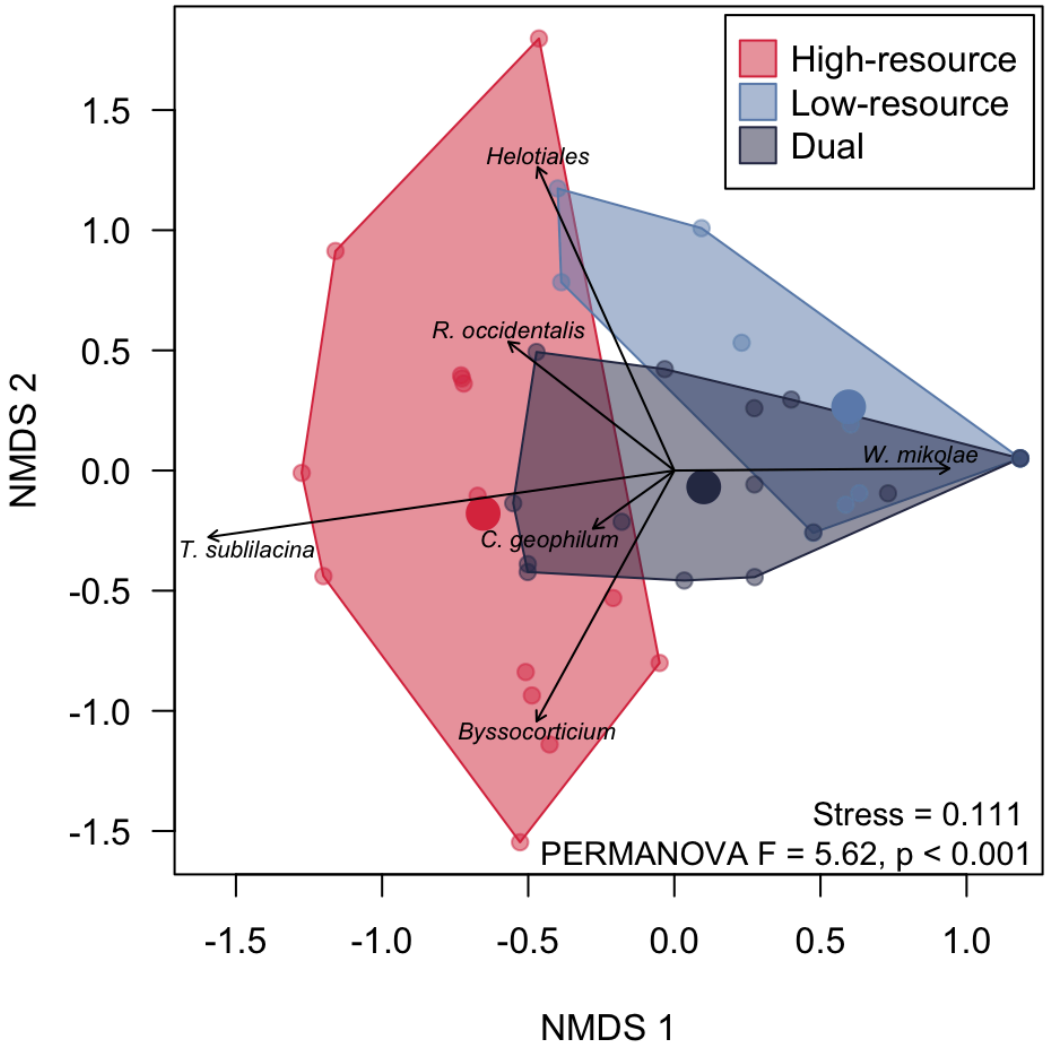

Figure 5: A non-metric multidimensional scaling (NMDS) ordination of individual fungal taxa in December shows distinct inoculation treatment-level clustering. Three large colored points represent centroids, or the average position on the ordination for high-resource (red), low-resource (blue), and dual (dark grey) treatments. Points that are farther away from each other are more dissimilar. Polygons were used to visually group treatments and outline the range between communities. Fungal communities within single inoculation treatments were distinct with minimal overlap, and dual treatment had clear overlap in both single inoculation groups (PERMANOVA, F = 5.617, R = .228, P < 0.001).

