Disentangling the context dependency of mycorrhizal relationships to better understand current and future forest structure

By

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DEDICATION

This dissertation is dedicated, whole-heartedly, to my sweet girl, Miss Phoebe Jean. Though I have learned so much from my time in the field and lab, it pales in comparison to the rich life lessons you have taught me during our many years together, particularly those in Madison. Through you, I've learned that things will be ok – even when they won't be. That change is what makes life beautiful, rich, and worth living – even if it breaks your heart. You taught me so much about trust, vulnerability, and how to live in the presence. But, most importantly, you reminded me that magic is real; that love is boundless, good medicine, and not something to be saved for tomorrow.

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ABSTRACT

Despite the prevalence and well-known importance of mycorrhizal symbioses, the factors influencing these plant-fungal interactions are not well understood. Part of this muddling is due to the highly context-dependent nature of these dynamic systems. Though, as global changes continue to ensue, understanding connections between nutrient cycling and forest composition becomes increasingly important. Through the use of a dually-colonized *Populus* system, which can associate with both ecto- (EM) and arbuscular mycorrhizal (AM) fungi simultaneously, this research program has investigated the role of environmental abiotic factors and genetics to determine which factors favor one mycorrhizal guild over the other. Additionally, this research has studied the nutrient economics of mycorrhizal interactions, which is an important consideration for predictive models of biogeochemical cycling and global change. Overall, we found 1) evidence that dually colonized *Populus deltoides* is able to shift its mycorrhizal investment strategies in adaptive ways across environments, highlighting the influences of environmentally dependent costs and benefits in shaping plant mycorrhizal strategies, 2) that *P. tremuloides* litter chemistry and mycorrhizal investment are genetically and functionally linked and that tree litter chemistry has the potential ability to promote nutrient acquisition feedback loops through its effects on EM functional diversity, and 3) that the plant nitrogen return rate per unit carbon invested in EM fungi is a function of nutrient quality and degree of symbiotic investment. In summation, the results of this work contribute to our

understanding of influences dictating the current ranges and geographic distributions of tree species – including non-dual species. Understanding factors controlling the current compositional structure and biogeochemical cycling of our forests is central to predicting future forest states and functions.

INTRODUCTION

The Mycorrhizal Symbiosis

It is estimated that more than 90% of all land plant families form unique symbiotic relationships with mycorrhizal fungi (Smith and Read 1997, Brundrett and Tedersoo 2019). This plant-fungal interaction primarily entails the trading of photosynthetically-assimilated carbon for resources such as mineral nutrients and access to water. Though the exchange is often beneficial for both partners involved, it is highly context dependent and can vary from mutualistic to parasitic depending on the associated conditions (Johnson et al. 1997). Of the multiple types of mycorrhizal fungi that exist, arbuscular mycorrhizal fungi (AM) and ectomycorrhizal (EM) fungi are the most abundant and widespread (Smith and Read 1997) and constitute the vast majority of mycorrhizal partnership with forest trees. Most tree species associate exclusively with members from a single mycorrhizal guild following ontogenetic development (Egerton-Warburton and Allen 2001). However, certain tree species – such as members of the genera *Populus*, *Salix*, *Alnus*, and *Eucalyptus* – have the unique ability to associate with both AM and EM fungi concomitantly throughout their lifespan (Teste et al. 2020). Factors influencing the degree to which "dually-colonized" individuals are inhabited with one mycorrhizal guild versus the other are currently poorly understood, though soil moisture (Lodge 1989, Gehring et al. 2006) and, more recently, decomposition estimates have been shown to play a role (Van Nuland et al. 2023).

Though both fungal guilds fill roles as mycorrhizal symbionts, AM fungi and EM fungi have considerably different evolutionary histories as well as morphology and physiology, and therefore maintain distinct partnerships with their plant hosts. A primary distinction between the two fungal guilds is their differential ability to access nutrients from organic material. EM fungi have, in general, retained a much larger and more variable repertoire of saprobic enzymes than their AM counterparts (Kohler et al. 2015). With the unique ability to enzymatically break down complex molecules and "mine" for nitrogen from organic matter (Hobbie et al. 2013), EM fungi may offer an advantage to plants in nitrogen-limited environments, such as boreal and temperate forests. However, it has been hypothesized that maintaining an EM symbiosis is more carbon-costly to the plant host (Lu and Hedin 2019, Smith and 2008 as cited by Phillips et al. 2013), therefore, the context dependency of a given mycorrhizal interaction is likely also a result of which fungal partners are involved.

Forest Structure and Nutrient Cycling

There currently exists a distinct latitudinal gradient with regards to mycorrhizal associations in forested systems: EM interactions dominate in the northern, boreal biomes whereas AM interactions dominate in the tropics, and both are common in temperate regions (Figure 1; Lu and Hedin 2019). This pattern is closely correlated with latitudinal patterns of soil organic matter (SOM) and nutrient limitation (Lu and Hedin, 2019, Read 1991, Averill et al. 2018), with

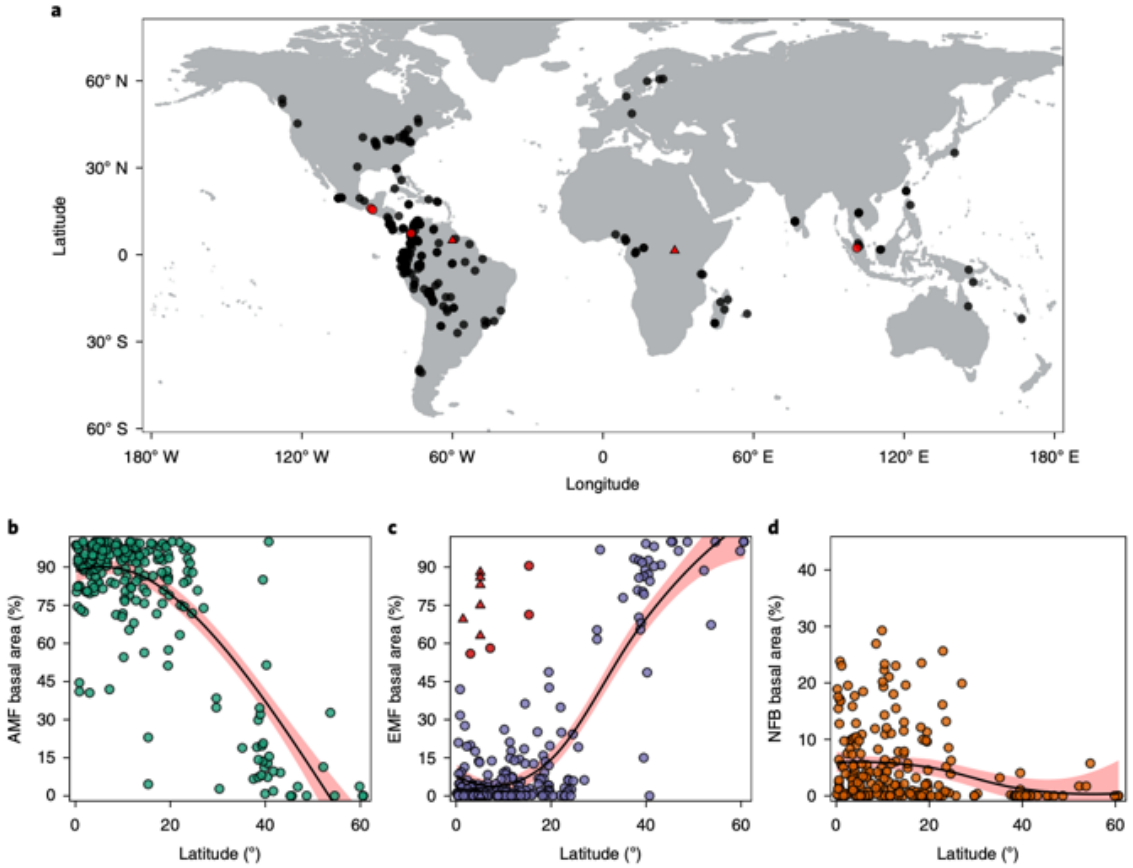

Figure 1. Changes in AM (subset a) and EM (subset b) dominance across latitudes. AM basal area decreases as distance from the equator increases, and EM basal area exhibits the opposite pattern. (Subset d shows latitudinal patterns of nitrogen fixing bacteria, though they are not addressed in this dissertation). Figure from Lu and Hedin 2019

boreal regions being characterized by high levels of SOM and nitrogen limitation, tropical regions by low levels of SOM and phosphorous limitation, and temperate zones being more site specific, at least with regard to nutrient limitation. These parallels are thought to be due, in part, to differences in litter quality between EM and AM tree species and their resultant rates of decomposition (Phillips et al. 2013, Ehrenfeld et al. 2005), which are further exacerbated by climatic factors.

Litter from AM-associated trees tends to be of higher quality and decompose more quickly than that of EM-associated trees (Cornelissen et al. 2001). Therefore, there is a well-established connection between mycorrhizal guild dominance (AM or EM) and associated biogeochemical signatures.

In addition, the ability of EM fungi to “mine” nitrogen from organic matter can alter carbon and nitrogen cycling in soil. When EM fungi access organic nitrogen for their host plants, they leave behind carbon-rich compounds, which eventually increases the C:N ratios of the soil organic matter. Conversely, when saprotrophic fungi decompose organic matter, they incorporate both nitrogen and carbon and respire carbon to the atmosphere maintaining a relatively lower C:N ratio. The phenomenon of suppressed decomposition due to EM and saprotrophic competition is known as the Gadgil Effect (Gadgil and Gadgil 1971, Fernandez and Kennedy 2015) and has gained renewed attention as concerns regarding global nutrient cycling has increased (Averill et al. 2014, Wurzburger et al. 2017, Zhu et al. 2018).

Chemical differences in litter, however, can interact with the different functional attributes of symbiotic fungi and, consequently, influence how plants access nitrogen. For example, Wurzberger and Hendrick (2009) documented a relatively closed nitrogen feedback loop, where litter chemistry promoted the acquisition of nitrogen by associated mycorrhizae and hindered acquisition in neighboring roots of co-occurring plants. In this sense, the soil biogeochemical signatures of

each mycorrhizal guild could be creating conditions that self-selects for that guild, and therefore selects for tree species that associate with that guild, creating a positive feedback loop (Phillips et al. 2013). These feedback loops are currently receiving more attention in the mycorrhizal literature and are thought to contribute to broadscale processes of nitrogen and carbon cycling (Phillips et al. 2013, Lu and Hedin, 2019). However, given that global changes are expected to affect both above and belowground processes, determining which factors are important to the maintenance of these feedback loops is critical to understanding how these systems might change in the near and distant future.

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CHAPTER ONE

Nutrients and light drive the ratio of ecto- to arbuscular mycorrhizal fungi in the roots of dually-colonized *Populus deltoides*

ABSTRACT

Forested systems currently exhibit a distinct latitudinal gradient in mycorrhizal dominance, which is thought to derive from the differing nutrient acquisition abilities and carbon costs of ecto- (EM) and arbuscular mycorrhizal (AM) fungi. Therefore, the cost and benefits of a particular fungal strategy to plants are predicted to depend on environmental conditions, especially nutrient accessibility and factors controlling the photosynthetic rate. Comparative mycorrhizal studies are challenging due to confounding effects of the host tree, but certain "dually-colonized" species, such as *Populus deltoides*, can associate with both mycorrhizal guilds simultaneously, making them useful models. Here, we conducted a greenhouse experiment using *P. deltoides* and variable nutrient, soil moisture, and light intensity treatments, to test two hypotheses: 1) *P. deltoides* will show the highest levels of total colonization in environments that yield the highest benefits from mycorrhizal association (nutrient limitation) but

without cost constraints due to reduced photosynthesis (high light and moisture), and 2) that plants will face a tradeoff in EM and AM investment when photosynthates are limited. Total mycorrhizal colonization was significantly and positively correlated with soil moisture and this relationship was mediated by plant growth. Across all nutrient, light, and soil moisture environments, there was a strong negative correlation between AM and EM colonization, providing evidence for a biological tradeoff between the two guilds. This tradeoff was driven by nutrients and light: plants showed increased EM (vs. AM) colonization in low nutrient and high light conditions, and increased AM (vs. EM) colonization in conditions of high nutrient accessibility and low light. Overall, this study provides evidence that dually colonized *Populus deltoides* is able to shift its mycorrhizal investment strategies in adaptive ways across environments and highlights the influences of environmentally dependent costs and benefits in shaping plant mycorrhizal strategies.

INTRODUCTION

Forested systems across the globe currently exhibit a distinct latitudinal gradient in mycorrhizal dominance, where ectomycorrhizal (EM) interactions dominate in northern, boreal biomes, arbuscular mycorrhizal (AM) interactions dominate in the tropics, and both are common in temperate regions (Lu and Hedin 2019). This distributional pattern is closely correlated with patterns of soil organic matter, decomposition rates, nutrient limitation, and general patterns of litter quality in EM and AM associated plants (Lu and Hedin, 2019, Read 1991, Averill et al. 2018). Overall, these correlations are thought to be the result of mycorrhizal fungal functional traits, namely those involved in the acquisition of nitrogen, paired with climate-driven differences in decomposition rates.

In general, EM fungi have retained a much larger and more variable repertoire of saprobic enzymes than their AM counterparts (Kohler et al. 2015), giving them the unique ability to “mine” for nitrogen from organic matter (Hobbie et al. 2013). AM fungi, on the other hand, are considered to only “scavenge” for dissolved mineral forms of nitrogen, phosphorus, and other soil nutrients. The ability of EM fungi to mine for nitrogen may provide an advantage to trees in higher latitudes, where much of the available nitrogen is bound up in organic matter and the climatic window for decomposition is limited. Therefore, increased selection for EM fungi in high latitudes may explain the dominance of EM-associated plants in these regions. In the tropics, however, forested systems

exhibit open, mineral-nutrient cycling economies due to less restricted windows for decomposition and increased litter quality inputs (Phillips et al. 2013). Therefore, in tropical areas, there may be reduced selection for the enzymatic abilities of EM fungi, especially if they impose a high carbon cost to the host tree.

It has been hypothesized that EM relationships incur a higher carbon cost per unit nitrogen acquired to the associated plant host than AM associations (Lu and Hedin 2019, Smith and Read 2008 as cited by Phillips et al. 2013). This differential cost is thought to be the result of the enzymatic capabilities possessed by most EM fungi, as well as differing morphologies between the two guilds and slower turnover of EM compared to AM structures (Lu and Hedin 2019, Read & Perez-Moreno, 2003, Sulman et al. 2017). Understanding the drivers of mycorrhizal strategies could have important implications for our ability to accurately predict future forest states and functions. In particular, the carbon cost per unit nitrogen gained is a central controlling variable in models predicting the carbon sink or source potential of forests in future climates (Sulman et al. 2017, Meyer et al. 2010, Fatichi et al. 2019 and 2014).

However, to our knowledge, the assumption of differential costs and benefits of AM vs. EM strategies has yet to be empirically tested. Quantifying differences between EM and AM associations is difficult as most plants associate with one fungal guild or the other, and evolutionary transitions between the states have been rare in plants (Koele et al. 2012). Comparative studies, therefore, necessarily

involve different and largely unrelated host taxa, which introduces many confounding traits and variables both above- and belowground (Phillips et al. 2013, Cornelissen et al. 2001, Comas and Eissenstat 2009, Midgley et al. 2015, Wurzburger and Brookshire 2017).

Dually-colonized species, however, are unique in their ability to associate with both EM and AM fungi and often do so simultaneously throughout their lifespan (Teste et al. 2020). Dually-colonized species commonly include members of the genera *Populus*, *Salix*, *Alnus*, and *Eucalyptus* (Teste et al. 2020, Brundrett and Tederoo 2020), though the strategy has also been documented in multiple herbaceous plants (Teste et al. 2020), including the prairie forb *Pulsatilla patens* (Eastern Pasqueflower; Hoeksema et al. 2018). Dual colonization was previously thought to be rare and atypical (Lodge 2000), though it is now considered to be more common than previously thought (Teste et al. 2020). The ability to associate with both AM and EM simultaneously makes dual hosts useful models for EM:AM comparative studies of respective costs and benefits, particularly because they avoid the confounding effects of differing host species. Additionally, employing dually colonized species as models for comparative studies may highlight the potentially unique – and perhaps ecologically relevant – flexibility of dual species in navigating cost-benefit tradeoffs.