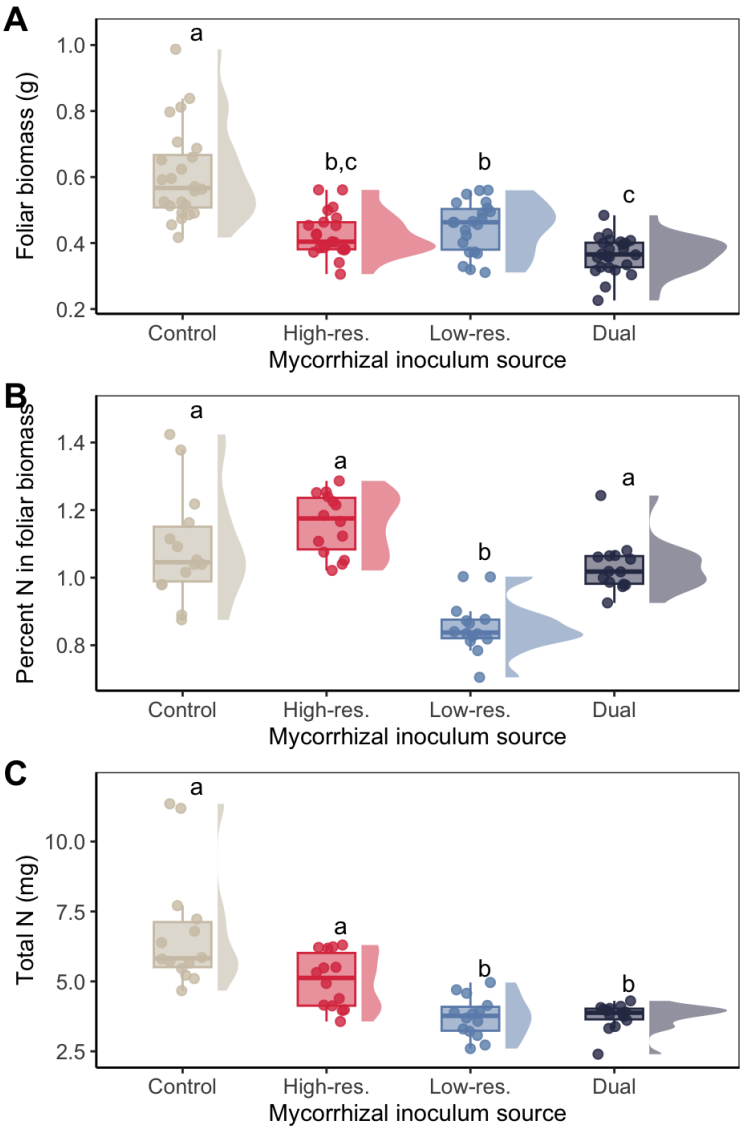

Figure 6: Foliar tissue and associated percent and total nitrogen content across treatments in December. Average (A) leaf tissue biomass, (B) percent nitrogen in leaf tissue, which was a product of leaf tissue and percent nitrogen, and (C) total nitrogen content. A two-way ANOVA was performed for foliar biomass (A) followed by a Tukey-HSD test for pairwise differences between groups. Assumptions of normality and equal variances were violated for total nitrogen (C), and only normality was violated for percent nitrogen (B), leading to a non-parametric Kruskal-Wallis test followed by reported results of Dunn's post-hoc analyses to identify differences in groups. Boxes are interquartile ranges with the horizontal line representing the median, and letters represent statistically significant differences at the $p < 0.05$ level.

Supplemental figures and figure captions

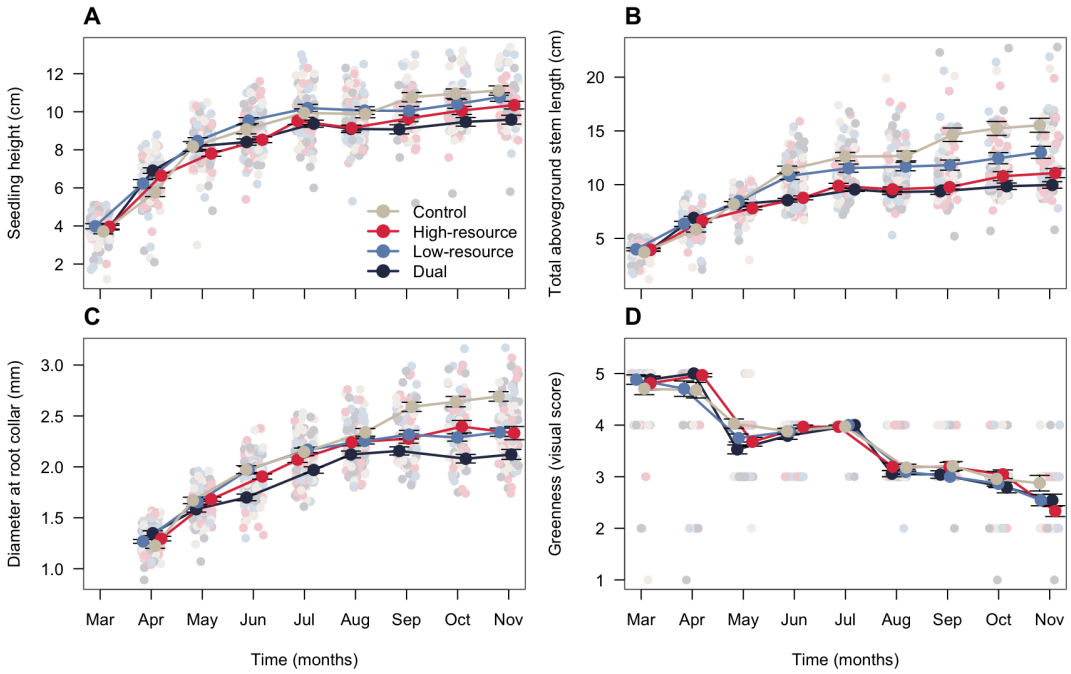

Figure S1: Growth and performance metrics of seedlings in respective inoculum treatments over the course of the nine-month experiment. We measured seedling performance using four non-destructive measures: (A) aboveground height, which increased rapidly over the first five months of the experiment before plateauing, (B) total aboveground stem length, which was greatest for control seedlings by the end of the experiment, (C) diameter at the soil surface (and approximate root collar), which increased most rapidly in control seedlings, and (D) seedling greenness, which generally decreased over the course of the experiment. Lighter points represent raw measurements and the darker points are the average of individual measurements, with error bars indicating standard error. Collectively, our non-destructive measures indicated greater aboveground growth of control seedlings.

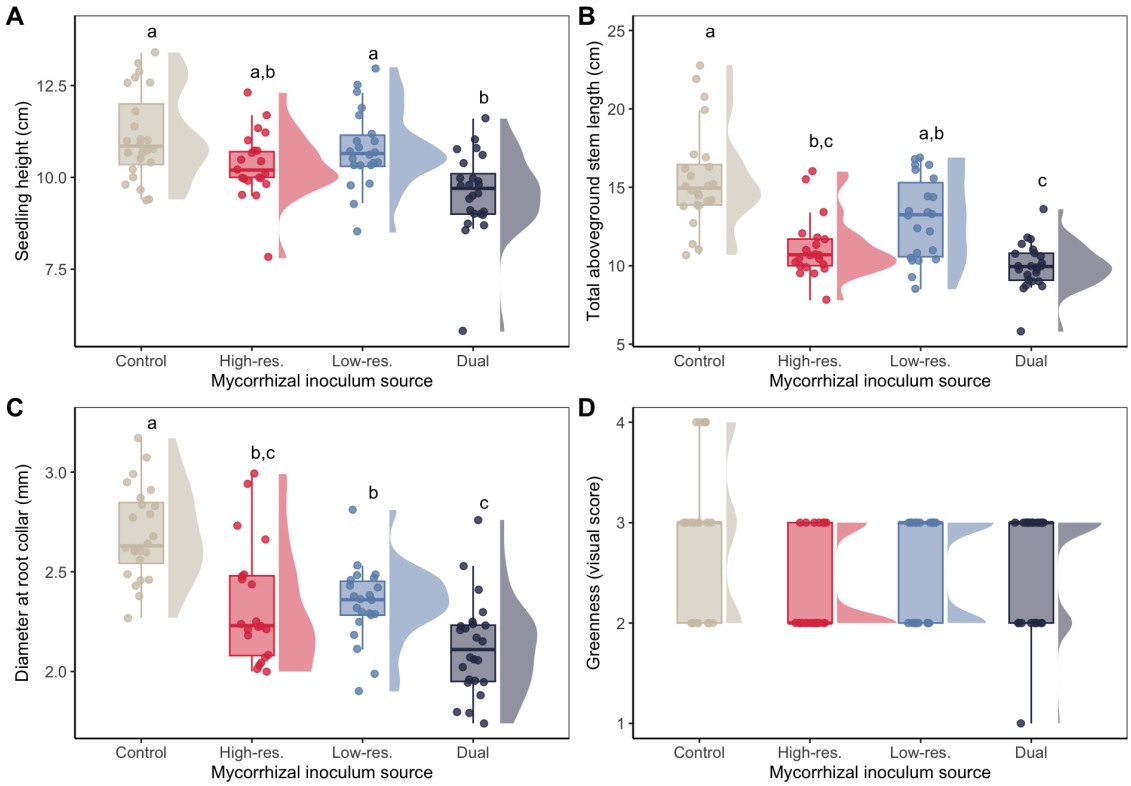

Figure S2: Terminal seedling aboveground performance decreased with increasing fungal inoculum. Across measures of (A) seedling height, (B) aboveground stem length, (C) diameter at the soil surface, and (D) greenness, a proxy for nutrient availability, control seedlings generally outperformed those with some form of fungal inoculum, especially when inoculum included fungi from high-resource soils. Boxes are interquartile ranges with the horizontal line representing the median, and letters represent statistically significant differences at the $p < 0.05$ level. A two-way ANOVA was performed for data in panel (A) and (D), followed by a Tukey-HSD test for pairwise differences between groups. Assumptions of normality and equal variances were violated for data in panels in (B), and (C) only violated assumptions of normality, leading to a non-parametric Kruskal-Wallis test followed by reported results of Dunn's post-hoc analyses to identify differences in groups.