Since dually colonized species can theoretically exist along a gradient from primarily AM to primarily EM nutrient acquisition strategies, they may reflect on

a microscale the patterns playing out on larger geographic and evolutionary scales between plant species. In particular, the ability of some EM fungi to acquire nitrogen directly from complex organic sources would be most beneficial when most of the nitrogen in the system is limiting (low nitrogen quantity) and bound in complex forms in soil organic matter or recalcitrant litter (low nitrogen "quality" for plants), with benefits diminishing as nutrients become more readily available and accessible. These benefits, however, are hypothesized to incur an increased carbon cost to plant hosts compared to AM symbionts; therefore, the ability of a dually-colonized host to invest in EM vs. AM symbioses may be affected by factors related to plant levels of non-structural carbon, such as photosynthetic rate.

The ability of a host plant to "afford" mycorrhizal symbionts with high carbon demands will be determined by environmental conditions determining photosynthetic rates. One obvious factor influencing photosynthetic rate is light availability. Mycorrhizal colonization has been shown to be positively correlated with increasing light quantity and quality (Johnson et al. 2015, Gamage et al. 2004, Konvalinková and Jansa 2016), suggesting that the carbon cost of mycorrhizal associations may be an important limiting factor to plant investment in the symbiosis. Soil moisture is another factor potentially influencing a host plant's ability to support mycorrhizal symbionts. Low moisture may present an orthogonal stress that influences the cost/benefit calculation for plant investment in mycorrhizal symbionts. Lower moisture could reduce plant

growth, and ultimately overall nitrogen demand, which may reduce the benefit conferred by mycorrhizae. Similarly, stomatal closure due to low moisture would reduce the photosynthetic rate as well as potential availability of non-structural carbon, similar to low light conditions. On the other hand, soil moisture levels may also affect the benefits received from mycorrhizal symbionts. Both AM and EM symbioses can improve plant performance under drought, in part through direct transfer of water to hosts (Lehto and Zwiazek 2011, Augé 2001 and 2004). Soil moisture can also affect nutrient availability, through leaching of mobile nutrients in saturated soils and reduced mobility in dry soils, both for dissolved mineral forms and the water-soluble enzymes that EM fungi use to access organic nitrogen. Soil moisture has already been shown to be influential to AM and EM colonization proportions in dually-colonized species (Lodge 1989, Lodge 1985, Gehring et al. 2006), though its role in interactions with other abiotic factors has yet to be examined.

Here, we used a dually-colonized *Populus deltoides* (Eastern cottonwood) system in a greenhouse experiment to identify how certain abiotic factors related to mycorrhizal costs and benefits affected the total and relative colonization of EM and AM fungi in tree roots. Potential benefits conferred by mycorrhizae were manipulated using different nutrient quantity and quality environments and costs (potential photosynthetic rates) were manipulated using gradients of light availability and soil moisture. If 1) EM symbionts incur higher photosynthate costs to host plants in return for unique benefits derived from their enzymatic

ability to acquire nutrients, and 2) *P. deltoides* individuals are able to optimize their mycorrhizal strategy to achieve the maximal benefit:cost ratio in a given environment, then we predict:

Prediction 1: Total mycorrhizal colonization

Total mycorrhizal colonization will be highest in environments where plants stand to benefit from mycorrhizae (low nutrient quantity or quality) and are not restricted by photosynthate costs (high light and moisture). *Corollary*: Plant growth will mediate the relationship between environmental conditions and total colonization; specifically, environments allowing rapid plant growth will promote higher total mycorrhizal colonization.

Prediction 2: EM/AM trade-off

When photosynthates are limited, plants will face a trade-off between investment in EM and AM colonization. The proportion of EM to AM colonization will be negatively correlated with nutrient quality and quantity, with the greatest EM colonization (and lowest AM colonization) in environments where nutrients are low in both quality and quantity.

METHODS

Experimental Design

Plant source: Cuttings of *Populus deltoides* were obtained from two different dormant individual parent trees located in the Lakeshore Nature Preserve (Madison, WI) in the fall of 2019. Each cutting was restricted to new growth from within the previous two growing seasons and rooted in rockwool propagation foam for two to three months. Rooted cuttings were measured for various morphological and developmental characteristics and transplanted to 1.6L tree pots containing autoclaved 40 % field soil (3.05% carbon, 0.07% nitrogen, 0.03% phosphorus; Table S1.17), 40% sand, and 20% whole inoculum. The inoculum served as the microbial source and was comprised of chopped environmental root samples (33.69% carbon, 1.03% nitrogen, 0.16% phosphorus; Table S1.17) and rhizosphere soil (12.71% carbon, 0.48% nitrogen, 0.03% phosphorus; Table S1.17) which was collected in the vicinity of adult *P. deltoides* individuals located in the UW-Arboretum. Inoculum was added 6 months prior to planting and again at the time of planting; pots were kept regularly hydrated between inoculation events. In both events, inoculum had been freshly collected and was distributed within the root zone area of the tree pot.

Experimental treatments: Planted cuttings were randomly assigned to factorial combinations of three light levels, three soil moisture levels, and three nutrient

treatments, yielding 3X3X3 = 27 treatment combinations. The treatment combinations were represented as equally as possible given the available *P. deltoides* cuttings, resulting in a total of 145 experimental cuttings (3-7 replicate pots per treatment combination following cutting transplant survival). Green-Tek (Janesville, WI) Aluminet shade cloths of 70% and 40% reflectance were used to create a light exposure gradient of 30%, 60%, and 100% (no cloth) by constructing PVC-pipe and shade cloth structures to house seedlings. Each of the nine light shelters contained between 10-22 final plants, with each combination of soil moisture and nutrient treatments assigned randomly to avoid spatial bias.

Soil moisture was manipulated by watering pots at varying frequencies: “high” soil moisture pots were watered every three to four days, “medium” moisture pots every seven days, and “low” moistures pots every 10 to 11 days. Preliminary measurements were made to ensure that light treatments were not confounding the soil moisture gradient, given that light level may influence rates of evaporation (Table S1.1). Soil moisture data was collected mid-experiment using a Meter ECH_2O volumetric moisture sensor (Meter Group, Inc., USA) to verify whether the watering treatment produced a gradient of soil moistures, which was confirmed ($P < 0.0001$; Table S1.2).

Nutrient treatments included an organic addition treatment in the form of dried, crushed *P. deltoides* leaf litter (44.37% carbon, 1.23% nitrogen, 0.2% phosphorus), an inorganic addition treatment in the form of a slow-release NPK (nitrogen,

phosphorus, potassium) mineral fertilizer (Nutricote 13-13-13), and a low-nutrient treatment where no additional nutrients were added. Nitrogen was of particular interest given its proposed difference in accessibility to AM and EM fungi; therefore, we balanced the total nitrogen added in the two nutrient addition treatments in order to create a chemically recalcitrant, organic nitrogen source (crushed *P. deltoides* leaf litter) and a more easily accessible inorganic mineral nitrogen source (NPK fertilizer) with equal amounts of total nitrogen. The concentration of nitrogen, phosphorus, and carbon present in the leaf litter of *P. deltoides* was analyzed by the UW Soil and Forage Lab (Marshfield, WI) and was used to inform how much mineral fertilizer was added to each pot. Equal amounts of nitrogen were added to pots containing leaf litter (0.369 g N = 30 g dried leaf mass) and mineral fertilizer (0.369 g N = 2.84 g Nutricote pellets) and each addition increased gross levels of nitrogen by ~35% relative to the low quantity treatment, which received no addition. Crushed leaves were mixed into the top two-thirds of the growth medium in select pots prior to the transplant of *P. deltoides* cuttings, and this soil disruption was replicated in inorganic-addition and no-addition pots to avoid differential impacts on the present microbial community following initial inoculation. The slow-release fertilizer pellets were added to the soil surface of selected pots following planting.

Measurements: Chlorophyll content was measured using a SPAD 502 chlorophyll meter (Spectrum Technologies, Inc., USA) by selecting a mid-canopy leaf that appeared representative of the individual. Cuttings were harvested after

approximately four months of growth in the greenhouse under the prescribed treatments. Fine root samples were collected from the upper, middle, and lower sections of each individual root mass and stored in tissue embedding cassettes for microscopic quantification of AM and EM colonization and in the freezer for molecular sequencing. The remaining root mass and aboveground biomass were dried, and aboveground components were further divided into leaves, new stem growth, and original cuttings. Plant growth, or the amount of biomass accumulated during the experimental timeframe, was calculated as the dried weight sum of the belowground biomass and new aboveground biomass (leaf biomass + new stem growth), without the inclusion of the original cutting mass.

Roots were cleared and stained according to lab-tested protocols based on standard methods (Phillips and Hayman 1970) that were further optimized for *Populus* roots. Samples were loaded into tissue embedding cassettes and autoclaved (121°C) in 10% KOH for 10 minutes; this step was repeated with new KOH solution until cortex cell contents were removed. Cassettes were then rinsed in water and bleached in a 3% H_2O_2 solution for one hour until cortex cells were colorless. Cassettes were rinsed and subsequently soaked in a 1% HCl solution for one hour to acidify roots and assist with their uptake of dye. Roots were then directly transferred into a 0.05% Trypan Blue solution that was diluted in a 1:1:1 lactic acid:glycerol:water background and autoclaved for 10 minutes (Deguchi et al. 2017). Cassettes were rinsed following, and roots were stored in DI water in the refrigerator until microscopic scoring.

Colonization was quantified according to Brundrett et al. 1996. Roots were initially assessed for EM colonization using the grid intersection method (Brundrett et al. 1996) under a dissection scope. However, certain EM structures, particularly thin and inconspicuous mantles, were easier to identify in the cleared and stained roots and were therefore quantified concomitantly with AM fungi. See Figure S1.1 in the supplement for structure images.

Sequencing of fungal communities: Fungal communities were sequenced via Illumina sequencing of the ITS2 rRNA gene region via methods described in Allsup et al. 2023. Roots were frozen with liquid nitrogen and ground using a SPEX Sample Prep 1600 MiniG tissue homogenizer (SPEX SamplePrep, Metuchen, NJ, USA). DNA extractions were prepared using the Omega E-Z 96 Plant DNA Kit (Omega Bio-Tek, Norcross, GA, USA) and the ITS2 region was amplified through polymerase chain reaction (PCR) using the ITS3-KYO2 (Toju et al. 2012) and ITS4 (White et al. 1990) primers. An additional round of PCR attached Illumina adapters and unique metagenomic barcodes to each sample, which were then sequenced via Illumina Miseq PE 2x300 chemistry. Sequences were denoised, clustered into exact amplicon sequence variants, and taxonomically identified using the DADA2 program (Callahan et al. 2016) as implemented in the QIIME2 pipeline. Fungal species were assigned to guilds based on the Fungal Traits database (Põlme et al. 2020).

Analysis

Plant growth, %AM colonization, %EM colonization, and total colonization (sum of %AM and %EM) were analyzed using linear mixed effect models in the lme4 package in R version 3.6.2. Metadata covariates accounting for the initial size of the *P. deltoides* cutting at the start of the experiment (stem diameter) and "Tree ID" (factor denoting from which of the two source trees the cutting originated) were significant explanatory variables in at least one of the following models and were, therefore, included in all models to avoid confounding interpretation between models. Differences in initial size and developmental state as well as plant genetics may have affected rates of colonization; therefore, these terms were included to statistically account for any differences. "Plot" (referencing the specific shade structure pots were grown under) was included as a random effect in all models to account for spatial structure in the greenhouse and the non-independence of samples sharing the same light manipulation structures. Graphs were made using the R package ggplot2.

Prediction 1: Total Mycorrhizal Investment

Prediction 1a – Environmental Conditions

To test the effect of abiotic factors on total mycorrhizal colonization, a linear mixed model and ANOVA test were used (R package lmerTest; Table S1.3). This model included the sum of %EM and %AM as the response variable, where %EM was calculated as the percent of root intersections scored that contained EM

structure(s), and %AM was calculated similarly. Explanatory variables included all experimental variables and all two and three-way interaction terms (Nutrient*Light*Moisture) as well as the aforementioned metadata covariates. Plot was included as a random effect. Differences between specific treatment groups were assessed using Tukey's HSD post hoc test using the R package emmeans, with Kenward-Roger estimated degrees of freedom (Lenth 2022; Table S1.4).

Prediction 1b – Plant Growth

A linear mixed model was used to determine how environmental conditions affected plant growth (Table S1.5). Plant growth was used as the response variable and was square root transformed to reduce heteroscedasticity and meet the homogeneity of variance assumption of ANOVA. Explanatory variables included the three treatment conditions (Light, Moisture, and Nutrient conditions), including all two- and three-way interactions, and the original stem cutting mass to account for variability in initial biomass.

To test the influence of plant growth on the relationship between environmental conditions and total mycorrhizal colonization, we used the same model structure as Prediction 1a but included plant growth as a covariate (Table S1.8). Because plant growth varied in response to the experimental treatments, the effects of experimental treatments on total mycorrhizal colonization could have been mediated by these differences in plant growth rate. If the inclusion of the plant

growth term in our model substantially reduced the significance or strength of the effect of environmental variables on total colonization (compared to the model without plant growth used for Prediction 1a), then we interpret this as evidence that the effects of those environmental variables on total colonization were mediated through plant growth.

Prediction 2: EM vs. AM Investment Tradeoff

Factors controlling AM colonization were tested using a linear mixed model and ANOVA test (Table S1.9). The response variable, %AM, was arcsin-square root transformed to reduce heteroscedasticity and meet the homogeneity of variance assumption of ANOVA. The explanatory variables included the experimental variables and all two- and three-way interaction terms (Nutrient*Light*Moisture), as well as the aforementioned metadata covariates. Differences between specific treatment groups were assessed and compared using the R package emmeans (Lenth 2022; Tables S1.10 and S1.11). EM colonization was analyzed using the same model structure as AM colonization and %EM was similarly arcsin-square root transformed for consistency and to facilitate comparison between models (Table S1.12).

Finally, to test whether plants faced a tradeoff in investment between AM and EM colonization, we used a modified linear mixed model. We used arcsin-square root transformed %AM as the response variable and included %EM as a quantitative

predictor along with three treatments (Light, Moisture, and Nutrient conditions) and all two- and three-way interactions between treatments (Table S1.14). Emmeans and Tukey's HSD post hoc tests were used to assess differences between treatment means. In this model, a strong and significant negative effect of the %EM variable would indicate a trade-off in investment. Significant effects of experimental treatments then demonstrate whether a given treatment level leads to higher or lower AM colonization than would be expected given the level of EM colonization in that treatment (i.e., whether the treatment allows for greater or lesser total colonization). This model also tests whether the effects of experimental conditions on AM colonization observed in the previous model (Table S1.9) represent the direct effects of those treatments or are instead mediated by the change in EM colonization. If a treatment was a significant predictor of AM colonization in the previous model but is no longer significant in the current model, this indicates that the treatment primarily affects AM colonization indirectly through its effect on EM colonization.

RESULTS

Prediction 1: Total Colonization

Prediction 1a – Environmental conditions

Total mycorrhizal colonization (AM + EM) was significantly and positively affected by soil moisture ($p < 0.0001$, Table S1.3, Figure 1), with colonization in

low moisture levels being significantly lower than both medium and high moisture levels (p = 0.0194, <.0001, respectively; Table S1.4). Medium and high moisture were mildly, and marginally significantly, different from each other (p = 0.0826, Table S1.4). The Light and Nutrient treatments did not have significant direct effects on total mycorrhizal colonization (p = 0.31701, p = 0.08654, respectively; Table S1.3).