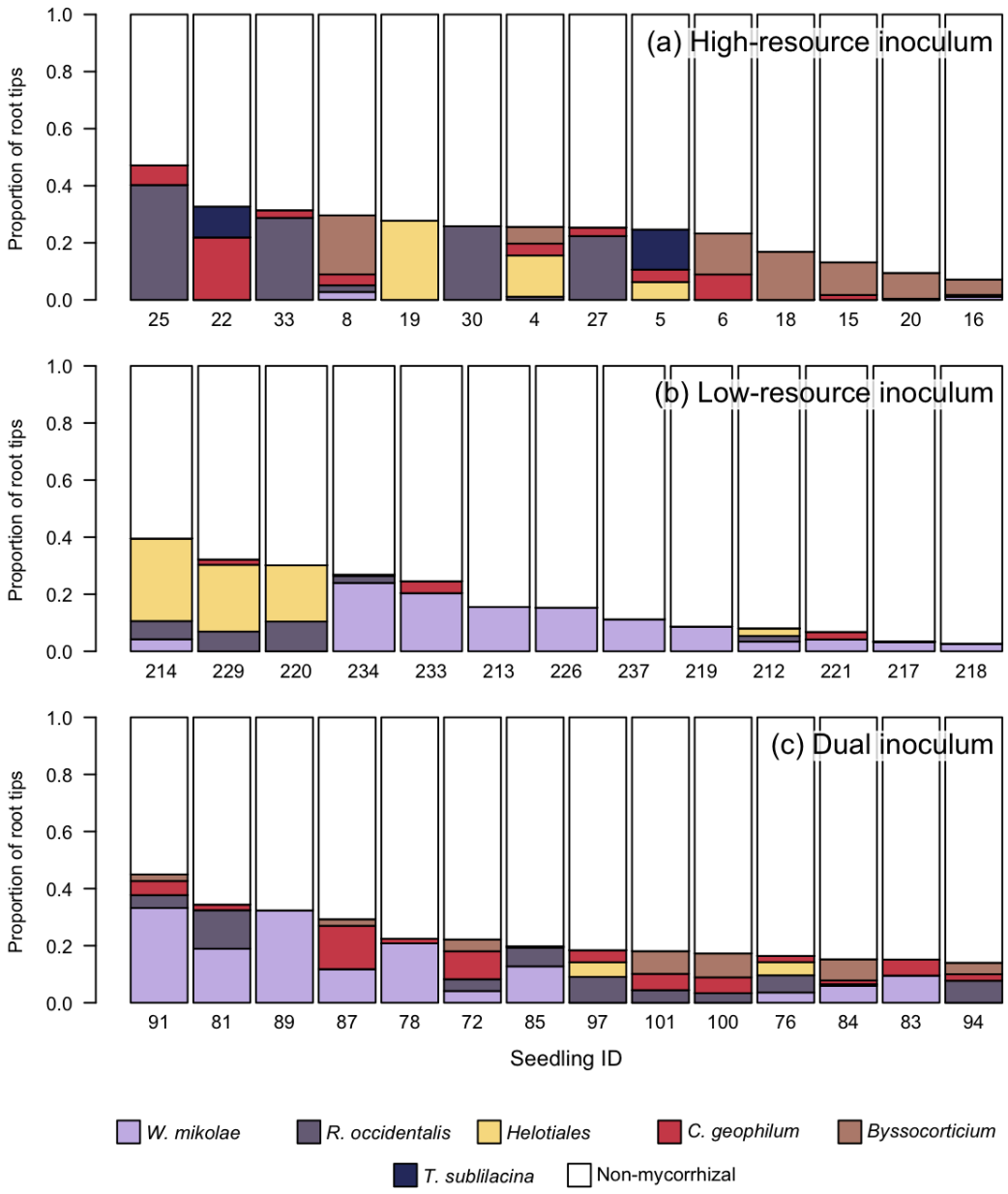

Figure S3. Relative abundance in fungal community composition varied across all inoculated treatments at the end of the experiment for sequenced seedlings. Barplots are filled with fungal taxa that are depicted in various colors for (A) high-resource inoculum, or fungi from T2 environments, which had dominance in *Byssocorticium* and *Rhizopogon occidentalis*, (B) low-resource inoculum, or fungi from T3 environments which had particular prominence of *Wilcoxina mikolae* and (C) dual inoculum, representing fungi from both high-resource and low-resource environments, depicting mixed populations from both inoculum sources.

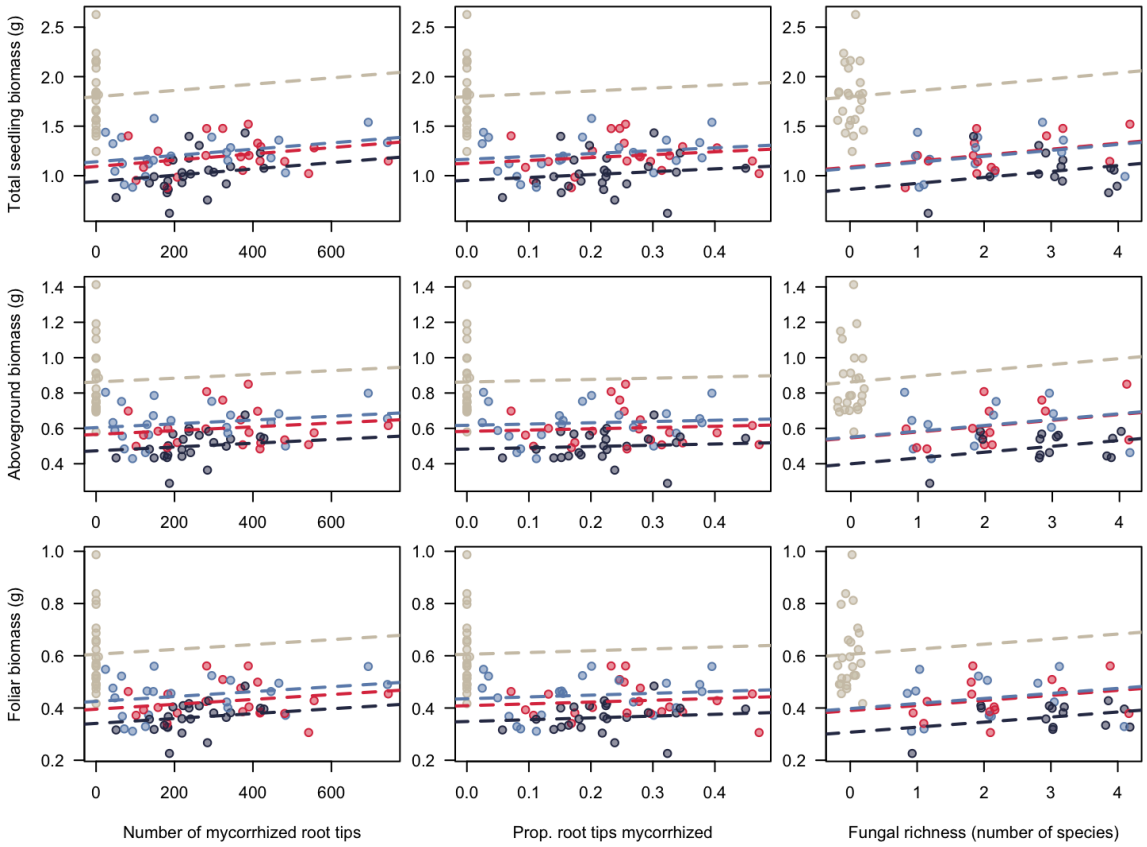

Figure S4: Relationship between seedling performance and mycorrhization. Points and trend lines represent control treatments in beige, high-resource in red, low-resource in blue, and dual treatments in black. Once treatment was accounted for (resulting in four different y-intercepts for the treatment effect), there was no significant relationship between mycorrhization and seedling performance (indicated by the dashed lines). There was no significant interaction between treatment and mycorrhizal response, so all lines are plotted with the same, non-significant slope.

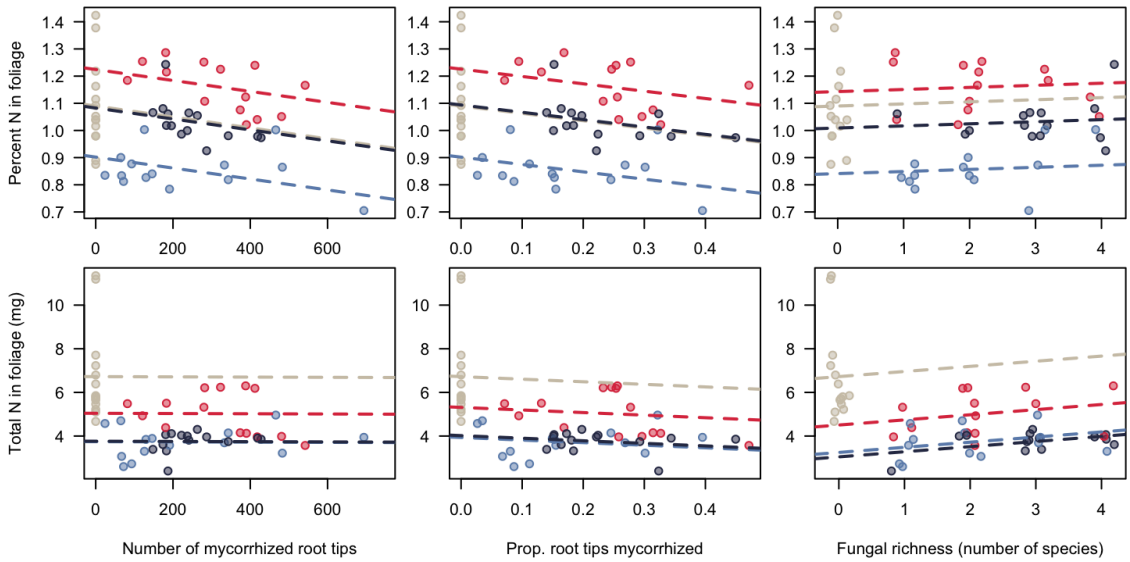

Figure S5: Relationship between seedling foliar nitrogen and mycorrhization. Points and trend lines represent control treatments in beige, high-resource in red, low-resource in blue, and dual treatments in black. Once treatment was accounted for (resulting in four different y-intercepts for the treatment effect), there was no significant relationship between mycorrhization and seedling nitrogen content (indicated by the dashed lines). There was no significant interaction between treatment and mycorrhizal response, so all lines are plotted with the same, non-significant slope.