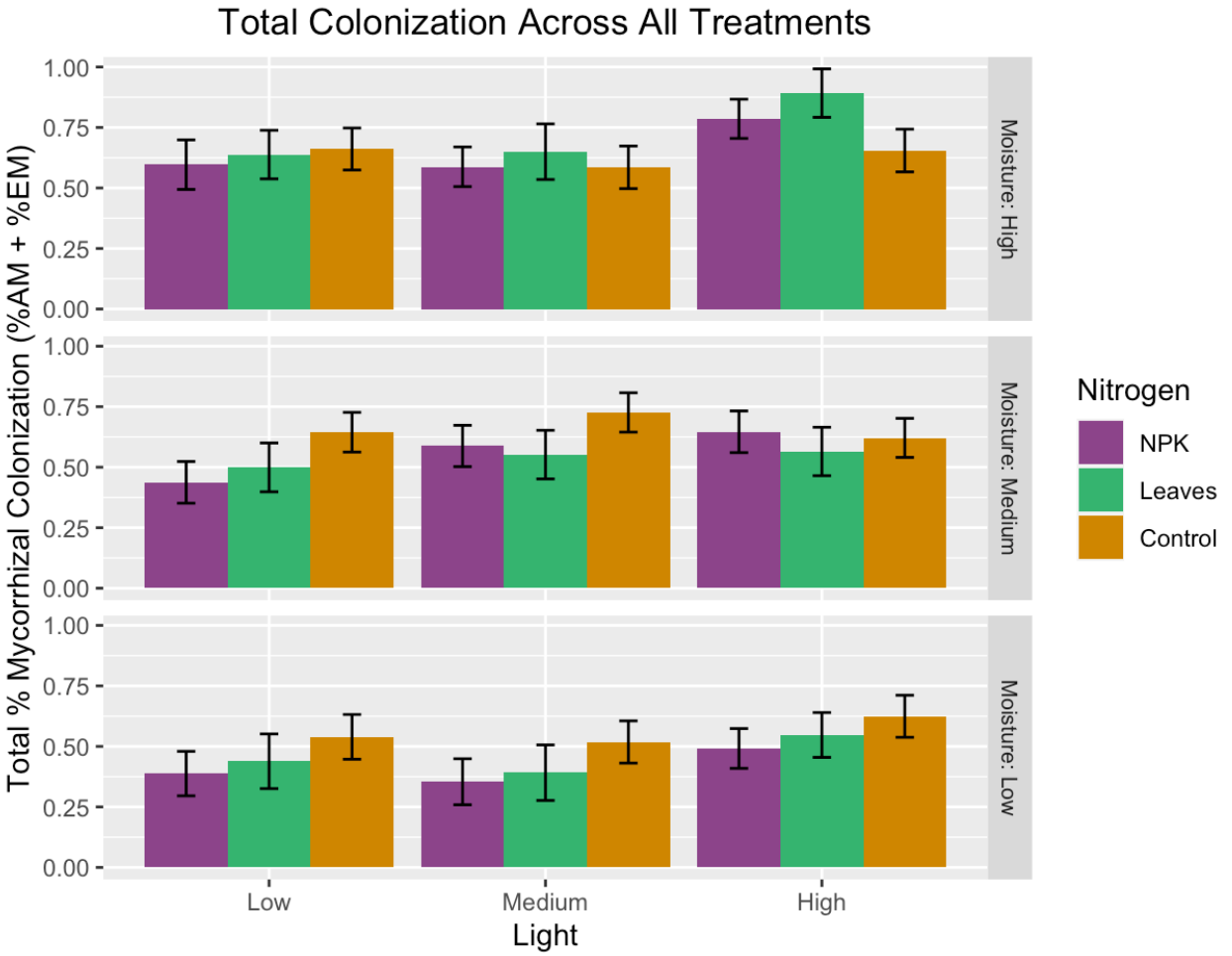

Figure 1. Proportion of total mycorrhizal colonization (%AM + %EM) by treatments. Moisture was significantly and positively correlated with total colonization. However, this signal was found to be mediated by plant growth (see Figure 2).

Prediction 1b – Plant Growth

Moisture (p = <.0001; Table S1.5) and Nutrient treatments (p = <.0001; Table S1.5) were the primary drivers of plant growth, with a strong interaction between Moisture and Nutrient treatments (p = <.0001; Table S1.5) and a mild interaction between Moisture, Nutrient, and Light treatments (p = 0.06473; Table S1.5). Overall, plants exhibited the greatest rates of growth in conditions of high moisture, high nutrient quantity and quality (inorganic-addition), and high light (Table S1.6). Conversely, conditions of low moisture, low nutrient quantity and quality (no-addition), and low light yielded low levels of new growth.

Plant growth was also found to be positively correlated with total colonization (p= 0.0001533; Table S1.7). Since plant growth was itself determined by Moisture and Nutrient treatments (Table S1.5), we tested whether plant growth mediated the effect of these treatments on total colonization. When we included plant growth as a covariate in the same model with our experimental treatments, we found that the significance and strength of the Moisture treatment effect on total colonization were reduced, indicated that the effect of Moisture on total colonization was largely mediated by its effect on plant growth. However, the strength and significance of the effect of the Nutrient treatment were not greatly affected, indicating that the Nutrient treatment effects total mycorrhizal colonization independently of changes in plant growth (Table S1.8; Figure 2).

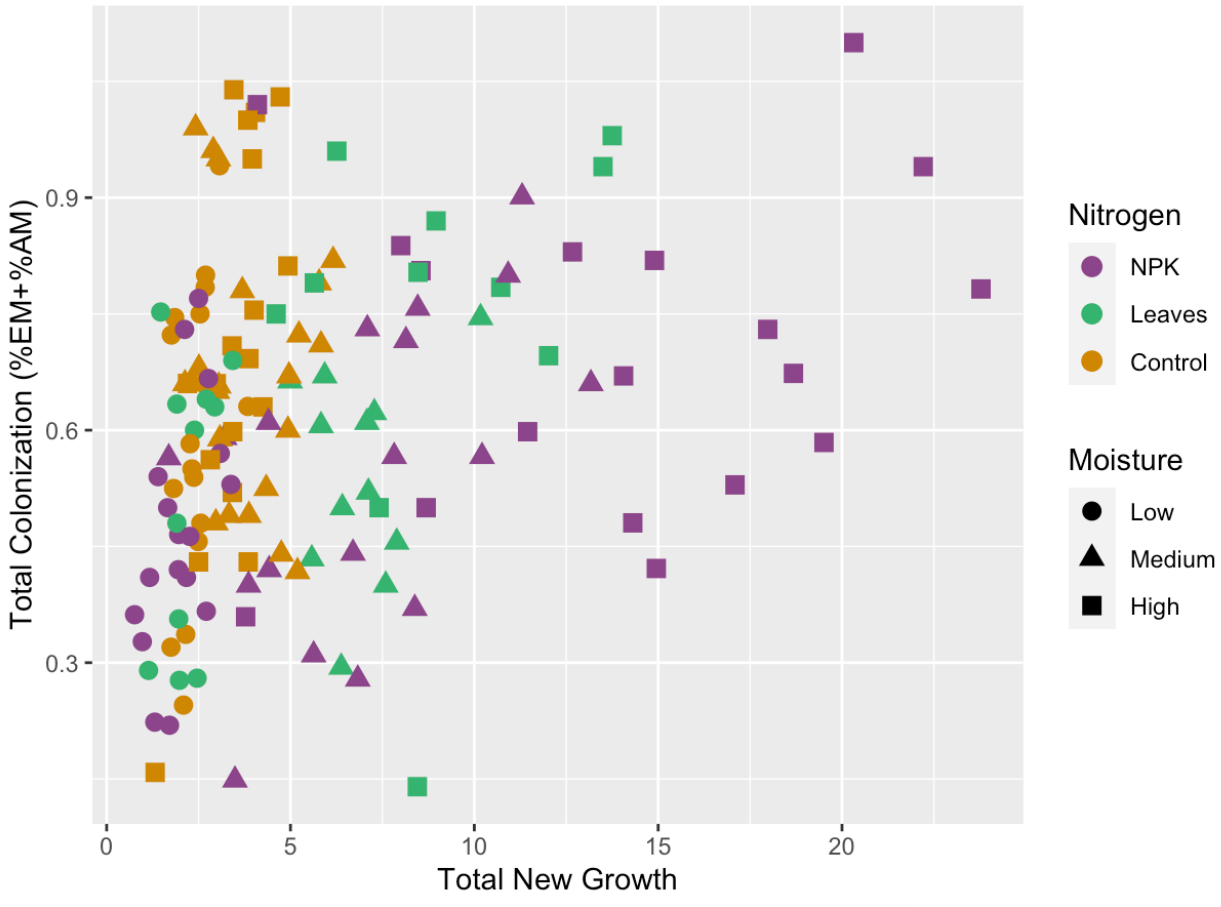

Figure 2. The relationship between moisture and total colonization was mediated by plant growth; high moisture treatment produced larger plants which exhibited higher levels of total colonization.

Prediction 2: EM vs. AM Investment Tradeoff

Across all Nutrient, Light, and Moisture environments, there was a strong negative correlation between AM and EM colonization ($p = 0.0007035$, Table S1.14), providing evidence for a biological tradeoff between the two guilds (Figure 3). This tradeoff was driven by Nutrients and Light, where low light/inorganic-

addition treatments clustered near the high AM/low EM investment end and high light/no nutrient addition treatments clustered near the low AM/high EM end of the tradeoff gradient (Figure 3). The response in the organic-addition treatment spanned the AM/EM tradeoff axis regardless of Moisture and Light conditions. As with the high light/no nutrient addition treatments, low and medium light/no nutrient addition treatment combinations were also restricted to high values of EM colonization, with all no-addition treatment means spanning between ~35% and ~60% EM colonization as opposed to the ~10% and ~55% for the two nutrient addition treatments (Figure 3). Nutrient status was, therefore, the primary factor influencing EM colonization ($p = <.0001$, Table S1.12), which was driven by these no nutrient addition treatments. The relationship between nutrient status and EM colonization was somewhat mediated by Moisture (N:M $p = 0.03064$, Table S1.12), where the difference in EM colonization between no-nutrient and nutrient addition treatments was only significant in low and medium moisture levels and lost in high moisture (Table S1.13; Figure 3).

AM colonization was explained by Nutrient status ($p = 0.001738$; Table S1.9), an interaction between Nutrients and Light ($p = 0.007714$; Table S1.9), and mildly by Moisture ($p = 0.066392$; Table S1.9). In both inorganic-addition and no-addition treatments, AM colonization was higher in low light; however, the effect of light levels in the organic-addition treatment was less straightforward (Table S1.11). AM colonization was positively affected by Moisture level (Table S1.10), which is consistent with our results presented above on total colonization.

The strength of the tradeoff between AM and EM colonization was driven by Moisture ($p = 0.0049499$, Table S1.14) as well as an interaction between Nutrients and Light ($p = 0.0174423$; Table S1.14). High Moisture levels led to higher values of AM colonization per unit EM colonization, which is consistent with our prior analysis showing higher total colonization in high moisture treatments (see Prediction 1a results). High light conditions led to less AM colonization per unit EM colonization in the no-addition treatment compared to nutrient addition treatments, which is consistent with our analysis showing enhanced EM and reduced AM colonization in these conditions.

Overall, these results support our general hypothesis: plants faced a tradeoff between investment in EM and AM colonization that was mediated by environmental factors controlling photosynthetic rates, and the greatest EM colonization (and lowest AM colonization) was in environments where nutrients were low in quantity and quality/organic (no addition treatment).

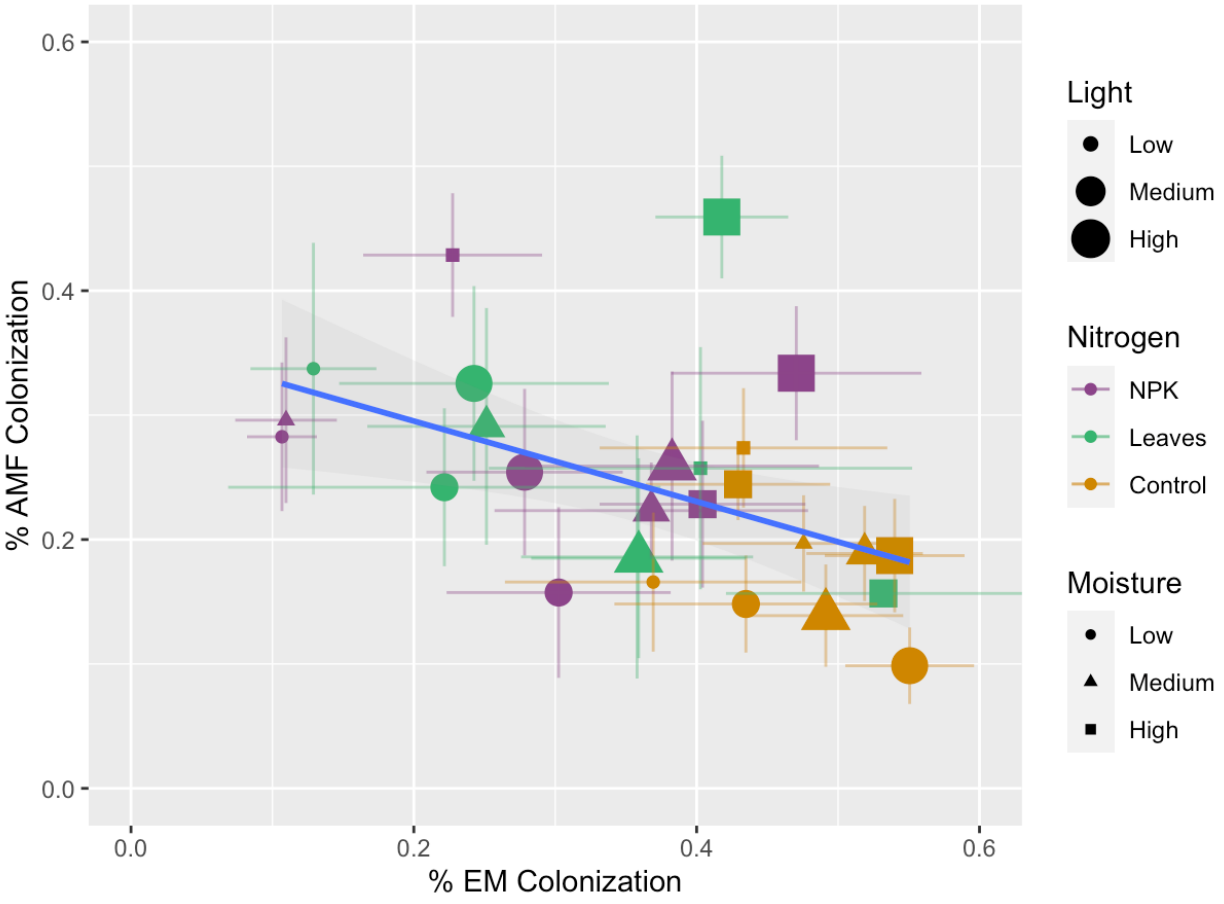

Figure 3. Investment tradeoff between %AM and %EM colonization. Points show mean values for each three-way treatment combination (nitrogen x moisture x light). The tradeoff is driven by nutrients and light, where low light/inorganic-addition treatments clustered near the high AM/low EM investment end and high light/no nutrient addition treatments clustered near the low AM/high EM end of the tradeoff gradient. No nutrient addition treatments were restricted to the low AM/high EM end of the tradeoff, regardless of light and moisture treatments.

DISCUSSION

Plants employ a range of strategies to acquire necessary soil nutrients, including uptake via their own roots and partnerships with different types of mycorrhizal fungi. Dually-colonized plants, which can interact simultaneously with both arbuscular and ecto-mycorrhizal fungi, offer a unique system in which to interrogate how plants navigate the demands for nutrient access with the costs of different nutrient acquisition. Here, we found that dually colonized *P. deltoides* plants employed flexible mycorrhizal strategies that were well predicted by the anticipated benefits of each mycorrhizal type and the ability of plants to pay the associated carbon costs.

We predicted that total mycorrhizal colonization (summing over both arbuscular and ectomycorrhizal symbioses) would increase in environments where the predicted benefits were high (low nutrient accessibility) and the ability of the plant to pay the carbon costs of the symbionts was also high (high light and high moisture environments). Our prediction was supported for soil moisture, though there were no consistent differences among our nutrient and light availability treatments. Moisture appears to have been a primary environmental filter limiting plant growth in our experiment. All plants grown in low moisture conditions were small: a pattern evident in the data but also documented observationally during experimental growth. Plants in the low moisture condition frequently lost leaves during the experiment and were regularly restricted to

their newest leaves, which were also very small. This further suggests that plants were water stressed and seeking ways to avoid transpiration loss. However, when moisture limitations were relieved, the effects of nutrient and light conditions on plant growth became evident. Plants that did not receive a nutrient addition, however, appeared to be co-limited by moisture and nutrients and acquired very little new growth even when provided with high levels of moisture and light.

One of the purported benefits of mycorrhizal associations to plants is drought tolerance through increased water acquisition (Lehto and Zwiazek 2011, Augé 2001 and 2004, RuizLozano and Azcon 1995, Kivlin et al. 2013). However, stomates close when moisture is low which reduces rates of photosynthesis and, ultimately, carbon budgets. Therefore, variations in soil moisture could have theoretically selected for increased colonization, due to increased symbiont investment to relieve moisture limitation, or decreased colonization, due to carbon constraints. Since moisture influences both the costs and benefits of mycorrhizal colonization, the effects of moisture on mycorrhizal investment are challenging to predict from a cost:benefits economic framework. In this experiment, however, total colonization was positively correlated with moisture, indicating that despite whatever water-acquisition benefits mycorrhizae may be able to offer to plants, carbon costs may have limited their ability to invest.

The relationship between soil moisture and total colonization was mediated by plant growth. Higher moisture levels produced larger plants, and larger plants

were able to increase their mycorrhizal colonization. This suggests that plants in high growth conditions, where photosynthetic rates were not limited, had adequate carbon to invest in mycorrhizal symbionts; conversely, plants experiencing growth limitation may have been less able to invest in symbionts.

The general relationship between plant growth and total colonization was maintained across nutrient treatments, though an interesting pattern emerged between the nutrient addition and no-addition treatments. Plants in the no-addition treatment displayed more colonization than would have been otherwise predicted by plant size, and this increased total colonization was driven by increased EM colonization. Therefore, although size (and correlated photosynthetic rate) may determine a plant's carbon "budget" for symbiotic investment, when nutrients are limiting growth, investing in the nutrient acquisition abilities of EM fungi appeared to be a necessity, despite the presumably high carbon cost (see below).

We detected evidence for a biological tradeoff between investment to EM versus AM symbioses in dually-colonized *P. deltoides*, where EM colonization increased as AM colonization decreased. Plants in the low nutrient quantity and quality conditions, where plants were reliant entirely on soil organic matter for nutrients, clustered at the high EM/low AM end of this tradeoff. Interestingly, plants in the lowest nutrient conditions appeared to be restricted to the high EM/low AM investment strategy regardless of light and moisture conditions. This indicates

that these plants were nutrient limited and lacked the flexibility to employ another nutrition acquisition strategy. This is consistent with our findings on the relationship between moisture, plant growth, and total colonization (Prediction 1b, discussed above). Even after plants in low nutrient conditions were relieved of moisture limitation, they remained small. Furthermore, they displayed high levels of colonization for their size, which was driven by increased EM colonization. Therefore, when nutrients are limiting plant growth and the source of nutrients is both limited and organic (soil organic matter), then the high EM/low AM strategy appeared to be the only viable nutrient acquisition strategy despite the high carbon costs.

This finding is consistent with the cost:benefit economic framework, where plants facing low levels of recalcitrant nutrients yet possessing an adequate carbon budget (not light limited) are able to invest in EM fungi and potentially benefit from their unique nutrient acquisition capabilities. On the other hand, plants given access to additional mineral nutrients while facing low light levels clustered at the low EM/high AM end of the trade-off spectrum. This suggests that plants with readily-available mineral nutrients coupled with carbon constraints likely had less to gain from the EM symbiosis, coupled with a reduced ability to “pay” for a more expensive interaction on an already limited carbon budget. These dually-colonized plants were thus able to flexibly switch to a lower cost symbioses, which was still adequate to meet their nutrient acquisition needs when these nutrients were available in an already mineralized form.

As a high quantity yet low quality (organic) nutrient source, our litter addition treatment provides an interesting comparison to the relatively accessible (high quantity-inorganic) and inaccessible (low quantity-organic) nutrient scenarios. Consistent with the cost:benefit economic model, leaf litter could be considered a recalcitrant nutrient source and select for the enzymatic capabilities of EM fungi, providing photosynthate costs are not restrictive. In this study, however, regardless of moisture and light conditions, plants in the leaf litter (organic addition) treatment spanned the EM/AM tradeoff gradient and were not restricted to a particular EM/AM ratio. This pattern differed from the no-addition nutrient treatment, where nutrients were present solely in soil organic matter (SOM) in the background soil and inoculum and plants were restricted to high EM/low AM values. The differential response between our leaf litter and no-addition treatments could be the result of both the different quantities of organic nitrogen as well as the different chemical profiles of leaf litter and SOM. All else being equal, the addition of more organic material would be expected to increase the net rate of nitrogen mineralization in our pots. Additionally, the leaf litter likely contained a more heterogenous mix of recalcitrant and more quickly mineralizable forms of nutrients (especially nitrogen) than SOM. However, the rate of mineralization is mediated by biological and chemical processes which are often controlled by environmental factors. The rate of nitrogen mineralization from our leaf litter additions likely varied among our experimental pots. Some of this variation was likely due to our experimental

treatments; for example, we expected higher mineralization rates in high versus low moisture conditions (Agehara and Warncke 2005). However, other environmental conditions may have varied even within our experimental treatments. For instance, temperature is known to influence rates of nitrogen mineralization (Agehara and Warncke 2005, Cassman and Munns 1980, Guntiñas et al. 2012). Although our greenhouse was temperature-controlled at the room-level, it is possible that "pot level" differences occurred that were not captured in our statistical models. This may have led to different experienced nutrient environments within the leaf litter addition treatment, producing the more variable response in colonization patterns seen in this study.

Overall, this study provides evidence that dually colonized *Populus deltoides* plants are able to shift their mycorrhizal investment strategies in adaptive ways. Though *P. deltoides* was the only species included in this study, it is plausible that other dual species are capable of similar adaptive shifts. This finding highlights a unique flexibility of *P. deltoides*, and potentially all dually-colonized species, in navigating cost:benefit tradeoffs in differing environmental conditions. This corroborates the findings of VanNuland et al. (2023) and Lodge (1989), who also report a negative relationship between EM and AM colonization in *P. deltoides*; however, VanNuland et al. (2023) found that this relationship was better explained by projected litter decomposition rate than soil nutrient conditions. Had our study measured nitrogen mineralization rates, perhaps we would find the same conclusion.

Populus deltoides boasts an extensive latitudinal range and grows robustly in dynamic and ephemeral habitats, such as flood plains, where many plant species struggle to survive (Segelquist et al. 1993, Rodríguez et al 2020). It is possible that *P. deltoides* is able to maintain a broad range and thrive in these challenging habitats due to their ability to associate with a broader range of fungal symbionts, which potentially offer a broader range of services to the plant. This is further emphasized given the recent prediction that most of the EM taxa associated with *Populus* may be at risk due to climatic changes (Van Nuland et al. 2023). Dual species have also been noted to be highly competitive and often limited by soil fertility (Brundrett and Tedersoo 2018).

The conditions in which *P. deltoides* invests more heavily in EM and AM fungi correlates with global latitudinal patterns of soil nutrient cycling and mycorrhizal guild dominance. In this study, *P. deltoides* exhibited increased EM (and decreased AM) investment when nutrients were limited in quantity and bound up in SOM; this is consistent with the patterns of EM-dominance in northern latitudes, where nutrients are bound up in SOM (Steidinger et al. 2019). Conversely, *P. deltoides* exhibited increased AM (and decreased EM) investment when nutrients were inorganic and relatively abundant, which is consistent with patterns of AM-dominance and open mineral nutrient cycling in tropical latitudes. This suggests that mechanisms driving the investment strategy in dually colonized plants may also be driving the current ranges and distributions

of non-dual plants at the global scale. Additionally, it highlights the value and potential of dually-colonized plants as models for mycorrhizal and ecological studies.

Models of global nutrient cycling and prediction of global change have recently begun to incorporate mycorrhizal interactions (Lu and Hedin 2019, Sulman et al. 2017, Fischer et al. 2010, Brozostek et al. 2014, Aas et al. 2023, Jo et al. 2019). Central to these predictions are the assumed benefits and costs of ecto- vs. arbuscular mycorrhizal strategies. Here, we show that in a dually-colonized species, shifts in investment between the mycorrhizal strategies were well predicted by an economic framework that assumes a higher carbon cost in return for unique nutrient acquisition traits of ecto- vs. arbuscular mycorrhizal strategies. As global changes ensue, the importance of understanding factors which drive current forest structure as well as species ranges and distributions becomes increasingly important in accurately predicting how forested systems will respond.

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CHAPTER TWO

Ectomycorrhizal colonization of *Populus tremuloides* is a function of genotypic and environmental factors

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ABSTRACT

Ectomycorrhizal (EM) and arbuscular mycorrhizal (AM) strategies exist along a latitudinal gradient, which is thought to be the result of their differential nutrient acquisition abilities and latitudinal gradients in nutrient cycling. EM fungi are able to enzymatically access nitrogen from organic matter, and plants associated with EM fungi tend to have more recalcitrant litter chemistry. This creates the potential for feedback loops in which trees with more recalcitrant litter chemistry benefit from greater EM investment, which could potentially lead to trait correlations that have been genetically co-selected as an adaptive trait complex. *Populus tremuloides* has high chemical and genetic variation as well as a notable life history strategy of persisting in extensive and long-lived clonal stands, where the primary nutrient inputs for trees are the stand's own litter. This temporal

and spatial consistency creates the exact conditions required for phenotypic feedbacks to become genetically fixed. Using a *P. tremuloides* common garden system, this study aims to determine 1) the degree of genetic variation in plant tissue chemistry and mycorrhizal traits among *P. tremuloides* genotypes, 2) whether correlations between tissue chemistry and mycorrhizal traits represent phenotypic responses to shared (or induced) environmental conditions, and 3) whether any correlations represent co-selected genetic complexes. Overall, broad-sense heritabilities were highest for plant tissue chemistry traits, which are most closely linked to plant gene products. However, the degree of EM colonization had a heritability of 0.26 and was the only aspect of fungal communities that was genetically variable among *P. tremuloides* clones. Abscised leaf C:N was the only trait correlated with EM colonization at both the phenotypic and genotypic level, indicating co-selection between the alleles for these traits. *Cortinarius* was the most abundant EM genera in root communities and its relative abundance was positively correlated with abscised leaf C:N as well as root phenolic glycosides. Overall, these results indicate that *P. tremuloides* litter chemistry and mycorrhizal investment are genetically and functionally linked and that tree litter chemistry has the potential ability to promote nutrient acquisition feedback loops through its effects on EM functional diversity.

INTRODUCTION

Plants access nutrients in two primary ways: through their own roots and through mycorrhizal symbionts, with ecto- (EM) and arbuscular mycorrhizal (AM) fungi being the two most common and widespread guilds (Kivlin 2020). As highlighted in Chapter 1, mycorrhizal nutrient acquisition strategies exist along a distinct latitudinal gradient (Lu and Hedin 2019): a pattern thought to be driven, in part, by the unique ability of EM fungi to enzymatically "mine" for nitrogen from organic matter (Hobbie et al. 2013). In high latitudes, where much of the available nitrogen is bound up in organic matter and the climatic window for decomposition is limited, the enzymatic capabilities of EM fungi may provide an advantage to EM-associated trees.

Differential litter quality between EM- and AM-associated plants may also perpetuate regional feedbacks. EM-associated trees tend to have lower quality litter than that of AM-associated trees (Cornelissen et al. 2001, Wurzburger and Brookshire 2017, Phillips et al. 2013). Over many generations, plant tissue chemistry can reflect adaptations to sustained resource conditions, and both leaf and root tissue chemical phenotypes can represent coordinated syndromes. The leaf economic spectrum is well established (Wright et al. 2004, Reich et al. 1997) and largely reflects a spectrum between resource conservation, where leaves are structurally expensive but longer lived and have low nitrogen content and photosynthetic rate, versus resource acquisition, where leaves have high specific

leaf area (SLA) and photosynthetic rate, but also high turnover and nitrogen content. Root economic traits are less well established but can also show coordinated syndromes reflective of resource conservative versus acquisitive traits (Ma et al. 2018b, Kong et al. 2019, Reich 2014, Eissenstat 1992, Freschet et al 2010, Ryser and Eek 2000, Ostonen et al 2007), which has recently been coupled with a resource collaboration gradient including mycorrhizal associations (Bergmann et al. 2020). Mycorrhizal strategies and plant tissue traits may thus reflect shared evolutionary responses to nutrient limitations, with the EM trait tending to co-occur with resource conservative tissue traits.

However, litter chemistry also varies within each mycorrhizal guild and even within plant species. Variation in plant chemistry can be a phenotypically plastic response to the current nutrient environment, where plants may shift their tissue chemistry in accordance with whether nutrients are high or low. Similarly, the degree of mycorrhizal colonization and the composition of the mycorrhizal fungal community on individual plants are also plastic and responsive to the nutrient environment (see Chapter 1, Frater et al 2018, Lilleskov and Bruns 2003). Though EM fungi are generally considered to possess the enzymatic capabilities to degrade organic matter, there is considerable intraguild variation in the type and number of enzymes produced by EM taxa (Kohler et al. 2015, Lindahl and Tunlid 2014, Pelletier and Zak 2018). Additionally, EM species are known to have different “exploration types”, or general patterns of morphological traits associated with root-tip colonization and extraradical hyphae (Agerer 2001).

These have often been considered functionally synonymous with foraging strategies, though recent evidence suggests that this relationship may be more complicated (Jörgensen et al. 2023). Regardless, variation in EM functional traits may result in differential nutrient acquisition abilities and efficiencies within the EM guild. Therefore, plant tissue chemistry and mycorrhizal traits may covary intraspecifically across landscapes as a result of shared phenotypic responses to nutrient limitations, again with nutrient conservation and acquisition traits corresponding to nutrient environment.

Plants also have the ability to shape their local nutrient environments through litter inputs (leaves, roots), which could, in turn, influence nutrient acquisition by the plant's associated mycorrhizae. This interplay between above and belowground nutrient acquisition processes has the potential to lead to closed plant-fungal nutrient feedbacks (Wurzburger and Hendrick 2009) and could further explain the latitudinal relationship between leaf litter quality and mycorrhizal dominance. In short, production of recalcitrant, low-quality litter may induce local nutrient limitation for a plant. Nutrient limitation could then induce a greater investment in mycorrhizal symbionts to alleviate this limitation, especially for ectomycorrhizal fungal symbionts with the enzymatic capabilities to mine nitrogen from the recalcitrant organic matter produced by low quality litter inputs. Plants may therefore be regularly associating with EM taxa that are best functionally equipped (enzymatically and morphologically) to acquire nutrients from their own litter chemistry, creating a feedback loop.

If plant chemistry-fungal acquisition feedbacks are 1) stable, 2) reflective – at least in part – of genetic variation in the underlying traits, and 3) selected for over many generations, it is possible that the phenotypic correlation between the two traits may become genetically fixed as part of an adaptive trait syndrome. Therefore, variation in leaf tissue chemistry as well as mycorrhizal investment and diversity could be the result of environmental conditions, genetic control, or a combination of both. Determining the influence of each, however, is confounded when certain traits that vary with genotype also influence the surrounding nutrient environment and mycorrhizal community.

To study the relationship between these interacting influences, we turned to a *Populus tremuloides* (aspen) model. *Populus tremuloides* is known to have a high degree of intraspecific genetic variation (Mitton and Grant 1996, Madritch et al. 2006), producing variability in ecologically relevant traits such as leaf litter quality (Lindroth et al. 2002). Two particularly strong axes of chemical variation in *P. tremuloides* are concentrations of phenolic glycosides (PGs) and condensed tannins (Stevens et al. 2014, Lindroth 1987, Lindroth 2001). PGs and CTs are the primary secondary metabolites produced by *P. tremuloides* (Stevens et al. 2014) and are generally considered to be herbivore defense compounds. However, CTs have been shown to be ineffective against insect herbivore attacks (Barbehenn and Constabel 2011, Barbehenn et al. 2006) and are instead considered to be mediators of herbivore tolerance (versus resistance) due to their relationship

with increased (15)N recovery following herbivore defoliation (Madritch and Lindroth 2015). Given that variability in plant CT production is tightly and negatively correlated with plant nutrient availability (Davies et al. 1964), CTs have been studied for their role in nutrient cycling (Talbot and Finzi 2008, Northup et al. 1995 and 1998, Schimel et al. 1998) and have been shown to directly influence endophyte colonization (Bailey et al. 2005) and soil microbial communities (Bradley et al. 2000, Schweitzer et al. 2008). The effectiveness of PGs as herbivory defense mechanisms has been well established (see Stevens et al. 2014) and their influence on associated fungal communities in *Populus* has been documented (Veach et al. 2019). However, connections between PGs and nutrient cycling have been less well studied.