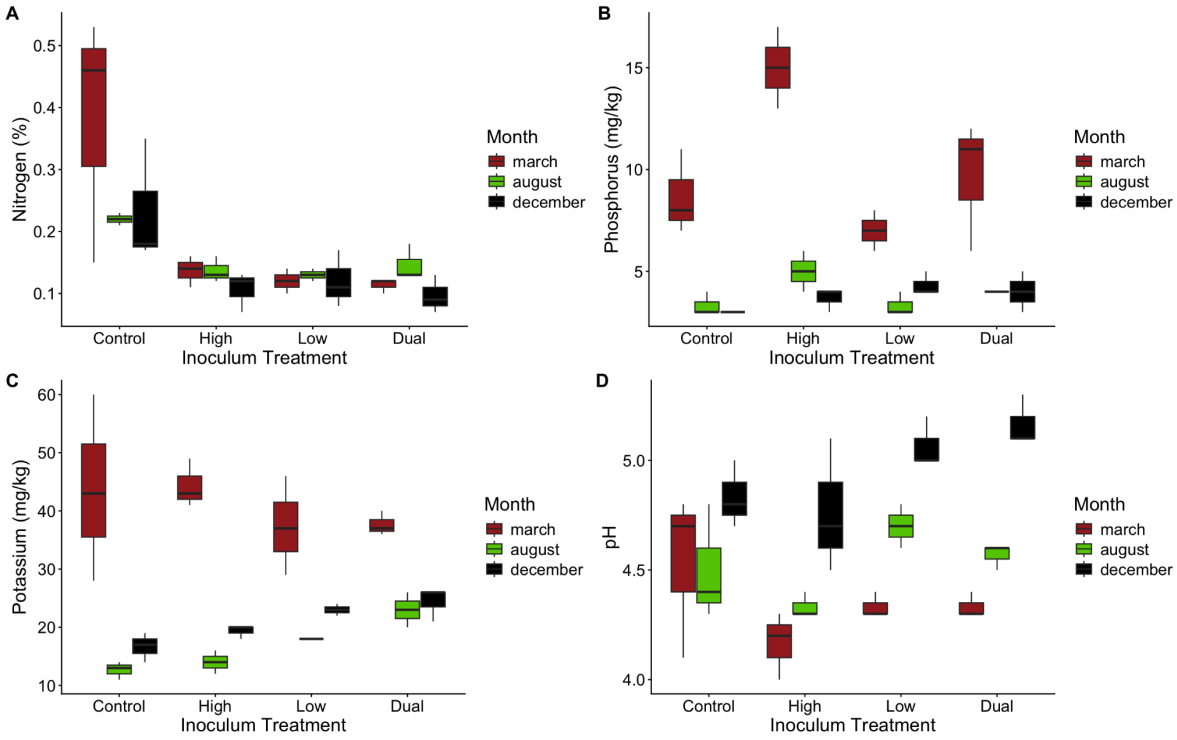

Fig. S6: Soil nutrient content from three timepoints throughout the experiment across the different treatment groups (n = 3 per inoculum treatment per timepoint). Boxplots are filled by month for (A) nitrogen percent, (B) phosphorus (mg/kg), (C) potassium (mg/kg), and (D) pH.

Fungal species	Presence in JHSR	Presence in sequencing data	Rhizomorph formation (DEEMY.de)	Exploration type
Rhizopogon occidentalis	T1, T2, T3 (Courty et al. 2018)	T2, T3, D	Present (information from closest taxa: *R. roseolus*)	Long-distance (DEEMY.de)
Cenococcum geophilum	T3 (Moeller et al. 2014)	T2, T3, D	Absent	Short-distance (DEEMY.de)
Wilcoxina mikolae	NA	T3, D	Absent	NA
Helotiales	T1, T3 (Courty et al. 2018)	T2, T3, D	NA	NA
Byssocorticium	Present (Moeller et al. 2014)	T2, D	Absent (information from different host spp.: *Fagus*)	Short-distance (information from different host spp.: *Fagus*) (DEEMY.de)
Tomentella sublilacina	Present (Moeller et al. 2014)	T2	Present (information from closest taxa: *T. albomarginata)*	Medium (Peay et al. 2011)

Table S7. Fungal ITS sequencing taxa present in this study and presence in the field from data generated by Moeller et al. 2014 and Courty et al. 2018. Fungal species and terraces where each of the taxa were found are provided, as well as physiological traits obtained from Determination of Ectomycorrhizae (DEEMY) database and Peay et al. 2011.