Populus species are also considered to be dually-colonized, or able to associate with both EM and AM fungi simultaneously (Teste et al. 2020), providing the potential for high flexibility in nutrient acquisition strategies. Additionally, a notable life history strategy of aspens is their ability to form clonal stands, which can grow to be quite large and persist for hundreds of years. This tendency to both reproduce clonally (which reduces the frequency of recombination scrambling co-adapted gene complexes) and exist in spatially-extensive and temporally-consistent stands may create the exact conditions necessary for the aforementioned feedback mechanism between litter chemistry and mycorrhizal strategy (Figure 1). Therefore, given the potential prevalence of these feedback loops over many generations, it is possible that the mycorrhizal associations of

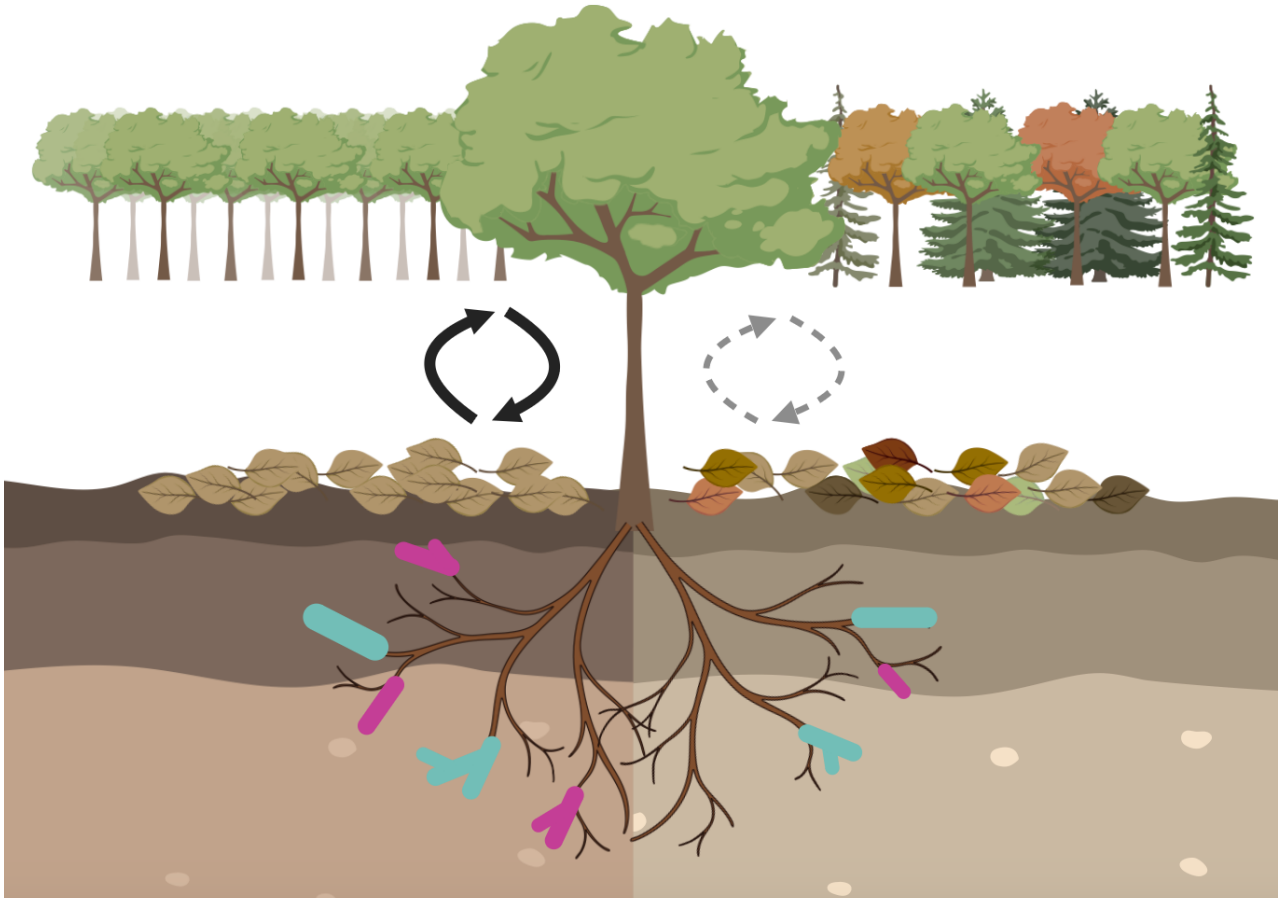

Figure 1. Schematic illustrating proposed feedbacks between Populus tremuloides leaf chemistry and ectomycorrhizal (EM) associations. The left pane shows a strong feedback between leaf litter chemistry and EM interactions as the result of relatively consistent chemical inputs over many generations in a clonal stand. The right pane illustrates an alternative scenario where a clonal stand is interspersed with trees from other taxonomic groups, resulting in mixed litter inputs and, ultimately, a weaker feedback between leaf litter chemistry and EM interactions. Created with BioRender.com.

aspens are under genetic control, at least in part. These clonal stands, however, undoubtedly experienced additional nutrient inputs throughout evolutionary time, such as those from individuals from other taxonomic groups interspersed within and around the stand (Figure 1). Therefore, the feedback between litter chemistry and fungal investment/partner selection may be weak, resulting in mycorrhizal associations that, instead, better represent a response to the current – and potentially transient – soil nutrient conditions.

This study aims to determine 1) the degree of genetic variation in plant tissue chemistry and mycorrhizal traits among *P. tremuloides* genotypes, 2) whether correlations between tissue chemistry and mycorrhizal traits represent phenotypic responses to shared (or induced) environmental conditions, and 3) whether any correlations represent co-selected genetic complexes. Specifically, we predict that:

Prediction 1: Heritability

Broad-sense heritabilities will be highest for traits most directly linked to plant gene products, with heritability values declining as traits become less directly linked to plant gene products.

Prediction 2: Phenotypic Correlations

If *P. tremuloides* plant litter chemistry and mycorrhizal traits covary in response to a shared nutrient environment, then we predict that plant chemistry and EM investment and functional diversity will be correlated at the phenotypic level.

Prediction 3: Genotypic Correlations

If *P. tremuloides* litter chemistry and mycorrhizal traits have experienced sustained feedbacks and become co-selected as part of an adaptive trait syndrome, then then we predict that plant tissue chemistry and EM investment and functional diversity will be correlated at the genotypic level.

METHODS

Experimental Design

WisAsp Common Garden: The WisAsp Garden is an aspen genotype common garden located in the Arlington Agricultural Research Station in Arlington, Wisconsin. The garden was planted in 2010 (with certain replacements replanted in 2011 and 2012) and contains approximately 3-5 replicates of roughly 500 different genotypes. Previous work has measured various chemical traits of each genotype, including phenolic glycoside (PG) and condensed tannin (CT) values (Cole et al. 2021, Barker et al. 2018 and 2019). This data was used to select 30 genotypes for this study that spanned the PG and CT ranges with low correlation

between the two traits based on prior measurements. Approximately three replicates for each genotype were sampled (with four genotypes having two replicates and five having four), for a total of 91 included individuals. The garden is rectangular in shape and sectioned into four blocks (referred to as “plot” in the analysis). Each block contained replicates of each genotype to account for environmental heterogeneity.

Tissue collection: Fine root samples were collected from each tree between July 13th and August 1st, 2020, by tracing roots back to the source individual to ensure proper genotypic identity. Soil samples were collected concomitantly from the soil directly surrounding the fine root collection in an attempt to best capture the nutrient environment of the roots collected for each individual. Both root and soil samples were stored on ice following collection. Roots were subsequently washed and processed for analysis. Roots allocated for EM and AM colonization metrics were refrigerated and air dried respectively; those for DNA sequencing were stored at -80˚F, and the remaining root material was vacuum dried for chemical analysis. Soil samples were dried for chemical analysis.

Within seven days of root collection, live green leaves were collected from each replicate according to lab-established protocols. This entailed selecting 3-6 leaves in an alternating pattern from 3-4 branches, each of which is connected to the main trunk of the tree and randomly selected from the bottom 2/3 of the canopy. Live leaves were subsequently stored on ice and vacuum dried. Naturally abscised

leaves were obtained between the dates of September 27th and October 16th, 2020, by gently shaking middle branches with a pole pruner and selectively collecting the leaves that fell to ensure genotypic identity. Abscised leaves were generally yellow to brown in color, and any potential variation in color between genotypes and individual replicates was not noticed. At least 20 individual leaves were collected for each replicate, which were stored on ice and subsequently vacuum dried.

Measurements: All tissues (roots, live leaves, and abscised leaves) were ground and analyzed for PG and CT concentrations as well %Carbon, %Nitrogen, and lignin using established methods. PGs were extracted in methanol, separated via reverse phase ultra performance liquid chromatography (UPLC), and quantified using electrospray ionization single quadrupole mass spectrometry (Acuity iClass UPLC/MS system from Waters; Milford, MA, USA) in negative ion mode (Rubert-Nason et al. 2018). CTs were extracted in a solution of 70% acetone and 10mM ascorbic acid, reacted with Fe(III) under acidic conditions, and concentrations were determined at 550nm using condensed tannins purified from *P. tremuloides* (Hagerman and Butler 1980, Porter et al. 1985). Carbon and nitrogen values were measured using combustion gas chromatography (Flash EA 1112 from Thermo Finnigan; Milan, Italy). Abscised leaves received an additional lignin assay, which was performed according the to the Acid Detergent Lignin protocol of the ANKOM 200 Fiber Analyzer (ANKOM; Macedon, NY, USA). Soil samples were analyzed for total nitrogen and C:N. Total and comparative (AM:EM)

colonization was quantified according to Brundrett et al. 1996, with AM roots being cleared and stained according to lab-tested protocols based on standard methods (Phillips and Hayman 1970, Deguchi et al. 2017).

Sequencing of fungal communities: Fungal communities were characterized via Illumina sequencing of the ITS2 rRNA gene region via methods described in Allsup et al. 2023 with some modifications. Briefly, roots were frozen with liquid nitrogen and ground using a SPEX Sample Prep 1600 MiniG tissue homogenizer (SPEX SamplePrep, Metuchen, NJ, USA). DNA extractions were prepared using the Omega E-Z 96 Plant DNA Kit (Omega Bio-Tek, Norcross, GA, USA) and the ITS2 region was amplified through polymerase chain reaction (PCR) using the ITS7 (Tedersoo et al. 2015) and ITS4 (White et al. 1990) primers. An additional round of PCR attached Illumina adapters and unique 12-base barcodes to each sample, which were then sequenced on one run using Illumina Miseq PE 2x300 chemistry. Sequences were denoised and clustered into exact amplicon sequence variants using the DADA2 program (Callahan et al. 2016) as implemented in the QIIME2 pipeline. Amplicon Sequence Variants were identified to the lowest possible taxonomic level using the RDP Naïve Bayesian classifier trained on the UNITE database (Abarenkov et al. 2010). Fungal species were assigned to guilds based on the Fungal Traits database (Polme et al. 2020). Fungi were assigned to one of six functional guilds based on genus identity (Arbuscular Mycorrhizal, Ectomycorrhizal, Plant Pathogen, Endophyte, Saprotroph, “Other”, and Unassigned). For genera with multiple lifestyles, we assigned guild with the

following priority: Ectomycorrhizal > Plant Pathogen > Endophyte > Saprotroph > Other.

Analysis

Predictions 1 and 2 were tested using linear mixed effect models in the lme4 package in R version 3.6.2. "Plot" was included a random effect in all models to account for non-independence of samples in the same plot and general spatial structure of the common garden. Graphs were made using the R package ggplot2.

Prediction 1: Heritability

Broad sense heritabilities for plant tissue and soil chemistries as well as fungal community structure were calculated using the following formula:

$$h_b = V_G / V_P = V_G / (V_E + V_G)$$

V_G represents genotypic variance, V_P represents phenotypic variance, and V_E represents environmental variance. To obtain these variance parameters, a linear mixed effect model was used which included the trait of interest as the response variable and both genet (genotype) and Plot as random effects. The genotypic variance was determined by the "genet" variance component, while the phenotypic variance was determined by the residual variance component (Table 1). Exact P-values for the genet variance component of each trait were generated via numerical simulation using the package RLRsim() and a series of three

models: the full model, which includes the trait of interest and both genotype and plot as random effects, as described previously; 2) a second model including the plant trait and genotype as a random effect, but excluding plot; and 3) a final model including the plant trait and plot as a random effect, but excluding genotype. These three models were then run through the command exactRLRT() to generate a p-value for the genotype random effect. Fungal community structure was represented using seven derived univariate variables: rarefied fungal richness (at a set sequence depth of 500 reads), Shannon and Simpson's diversity indices, and the proportion of four fungal guilds (AM fungi, EM fungi, plant pathogens, endophytes, and saprotrophs).

Prediction 2: Phenotypic Correlations

To test whether there were phenotypic correlations between heritable plant tissue chemistries and mycorrhizal investment, we used linear mixed effect models and ANOVA tests (R package lmerTest). Each model included EM colonization as the response variable and independent variables included the plant trait of interest, soil C:N, and both genotype and plot as random effects. Soil C:N was included to account for the surrounding nutrient environment, including any potential connection it had to plant tissue inputs. Both genotype and plot were included as crossed random effects to account for the non-independence of samples due to genetics and garden spatial structure. The summary() function was used to obtain effect estimates (Table 2). P-values were determined using Satterthwaite estimated denominator degrees of freedom

using the lmerTest package in R. Significant correlations between EM colonization and soil metrics were driven by three influential points (Figure S2.2, Table S2.1); when removing these points all signal was lost (Figure S2.3, Table S2.2). Therefore, soil chemistry metrics (soil C, N, C:N) were removed from the remaining correlation analyses to avoid misleading information.

To test whether there were phenotypic correlations between plant chemical traits and specific EM taxa, we used differential abundance analysis based on generalized linear models (GLM) to quantify the association of individual EM taxa relative abundance with plant tissue chemistry using DESeq2 within the Phyloseq package in R (Love et al. 2014). Based on rarefaction curves (function rarecurve() in R package vegan), we first omitted any samples that had less than 500 total fungal sequences, which reduced our dataset from 91 to 87 replicates. We then restricted the analysis to samples with total EM presence greater than zero and summarized data to the genus level, since many ASVs could not be identified to specific species and because our guild- assignments were based on genus identities. Plant chemical traits used as predictors in the GLM were restricted to primary litter sources (abscised leaves and roots) and included CTs, PGs, and C:N of both abscised leaves and roots, respectively. Models generated coefficients for plant tissue chemistries on the log(2) scale and we adjusted corresponding P values for multiple comparisons using the False Discovery Rate (Benjamini and Hochberg 1995).

To test whether EM fungi sharing specific functional traits (lignin modifying enzymes or exploration types) showed consistent correlations with plant tissue chemistry, we performed a meta-analysis of differences in the mean taxa response of each fungal guild. Linear models using the log(2) Fold Change coefficients for each EM genus (generated in the differential abundance analysis described above) were included as dependent variables with EM functional traits included as independent variables. A linear mixed model was used when exploration "sub type" was included as an independent variable, and Distance was included as a random effect.

Prediction 3: Genotypic Correlations

To test whether there were genotypic correlations between EM colonization and heritable plant tissue chemistries, we used linear mixed effect models with genotype mean values for traits. First, we calculated genotype mean values for all traits as the estimated marginal means from linear mixed effects models that included EM colonization or each plant trait of interest as the response variables and soil C:N as an independent variable and genotype and plot as random effects. We used the emmeans() function to generate estimated marginal mean values of each trait for each genotype, controlling for the effects of plot and soil C:N. A series of linear models (R function lm()) and ANOVA tests were then used to test for genotypic correlations, where genotype mean EM colonization was the response variable in each model and genotype mean plant tissue chemistry values served as the explanatory variable in each respective model. The

summary() function was used to obtain effect estimates (Table 2). None of the relative abundance values for individual EM taxa or functional groups based on the ITS sequencing data displayed heritability values statistically greater than zero, therefore they were not included in this analysis.