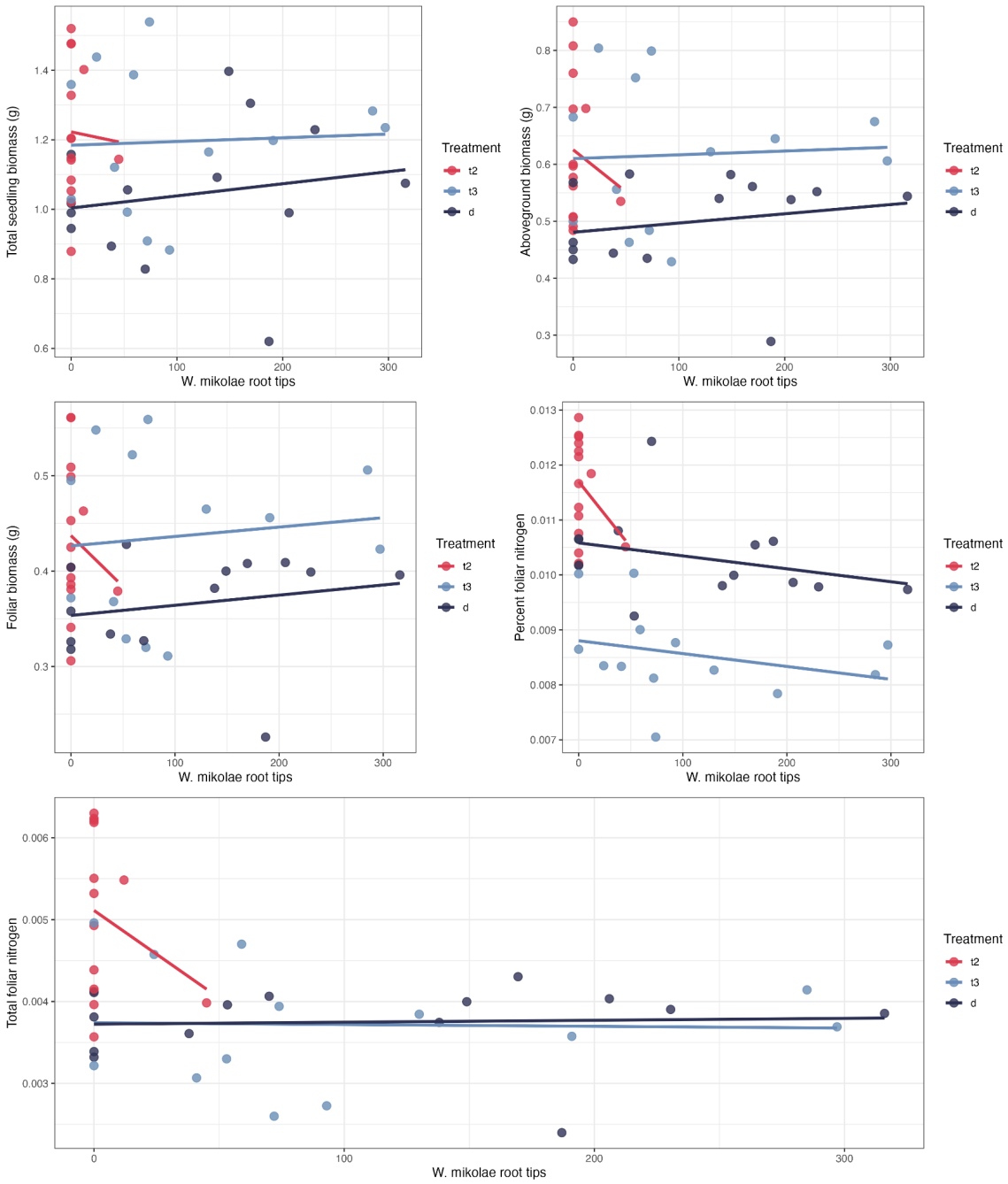

Fig. S8: Relationship between seedling performance and total tips colonized by *Wilcoxina mikolae* for the three inoculation treatment groups. No relationships were significantly different from zero.