RESULTS

AM colonization was only present in ~10% of samples and only in very low frequencies. This corroborates previously established findings that, despite the potential for dual colonization, the AM strategy is not common in field populations of *P. tremuloides* (VanNuland et al. 2023) and, when present, is primarily restricted to roots in deeper mineral soils (Neville et al. 2002). Therefore, we focus on ectomycorrhizal colonization and community composition hereafter.

Prediction 1: Heritability

Broad-sense heritabilities were, generally speaking, highest for traits most directly linked to plant gene products, which included live plant tissues (roots and green leaves). Green leaf chemistry showed consistently high heritabilities, ranging between 0.89 and 0.51 (Table 1). Root chemistry heritabilities were more variable, varying from 0.47 to 0 (Table 1). Most plant litter chemistry (abscised leaves) displayed intermediate levels of heritability, with the exception of PG

concentrations, which marked the highest heritability measured (0.91, P value < 0.0001). Ectomycorrhizal colonization had a broad-sense heritability of 0.26 (P value = 0.0164) and was the only fungal symbiont measurement with a broad-sense heritability statistically different than zero (Table 1).

Prediction 2: Phenotypic Correlations

Three plant tissue traits were significantly and positively correlated with EM colonization at the phenotypic level: Green Leaf %C (Estimate = 0.0339, P = 0.008), Abscised Leaf %C (Estimate = 0.025, P = 0.033), and Abscised Leaf C:N (Estimate = 0.0032, P = 0.029; Figure 2); Green Leaf C:N was mildly positively correlated (P = 0.062; Table 2). Although not statistically significant, both green and abscised leaf nitrogen content (%N) were negatively correlated with EM colonization (green leaf %N: Estimate = -0.107, P = 0.155; abscised leaf %N: Estimate = -0.0592, P = 0.229; Table 2). Therefore, the positive relationship between EM colonization and foliar C:N values was driven primarily by carbon content, but also partially by nitrogen content. A table of how plant tissue chemistry traits correlated to each other is included in the Supplement (Figure S2.1).

Ectomycorrhizal fungal community composition diverged along various axes of litter (abscised leaf and root) chemistry (Table 3). *Cortinarius* was the most abundant EM genus observed in root communities and positively correlated with Abscised Leaf C:N (log2 fold change: 0.878, P = 0.035) and Root PGs (log2 fold change: 0.607, P = 0.036), and negatively correlated with Abscised Leaf PGs (log2

fold change: -0.776, P = 0.03; Table 3). *Hymenogaster* and *Hebeloma* were the second and third most abundant EM genera, respectively, but neither was significantly correlated with any leaf or root chemistries (Table 3).

To test whether the variation in individual EM genera response to plant tissue chemistry reflected consistent responses among functional groups, we compared the average response of each genus (fold change across a tissue chemistry gradient) between groupings based on functional traits. The EM genera known to contain lignin-degrading (or modifying) enzymes (Table 3), had consistently more positive correlations with Abscised Leaf C:N compared to the genera that do not produce these enzymes (P = 0.00787, Table S3; Figure 3). EM exploration distance also explained the EM genera response to Abscised Leaf C:N (P = 0.02536, Table S2.4), with the long-distance foraging taxa displaying higher average correlations to abscised leaf C:N compared to short (contrast, P = 0.0389, Table S2.5) and medium (contrast, P = 0.0578, Table S2.5) distance foraging taxa (Figure S2.4). However, there were only two long-distance foragers (*Scleroderma* and *Paxillus*) present in the community and at relatively low abundance levels (Table 3). Additionally, the one contact foraging taxon (*Russula*) also exhibited a high correlation to Abscised Leaf C:N value (Figure S2.4); therefore the relationship between exploration distance and the Abscised Leaf C:N was not quantitatively linear.

Prediction 3: Genotypic Correlations

Of the traits with significant genetic variation (genotype random effect statistically different than zero; Table 1), two plant tissue traits were significantly correlated with EM colonization at the genotypic level: Abscised Leaf %N (Estimate = -0.173, P = 0.041) and Abscised Leaf C:N (Estimate = 0.00612, P = 0.0069); Abscised Leaf %C was mildly correlated (Estimate = 0.0317, P = 0.097; Table 2). Abscised Leaf C:N was the only trait that correlated with EM colonization at both the phenotypic and genotypic level (Figure 2). EM taxa and functional guilds did not show genetic variability (Table 1) and were therefore not included in this analysis.

Sample Type	Trait	Genetic Variance	Residual Variance	Broad Sense Heritability	P value	
Plant Litter	AbsLeaf Total PG %dw	3.10297	0.32026	0.906445083	2.20E-16	*
Live Plant Tissue	Total GLeaf PG %DW	8.869	1.132	0.886811319	2.20E-16	*
Live Plant Tissue	Green Leaf CT (%dw)	4.919	2.093	0.701511694	2.20E-16	*
Live Plant Tissue	GreenLeaf_%C	0.55909	0.49927	0.528260705	2.20E-16	*
Live Plant Tissue	GreenLeaf Ratio C:N	1.8637	1.7264	0.51912203	2.20E-16	*
Live Plant Tissue	GreenLeaf_%N	0.015813	0.015335	0.507673045	2.20E-16	*
Live Plant Tissue	Total Root PG %DW	0.34007	0.38126	0.471448574	2.20E-16	*
Plant Litter	Abscised Leaf CT (%dw)	2.8178	3.8694	0.421372174	2.20E-16	*
Plant Litter	AbsLeaf_%C	0.46302	0.72542	0.389603177	3.00E-04	*
Plant Litter	AbsLeaf Ratio C:N	25.39	51.334	0.330926438	0.0013	*
Live Plant Tissue	Root Ratio C:N	23.87	49.66	0.324629403	0.0027	*
Live Plant Tissue	Root_%N	0.01346	0.02807	0.324103058	0.0068	*
Plant Litter	Abscised.Leaf.ADL...d.w..	2.8444	9.3065	0.234089656	0.0099	*
Fungal Symbionts	EM Colonization	0.003476	0.010027	0.257424276	0.0164	*
Plant Litter	AbsLeaf_%N	0.014356	0.050744	0.220522273	0.0203	*
Live Plant Tissue	Root CT (%dw)	0.02422	0.08271	0.22650332	0.0289	*
Fungal Symbionts	%Plant Pathogens/total fungi	0.002338	0.037249	0.059059792	0.3102	
Fungal Symbionts	%EM/total fungi	0.000632	0.040881	0.015224147	0.42	
Soil	Soil Ratio C:N	0.02078	1.9846	0.010362126	0.4434	
Soil	Soil_%C	1.268E-18	0.2119	5.98395E-18	0.4751	
Fungal Symbionts	%Saprotrophs/total fungi	0	0.02305	0	0.4815	
Fungal Symbionts	Fungal Richness	0	201.36	0	0.4931	
Live Plant Tissue	Root_%C	9.765E-08	4.788	2.03947E-08	1	
Fungal Symbionts	Simpson Diversity	8.905E-09	3.544	2.5127E-09	1	
Fungal Symbionts	Fungal Rarified Richness	1.474E-07	73.65	2.00136E-09	1	
Soil	Soil_%N	0	0.001701	0	1	
Fungal Symbionts	Shannon Diversity	0	0.2042	0	1	
Fungal Symbionts	%AM/total fungi	0	1.221E-07	0	1	

Table 1. Broad-sense heritability values of plant and soil chemical traits and fungal symbiont traits. Plant traits most closely linked to plant gene products, such as live plant tissue and plant litter chemistries, tended to show the highest heritability values, whereas fungal symbiont and soil traits showed lower heritability values. Ectomycorrhizal (EM) colonization was the only fungal trait with a heritability statistically different than zero.

	EM Colonization					
Plant trait	Phenotypic Correlation			Genotypic Correlation		
Abscised Leaf %C	**0.033376**	+	*	*0.0972*	+	.
Abscised Leaf %CT	0.659161	+		0.59577	+	
Abscised Leaf %N	0.228528	-		**0.0413**	-	*
Abscised Leaf %PG	0.2923084	+		0.506	+	
Abscised Leaf C:N	**0.028946**	+	*	**0.00693**	+	*
Abscised Leaf Lignin (ADL)	0.7569802	+		0.6594	-	
Green Leaf %C	**0.008016**	+	*	0.124	+	
Green Leaf %CT	0.15866	+		0.933645	-	
Green Leaf %N	0.155106	-		0.4144	-	
Green Leaf %PG	0.5161497	+		0.811	+	
Green Leaf C:N	*0.061908*	+	.	0.309	+	
Root %CT	0.7539128	+		0.9003	+	
Root %N	0.3530711	-		0.3737	-	
Root %PG	0.5184498	-		0.75509	-	
Root C:N	0.271376	+		0.665	+	
Root %C	0.3594637	+				
Fungal Richness	0.405669	+				
Fungal Rarified Richness	0.2988727	+				
Shannon Diversity	0.3827658	+				
Simpson Diversity	0.736684	+				
%EM/total fungi	0.202461	+				
%AM/total fungi	0.874291	+				
%Endophytes/total fungi	0.319432	+				
%Plant Pathogens/total fungi	0.697846	+				
%Saprotrophs/total fungi	0.793104	+				

Table 2. Phenotypic and genotypic correlation P-values between plant traits and EM colonization. Those with values < 0.05 indicate a statistically significant interaction. Directional symbols for whether interactions were positive (+) or negative (-) are included. Missing genotypic correlations are due to lack of genetic variance (see Table 1).

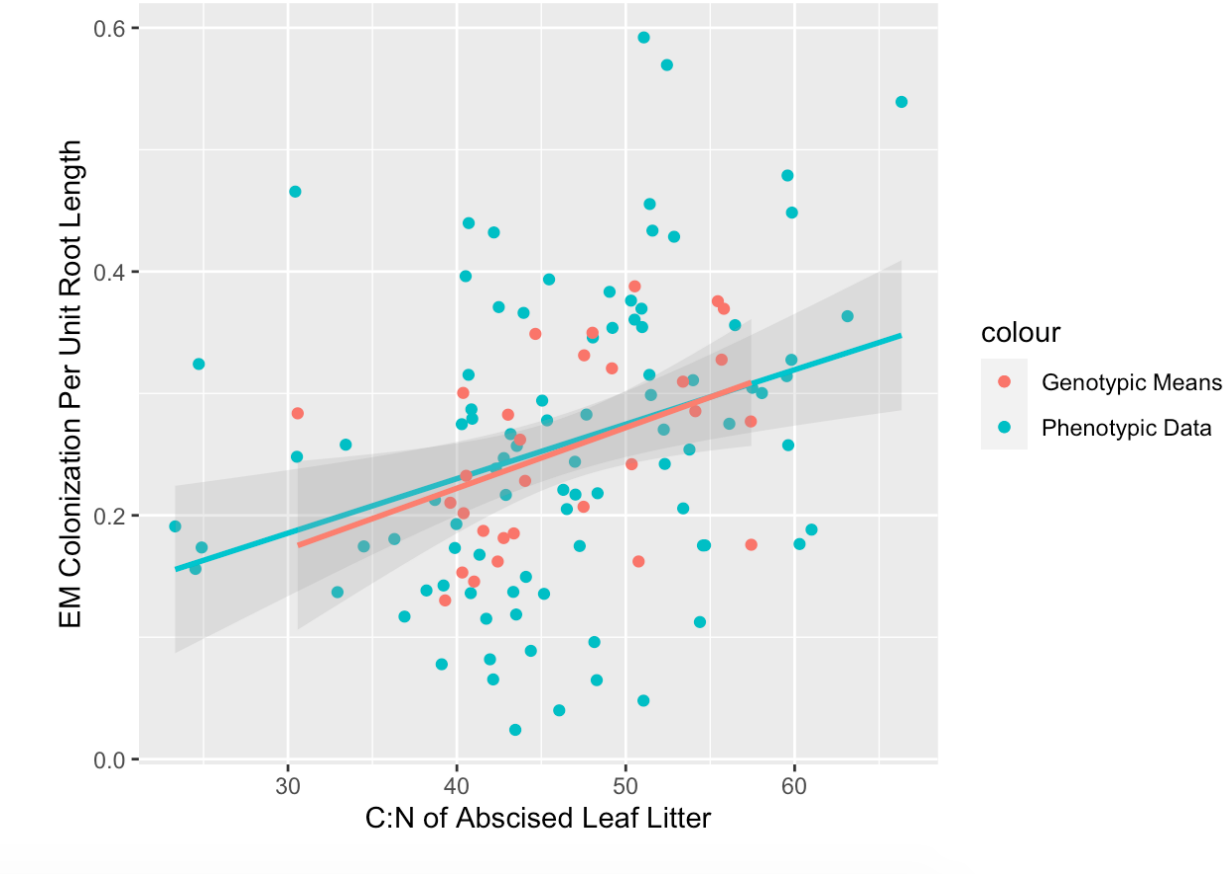

Figure 2. Phenotypic and genotypic correlation between the C:N of abscised leaf litter and EM colonization. Phenotypic data, or data from all individual trees sampled, are colored in blue. Genotypic means, or average values across replicates of the same genotypes, are represented in red.

														Exploration Type		
Genus	baseMean	AbsLeafCN log2FoldChange	AbsLeaf CN Pval	LeafPG log2FoldChange	AbsLeaf PG Pval	LeafCT log2FoldChange	AbsLeaf CT Pval	RootCN log2FoldChange	Root CN Pval	RootPG log2FoldChange	Root PG Pval	RootCT log2FoldChange	Root CT Pval	Distance	SubType	Enzymatic Capabilities
Cortinarius	3190.225147	0.8777275	0.034592696	-0.775514934	3.30E-02	-0.47063917	2.74E-01	0.34904433	0.375999914	0.607492	3.60E-02	0.32328927	0.311452529	Medium	Fringe	Yes
Hymenogaster	1708.463863	0.1274915	0.729343484	0.472698891	2.90E-01	0.41588373	3.94E-01	0.21363346	0.685343586	0.4873271	1.74E-01	-0.01538506	0.963499952	Short	Delicate	No
Hebeloma	583.866994	-0.5247918	0.355153573	-0.327770726	5.52E-01	0.53854679	3.94E-01	-0.03489235	0.92332969	0.5381206	2.16E-01	0.01777219	0.963499952	Short	Delicate	Yes
Inocybe	583.4470187	-0.5223336	0.360589046	0.88576558	1.03E-01	1.17025832	4.97E-02	0.0510424	0.92332969	-0.5303895	2.35E-01	-1.21185343	0.01889973	Short	Delicate	No
Thelephora	170.7045508	-0.3574568	0.708912477	1.201243054	2.26E-01	0.901476	3.94E-01	2.48975261	0.001372982	0.5168203	4.93E-01	-1.30028566	0.118708129	Medium	Smooth	Yes
Tuber	110.6145915	2.1051225	0.15497689	-2.42846514	5.89E-02	-2.45511187	7.73E-02	-0.39373587	0.886133678	-3.2842187	9.43E-04	1.62823908	0.13796406	Short	Delicate	Yes
Tomentella	75.3090056	-2.5041428	0.034592696	3.071093692	2.58E-03	3.82237779	3.19E-04	-0.97382947	0.375999914	-0.6865357	4.33E-01	0.27517962	0.83272871	Medium	Smooth	No
Lactarius	66.1765068	2.8826189	0.230146038	-30	1.56E-59	-13.742355	8.50E-11	-6.54887051	0.000290053	12.5952378	1.64E-15	4.69206776	0.01889973	Medium	Smooth	Yes
Laccaria	32.0653212	-1.1142219	0.685796735	-5.416321369	1.20E-02	-7.31353021	7.62E-04	5.34253706	0.002501398	5.7926843	7.27E-04	3.01081479	0.118708129	Short	Delicate	Yes
Paxillus	22.3023225	2.1864524	0.355153573	1.731773167	4.23E-01	0.10722093	9.76E-01	4.4206888	0.007486788	0.9648423	5.37E-01	-3.25543354	0.083399374	Long		Yes
Wilcoxina	19.1837546	-2.0199013	0.15497689	0.001114098	9.99E-01	0.3795125	8.67E-01	0.20295624	0.92332969	1.5610916	1.74E-01	2.46789526	0.039587439	Short	Coarse	No
Geopora	10.0762445	-3.3604898	0.15497689	0.636026803	7.75E-01	-4.45749681	5.68E-02	-1.30489513	0.628118012	5.7266091	7.27E-04	3.96980591	0.040405556	Short	Coarse	No
Scleroderma	5.3957016	7.4892453	0.001278346	-8.104632524	7.03E-05	-9.34481185	1.40E-05	4.40355821	0.010595265	5.6664286	7.27E-04	-6.65421825	0.000755938	Long		Yes
Sphaerosporella	3.0147206	-0.6256656	0.708912477	-0.563393531	7.75E-01	0.46531875	8.67E-01	0.31670415	0.92332969	0.4550891	6.94E-01	0.87029319	0.596752631	Short	Coarse	Yes
Russula	2.812658	4.0940591	0.102976495	-2.138609048	3.51E-01	-9.66315409	9.64E-06	5.00274082	0.004175243	-4.0193085	2.15E-02	2.74759703	0.13796406	Contact		Yes
Tricholoma	0.2448578	-4.0614177	0.102976495	4.318320149	4.73E-02	0.06052977	9.76E-01	1.88858999	0.384546965	1.6330749	3.76E-01	4.61903516	0.01889973	Medium	Fringe	No

Table 3. Differential abundance values for EM taxa and their correlation values with litter (abscised leaf and root) chemistries along with morphological and enzymatic functional data. Log2 fold change values indicate the change in fungal taxa abundance along the axis of that particular litter trait. The sign and magnitude of the log2 fold change value indicate the direction and strength of the correlation. Statistically significant correlations are highlighted in green.

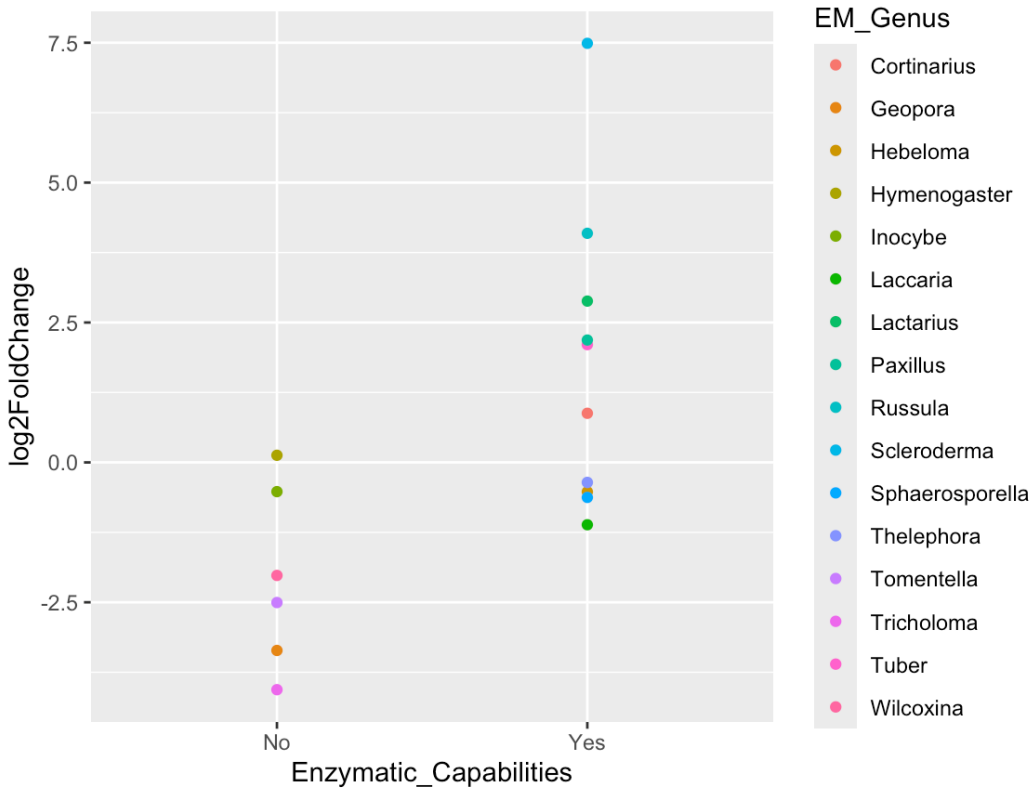

Figure 3. Change in the log2 fold change of abscised leaf C:N in relation to EM taxa with enzymatic capabilities. EM genera known to contain lignin-modifying enzymes had consistently more positive correlations with abscised leaf C:N.

DISCUSSION

Globally, plant nutrient acquisition strategies and nutrient use strategies both reflect adaptations to their nutrient environment. Across species, this can result in adaptive trait syndromes, such as the leaf economic spectrum. Moreover, plant tissue traits, shaped by nutrient use strategies, can in turn shape the local nutrient environment, affecting selection on nutrient acquisition traits like mycorrhizal associations in a positive feedback. To understand the evolution of these interspecific nutrient use – nutrient acquisition syndromes, we investigated the intraspecific correlations and genetic control over plant tissue chemical traits and ectomycorrhizal colonization and community structure among 30 *P. tremuloides* genotypes. We found that both tissue chemistry and EM fungal colonization were genetically controlled, and both the extent of EM colonization and make-up of the EM fungal community correlated with plant tissue traits. Specifically, we found that EM colonization and leaf litter quality (measured as the C:N ratio) were genetically linked, indicating the possibility of an adaptive nutrient use-nutrient acquisition syndrome within the species.

Heritability of plant tissue and fungal symbioses traits

As we predicted, broad-sense heritabilities were generally highest for traits most directly linked to plant gene products, which aligns with decades of research on the genetic and phenotypic variation of *P. tremuloides* traits (Schweitzer et al. 2008, Barker et al. 2018 and 2019, Mitton and Grant 1996, Madritch et al. 2006,

Lindroth et al. 2002, Stevens et al. 2014, Lindroth 1987, Lindroth 2001, Madritch and Lindroth 2015). More interestingly, however, was that EM colonization was a heritable trait, with genotype explaining over 25% of the variation in EM colonization. Additionally, EM colonization was the only heritable fungal symbiont trait of those considered. That EM colonization was heritable, but fungal community metrics such as richness and diversity were not, suggests that plant control of the symbiosis may be more quantitative and generic, ramping investment to mycorrhizal associations up and down, but that *P. tremuloides* may not have genetically variable traits to control the assembly of fungal communities in their roots. Heritabilities of certain EM taxa and EM community structure have been shown to be significant in tree species (*Picea abies*), but values were low (Velmala et al. 2012).

Phenotypic correlations between plant tissue and ectomycorrhizal colonization

At the phenotypic level, EM investment was positively correlated with the nitrogen and carbon content of leaves, but generally not with specific secondary compounds or root tissue values. Specifically, EM colonization was positively correlated with green leaf %C, abscised leaf %C, and abscised leaf C:N, and mildly positively correlated with green leaf C:N. Green and abscised leaf carbon values were tightly correlated, though their C:N ratios were less so due to the relatively low nitrogen content of abscised leaves. This is a well-documented relationship between green and abscised leaf chemistry due to the resorption of nitrogen from leaves prior to abscission (Vitousek 1982, Chapin 1980, Bloom et al. 1985).

Additionally, though not statistically significant, both green and abscised leaf nitrogen content (%N) were negatively correlated with EM colonization. Therefore, the positive relationship between EM colonization and foliar C:N values was driven primarily by carbon content, but also partially by nitrogen content. Furthermore, although not statistically significant, the relationships between EM colonization and %N of each tissue type (roots, leaves, and stem) were negative. This corroborates the negative relationship between EM fungi and nitrogen presence (or positive relationship between EM fungi and nitrogen limitation) that has been well established in the literature as well as this dissertation (see Chapter 1, Read 1991, Wallenda and Kottke 1998, Lilleskov and Bruns 2003).

Phenotypic correlations between plant tissue and ectomycorrhizal community composition

Plant tissue chemistries also favored and restricted the relative abundance of certain EM taxa. *Cortinarius* was the most relatively abundant taxa across all root samples, and had the strongest relationship (highest fold change value) with abscised leaf C:N. Members of the genus *Cortinarius* are known to possess Class II peroxidases (Lindahl and Tunlid 2014, Shah et al. 2016), which are key enzymes historically central to the process of lignin degradation by white rot fungi (Ayuso-Fernández et al. 2019, Sinsabaugh 2010). Furthermore, across all EM genera present, those known to contain lignin-degrading (or modifying) enzymes were more likely to be positively correlated with abscised leaf C:N. This may be the result of litter chemistry promoting nutrient acquisition feedbacks with EM

functional guilds. Moreover, the abundance of long-distance foraging taxa were associated with higher values of abscised leaf C:N than short or medium distance types. However, this interpretation is less straightforward given the low number of long-distance exploration taxa present and that the only contact exploration type also correlated with high abscised leaf C:N. Regardless, the general patterns with exploration type further corroborate the idea that tree litter chemistry can promote and reinforce closed nutrient feedbacks.

Relativized sequencing data vs. EM colonization

In this study, we used variation in both EM colonization and community composition to measure the potential of functional feedbacks between *P. tremuloides* tissue traits and EM symbionts. However, these two sources of data should be interpreted carefully. EM colonization abundance can be quantified directly, making it a useful proxy of plant investment to the EM symbiosis. Sequence counts, however, are relativized given the nature of metabarcoding and community sequencing. Each sample is subjected to different efficiencies in the PCR and sequencing process, so sequence counts for each taxon are relativized as proportions of the total number of sequences for each sample prior to analysis. Therefore, as one taxon increases in abundance, others must decrease in abundance (and vice versa) given the compositional nature of the data. However, this may not accurately reflect how the community is responding within the roots. In reality, as one taxon increases (or decreases) in abundance, other taxa may be maintaining their abundance levels. However, this would

appear as change in the relative abundance of the other taxa given the constraints of proportional data. Pairing quantitative PCR (qPCR) measures with this experiment would allow us to see not just that communities "shift" with different plant traits, but whether changes are restricted to a certain taxon, or taxa. Overall, this suggests that variation in EM colonization is our strongest measure of relationship between plant traits and fungal symbionts, with community change being an interesting, though less reliable, measure of plant-fungal feedbacks in this system.

Broad determinants of litter quality vs. specific secondary chemistry

The C:N ratio has long been used as measure of litter quality and decomposability (Aber et al 1990, Aerts 1997, Scott and Binkley 1997). A large portion of the carbon found in plant tissues, particularly those of woody species, is composed of lignin. As plant-tissue percent carbon increases, so does percent lignin (Ma et al. 2018a), suggesting that high-carbon containing tissues likely contain a disproportionately high amount of lignin. Lignin is a recalcitrant polymer that is not easily broken down and, therefore, makes high-carbon containing plant tissues challenging to decompose. In order for fungi, including EM fungi, to access nutrients from tissues containing lignin, they need to possess appropriate enzymatic capabilities, such as peroxidases, laccases, or Fenton chemistry to first degrade or modify lignin and access the nitrogen containing compounds. PGs, however, are considered to have antifungal properties (Osbourn 1996), and CTs bind nutrient-rich proteins (see Schweitzer et al. 2008). Therefore, although litter

C:N, PG and CT content may all result in slow decomposability and litter recalcitrance, they are likely doing so via different mechanisms. Furthermore, if fungal symbionts are serving as nutrient acquisition conduits, we may expect that each avenue of "litter recalcitrance" would select for different taxa or functional groups. The aforementioned enzymatic capabilities of *Cortinarius* may explain why we see a positive relationship between this genus with abscised leaf C:N, where lignin-degrading enzymes would be useful for nutrient acquisition in lignin-bound substrates. However, the negative relationship between *Cortinarius* and abscised leaf PGs may speak to the antifungal activity of those compounds.

Genotypic correlations

Abscised leaf %N and abscised leaf C:N were the only two plant tissue chemistries genetically correlated with EM colonization, though abscised leaf %C was mildly correlated. Similar to phenotypic correlations, the positive relationship between EM colonization and abscised leaf C:N was driven but both the negative relationship between EM colonization and abscised leaf nitrogen and the positive relationship with abscised leaf carbon. Contrary to the phenotypic correlation, however, nitrogen was the primary driver of the genetic correlation. The fact that the genetic correlation to leaf nitrogen was stronger than the phenotypic correlation, but the reverse was seen for leaf % carbon, suggests some potential mechanisms for the genetic link. Specifically, the ability of a *P. tremuloides* individual to effectively resorb nitrogen from its leaves prior to abscission may

be a genetically controlled trait, and higher resorption ability would likely be selectively favored in nutrient-constrained environments. These same nutrient constrained environments would also be expected to select for whatever genetic traits lead to higher EM fungal investment. Abscised Leaf C:N was the only trait that correlated with EM colonization at both the phenotypic and genotypic level, indicating co-selection between the alleles for these traits. This result suggests that clonal stands of *P. tremuloides* may have experienced nutrient conservation/acquisition feedbacks over their evolutionary history, leading to genetic fixation of traits related to foliar nutrient conservation and symbiotic nutrient acquisition through increased association with EM fungi.

Environmentally induced feedbacks between litter quality and mycorrhizal symbioses

Although the common garden system was useful for determining genetic correlations between plant traits and EM investment and diversity, it was less useful for studying induced environmental feedbacks. Since plant litter chemistry can influence the local nutrient environment and soil microbial community, it is possible that in clonal stands of *P. tremuloides,* litter chemistry creates biogeochemical hotspots. However, given that individuals of similar genotype are intentionally spaced in a common garden and other sources of environmental variation are intentionally reduced, a common garden was not able to provide a set up capable of showing these potential feedbacks. Based on our soil measurements of C, N, and C:N, there was relatively little environmental

variation in soil properties throughout the garden. However, three individuals did have C:N values that fell moderately outside of the range of other points, and, when considered, influenced soil relationships with plant and fungal data. However, to feel confident about the true nature of soil influence on these parameters, we would need a wider range of soil C:N values. Had this study been conducted in a different set up, such as looking across clonal stands where litter inputs were not mixed and had the potential to create biogeochemical hotspots over time, it is possible that we may have seen a stronger an even connection between the relationship between plant-soil-fungal traits, specifically in the influence of abscised leaf C:N on soil chemistry, and soil chemistry on EM communities.

Conclusions

Overall, this study showed that *P. tremuloides* litter, specifically the C:N ratio of abscised leaves, is genetically linked to EM investment, potentially as part of an adaptive trait syndrome. Although the common garden set up did not provide ample environmental variation to test for soil chemistry drivers of EM investment and diversity or induced environmental feedbacks between plant tissues and soil chemistry, we did find that EM taxa abundances varied with different plant tissue chemistries. In total, these results indicate that both nutrient use and nutrient acquisition strategies, specifically involving mycorrhizal symbionts, can be coordinated at the whole plant level even among genotypes of single species. Such genetic variation in individual traits and in the correlation between traits

provides the raw materials for the evolution of the plant lifestyle strategies that shape the functioning of ecosystems globally.

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CHAPTER THREE

Assessing the carbon-nitrogen economics of ectomycorrhizal interactions in *Populus deltoides* and how they vary with nutrient quality

ABSTRACT

Forested systems currently exhibit a distinct latitudinal gradient in mycorrhizal dominance, and this pattern is closely correlated with patterns of soil organic matter, nutrient limitation, and litter quality. Climate-driven differences in decomposition paired with mycorrhizal fungal functional traits drive this pattern. This primarily includes the ability of EM fungi to access nitrogen from organic matter, which is thought to come at a high carbon cost to plants. Mycorrhizal interactions have recently been incorporated into global models of nutrient cycling and predicted global change, requiring that the rate of plant nitrogen return per unit carbon investment in mycorrhizae be quantitatively defined. Currently, the parameters used to represent the nitrogen-carbon exchange rate are estimated values and fixed rates that do not account for ecological variability, such as organic matter quality or degree of EM investment. Here, we conducted a manipulative lab experiment using dually-colonized *Populus deltoides*, two ^{15}N-labeled organic nutrient sources of differing C:N values, and a ^{13}C pulse-chase set up to test the following hypotheses: 1) if reduced

nutrient quality requires increased EM enzymatic production, then more recalcitrant litter sources will result in a lower plant nitrogen return rate per unit carbon invested (N:C exchange rate), 2) if plant nutrient acquisition efficiency increases with increasing EM investment, then increased EM colonization will result in a higher plant N:C exchange rate, and 3) If EM associations require a period of morphological infrastructure "investment" before a maximum rate of plant N:C exchange can be achieved, then we predict that the relationship between N:C exchange rate and EM relative abundance will be positive and non-linear, and that the relationship will be mediated by EM colonization. Overall, we found that both litter quality and degree of EM investment were significantly and positively correlated with the plant nitrogen return rate per unit carbon invested. Additionally, we found that relative abundance of EM fungi correlated with N:C exchange rate in a logarithmic relationship that was also correlated with EM investment, supporting our hypotheses. Given that differences in litter quality and carbon costs between the two mycorrhizal guilds are thought to drive latitudinal patterns in forested systems as well as influence predictions of whether systems will serve as carbon sources or sinks in future climates, understanding factors influencing the rate of above- and belowground transfer of nitrogen and carbon is central to predicting future forest states and functions.