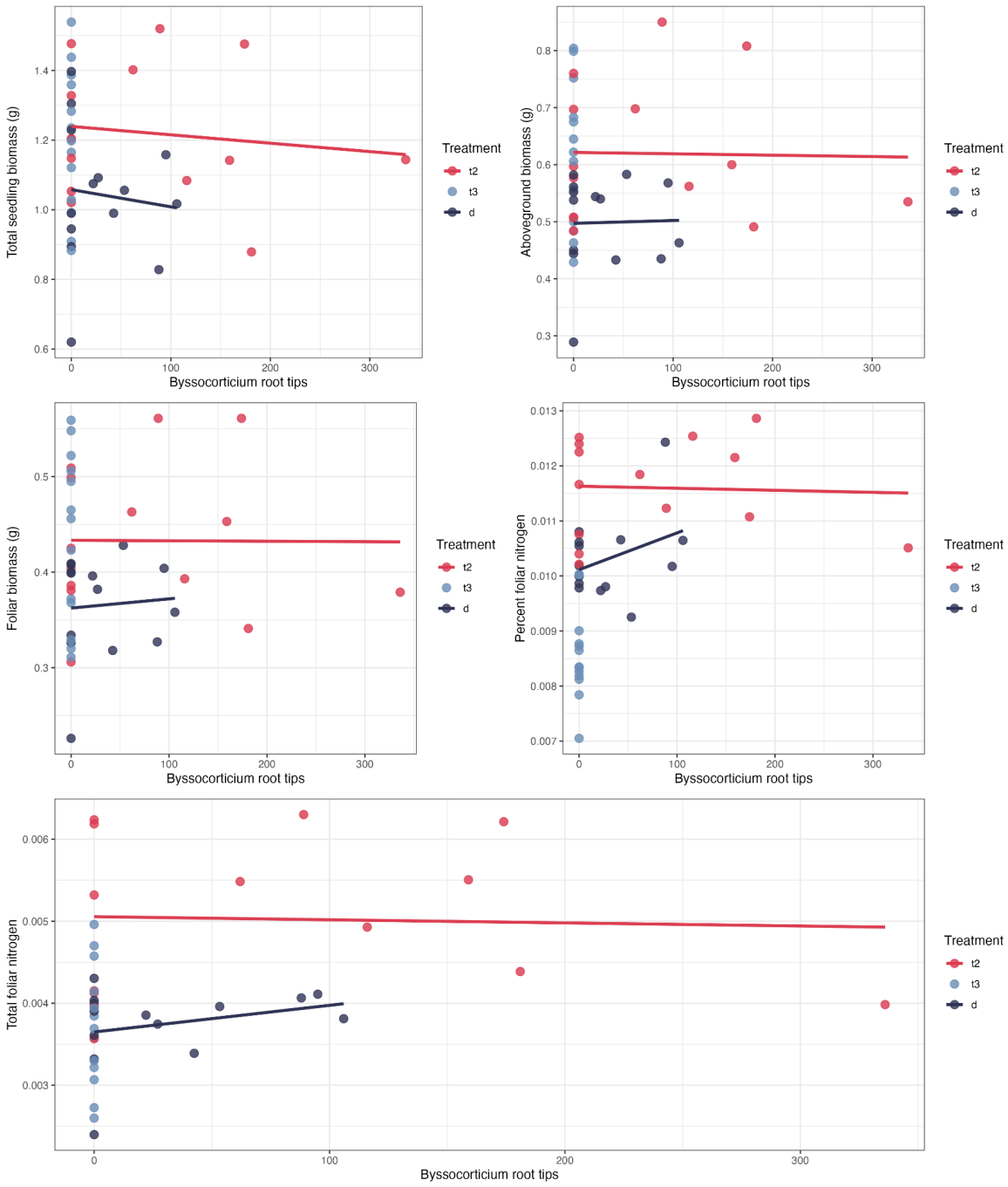

Fig. S9: Relationship between seedling performance and total tips colonized by *Byssocorticium* for the three inoculation treatment groups. No relationships were significantly different from zero.

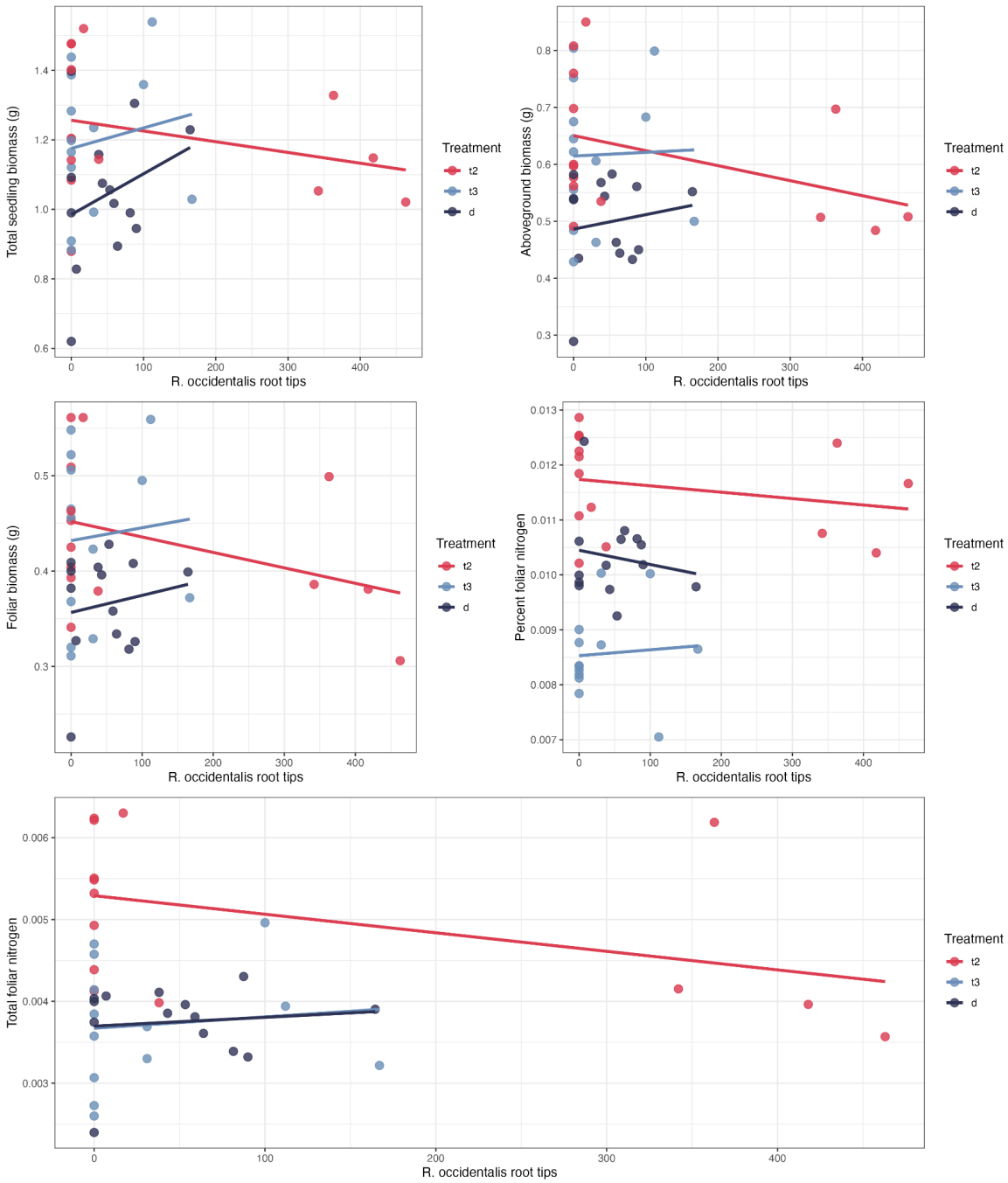

Fig. S10: Relationship between seedling performance and total tips colonized by *Rhizopogon occidentalis* for the three inoculation treatment groups. No relationships were significantly different from zero.

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