INTRODUCTION

Forested systems across the globe currently exhibit a distinct latitudinal gradient in mycorrhizal dominance, where ectomycorrhizal (EM) interactions dominate in northern, boreal biomes, arbuscular mycorrhizal (AM) interactions dominate in the tropics, and both are common in temperate regions (Lu and Hedin 2019). This distributional pattern is closely correlated with patterns of soil organic matter, decomposition rates, nutrient limitation, and general patterns of litter quality in EM and AM associated plants (Lu and Hedin, 2019, Read 1991, Averill et al. 2018). Overall, these correlations are thought to be the result of mycorrhizal fungal functional traits, namely those involved in the acquisition of nitrogen, paired with climate-driven differences in decomposition rates.

In general, EM fungi have retained a much larger and more variable repertoire of saprobic enzymes than their AM counterparts (Kohler et al. 2015), giving them the unique ability to “mine” for nitrogen from organic matter (Hobbie et al. 2013). AM fungi, on the other hand, are considered to only “scavenge” for dissolved mineral forms of nitrogen, phosphorus, and other soil nutrients. The ability of EM fungi to mine for nitrogen may provide an advantage to trees in higher latitudes, where much of the available nitrogen is bound up in organic matter and the climatic window for decomposition is limited. Therefore, increased selection for EM fungi in high latitudes may explain the dominance of EM-associated plants in these regions. In the tropics, however, forested systems exhibit open, mineral-nutrient cycling economies due to less restricted windows

for decomposition and increased litter quality inputs (Phillips et al. 2013). Therefore, in tropical areas, there may be reduced selection for the enzymatic abilities of EM fungi, especially if they impose a high carbon cost to the host tree.

It has been hypothesized that EM relationships incur a higher carbon cost per unit nitrogen acquired to the associated plant host than AM associations (Lu and Hedin 2019, Smith and Read 2008 as cited by Phillips et al. 2013). This differential cost is thought to be the result of the enzymatic capabilities possessed by most EM fungi, as well as differing morphologies between the two guilds and slower turnover of EM compared to AM structures (Lu and Hedin 2019, Read & Perez-Moreno, 2003, Sulman et al. 2017).

Given their connection to forest structure, biogeochemical signatures, and carbon sequestration (Gadgil and Gadgil 1971, Fernandez and Kennedy 2015, Averill et al. 2014), models of global nutrient cycling and predictions of global change have recently begun to incorporate mycorrhizal associations (Lu and Hedin 2019, Sulman et al. 2017). In order to do so, the rate of plant nitrogen return per unit carbon investment in mycorrhizal associations had to be quantitatively defined as cost coefficients. Currently, the parameters used to represent the nitrogen-carbon exchange rate are estimated values and fixed rates that do not account for ecological variability, such as organic matter quality. Differences in litter quality and carbon costs between the two mycorrhizal guilds are thought to drive latitudinal patterns in forested systems as well as influence predictions of whether systems will serve as carbon sources or sinks in future

climates and are therefore central to our understanding of future forest states and functions.

Here, we used a manipulative lab experiment involving two ^{15}N-labeled organic nutrient sources of variable quality and a gaseous ^{13}C pulse-chase to address the following question: What is the plant nitrogen return rate per unit carbon invested (N:C exchange rate) for EM interactions in *Populus deltoides*, and how does that rate vary with nutrient environment and degree of plant-fungal association? We used a dually-colonized *Populus deltoides* system, which, given its ability to associate with both EM and AM fungi simultaneously, may exhibit a greater degree of plasticity in the N:C exchange rate than non-dual species, who may have higher degrees of genetic fixation. More specifically, we hypothesized:

Hypothesis 1: Economic exchange rate vs. nutrient quality

If reduced nutrient quality requires increased EM enzymatic production, then more recalcitrant litter sources (higher C:N) will result in a lower plant nitrogen return rate per unit carbon invested.

Hypothesis 2: Economic exchange rate vs. EM investment

If plant nutrient acquisition efficiency increases with increasing EM investment, then increased EM colonization will result in a higher plant nitrogen return rate per unit carbon invested.

Hypothesis 3: Economic exchange rate vs. EM abundance in nutrient patches

If EM associations require a period of morphological infrastructure "investment" before a maximum rate of plant nitrogen return per unit carbon investment can be achieved, then we predict that 1) the relationship between N:C and the relative abundance of EM fungi in hyphal-only nutrient bags will be positive and non-linear, and 2) this relationship will be mediated by the degree of EM colonization in roots.

METHODS

Plant source: Populus deltoides seeds were collected during spring of 2023 from two locations in Madison, WI. In each area, there were multiple *P. deltoides* individuals, therefore each collection was expected to possess multiple genotypes but of unknown ratios. Seeds were germinated in flats containing a 50:50 mix of sand:vermiculite in a greenhouse setting for roughly two months, and flats were labelled by collection event to later account for any potential

genotypic signal in our models. Flats were transferred to a growth chamber for roughly three months prior to being planted into treatments pots.

^{15}N-labeled litter: Seeds of red clover (*Trifolium pratense*) and annual ryegrass (*Lolium multiflorum*; Sheffield's Seed Co., Inc; Locke, NY, USA) were grown in flats containing a 50/50 sand:vermiculite mix in a greenhouse setting. Extrapolating from red clover values in Gentry et al. 2013, flats were seeded at a rate of 0.28 g seeds/flat (~110 seeds) and fertilized with ^{15}N-labeled ammonium-sulfate (formula, Sigma-Aldrich info) at a rate of 1 g N/flat/week, matching the calculated expected rate of nitrogen accumulation. This was split between three fertilization events per week, so ~0.33 g N were administered at each event along with ~0.66 g of a PK fertilizer (Greenway Biotech, Inc; Santa Fe Springs, CA, USA) dissolved in 500 mL water. This amount of 500 mL was chosen to limit the amount of fertilizer leaking through the bottom of the flat. Flats with drain holes were elevated within flats with no drain holes to monitor leaking and readminister any lost fertilizer. Given that these calculations were done for a high-nitrogen plant and did not account for any microbial fixation, these same rates were used to fertilize rye as well. Plants were supplemented with additional watering events as they grew. Red clover and rye were grown for ~3.5 months prior to harvest. At harvest, plants were rinsed, separated into above- and belowground tissues, and subsequently dried. Tissues were then powderized using a coffee grinder and homogenized across flats for each species and tissue type. Samples were sent to The Stable Isotope Facility (SIF) at UC-Davis to quantify

atm% ^{15}N (as well as total %N, %C, and amt% ^{13}C) labeling of each species/tissue type. *Trifolium pratense* (aboveground) and *Lolium multiflorum* (belowground) tissues had a ^{15}N label of 8.29atm% and 8.87atm% and C:N ratio of 20.95 and 45.89, respectively. The C:N ratio was used to define litter quality in this study.

Experimental Treatments: Rooted seedlings were measured for various morphological and developmental characteristics and transplanted into 8.5 cm^3 acrylic plastic boxes (Amazon.com) containing a total of 400 mL of 50/50 autoclaved sand:vermiculite (200 mL of each, mixed). The clear plastic allowed for potential monitoring of root and hyphal development as well as regulation of watering events, as treatment pots had no drainage holes. Pots were soaked in a 10% bleach solution for 30 minutes, rinsed, and air dried prior to use. At planting, each pot received two duplicate nutrient bags, which consisted of powderized ^{15}N-labeled litter (both bags filled with either *T. pratense* leaf or *L. multiflorum* root material; see above) enclosed in a 40µm-opening mesh bag (Miami Aquaculture, Inc., Boynton Beach, Florida, USA) that had been sealed using an impact heat sealer. This opening size was chosen to allow for the passage of fungal hyphae but restrict the entry of *P. deltoides* roots (Johansen and Jakobsen 1992). Using known %N values, the amount of ^{15}N-labeled litter included in each bag was calculated to add a total of 0.027 g N/pot, which was split between the two bags (see Table S3.1). Bags were placed perpendicularly left and right of the seedling rooting zone and equidistant between the seedling and the pot edge (see Figure 1). Each pot received either an EM, AM, or no fungal inoculum (controls).

EM inoculum consisted of ¼ culture plate of *Laccaria bicolor* (strain S238N, Oak Ridge National Laboratory) grown on PDA cut into six triangular plugs. Plugs were dispersed around the pot at the level of the root zone and put in direct contact with roots and nutrient bags. AM inoculum consisted of a liquid suspension of *Rhizophagus irregularis* spores (Premier Tech; Quebec, Canada), applied at rate of 3 mL of an initial spore concentration of 2,000 spores/mL, totaling ~6,000 spores per pot. A viability test was conducted prior to administration on water-agar. AM spores were administered around the root-level zone of the pot and put in direct contact with both roots and nutrient bags. Each seedling was given an initial watering of 125 mL water. Pots were grown with their ^{15}N and inoculum treatments for ~7 weeks in a growth chamber with a 16-hour photoperiod and day and night temperatures oscillating between 23°C and 20°C, respectively. Plants were watered ~50 mL 3x/week and supplemented as needed.

^{13}C Pulse Labeling: P. deltoides individuals were pulsed with 5L of 99.0 atm% $^{13}CO_2$ gas for three days in a ~1x1x1.5-meter sealed chamber designed for isotopic labelling (Whitman Lab, UW-Madison). Gas was added over the first two days of the three-day pulse to allow plants to have at least one full day of access to the total volume of $^{13}CO_2$. Given their transparency, plant pots were covered in tin foil to prevent any photosynthesis from potentially present algae or cyanobacteria. After three days in the labeling chamber, pots were transported back to the growth chamber. The CO_2 threshold value was set to 1000ppmv and slowly raised to 2500ppmv to allow timely entry of CO_2 into the chamber. This

value was deemed to be high enough to allow consistent inward flow, but not too high to cause stomatal closure or abnormally high respiration.

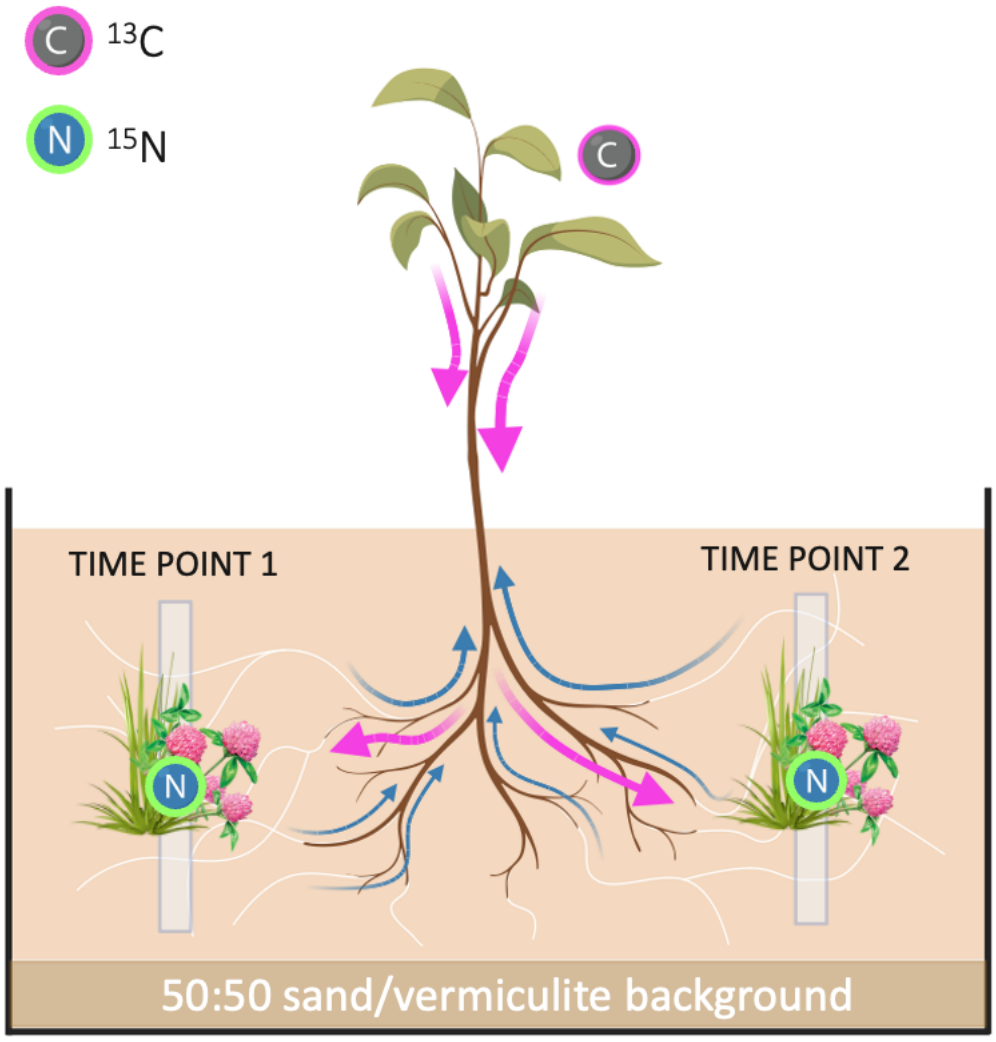

Figure 1. Experimental set up using to track ^{13}C and ^{15}N in Populus deltoides. Each P. deltoides individual was planted with mesh hyphal-only bags containing either ^{15}N-labeled rye roots (high C:N) or ^{15}N-labeled clover leaves/stems (low C:N). Plants were pulsed with gaseous ^{13}C for three days. Isotopic ^{15}N was traced into plant tissues and ^{13}C was traced to hyphal-only bags. Figured created with BioRender.com.

Harvest: On Day 5 of the chase period, one of the two nutrient bags was harvested, and a media sample was collected. The nutrient bag contents were sampled for sequencing, with sampling efforts focused on the outside surfaces of the litter mass where hyphal presence was expected to be highest. The remainder of the litter mass was dried and subsequently weighed. Media samples were collected using a metal scoopula and spatula to obtain a representative sample through the media profile, similar to a soil core. Three samples were taken: two flanking the harvested nutrient bag and one on the opposite side of the seedling with the aim to obtain a sample that would capture any ^{15}N leaching from the harvested bag but also represent the pot as a whole. Any plant root fragments present were removed, and the three subsamples were combined into a single representative sample and dried.

On Day 10 of the chase period, the same procedure for Day 5 was repeated for the second nutrient bag and media sample, though a subsample of the combined media sample was allocated for sequencing. The seedling was harvested, with care to not sever fine roots. Plant tissues were separated into leaves, stem, roots and were measured, weighed and photographed for subsequent morphometry. Representative subsamples of fine roots, taken from around the root mass as best possible, were allocated for sequencing and quantitative microscopy (clearing/staining). All remaining roots and other plant tissues were dried and weighed for dry mass. Central tap roots were excluded from homogenized samples for isotopic analysis if excessively large.

Isotopic Analysis (Sample Preparation): All dried samples (respective plant tissues, nutrient bag contents, and media samples) were powderized and homogenized using a SPEX Sample Prep 1600 MiniG tissue homogenizer (SPEX SamplePrep; Metuchen, NJ, USA). Target weights for each sample type were determined using the SIF online sample calculator tool using estimated or known %C and %N values. Samples were weighed using a microbalance and rolled into 5x9mm tin (Sn) capsules (CE Elantech, Inc.; Lakewood, NJ). Each capsule was folded according to SIF and standard protocols. Tin capsules containing media samples, which were larger in weight, were double tinned to avoid sample loss. Samples were submitted to SIF for ^{13}C and ^{15}N analysis according to their outlined procedures.

Sequencing of fungal communities: Fungal communities were characterized from both *P. deltoides* roots and from the litter material in the hyphal-only nutrient bags via Illumina sequencing of the ITS2 rRNA gene region via methods described in Allsup et al. 2023 with some modifications. Briefly, roots and litter tissues were frozen with liquid nitrogen and ground using a SPEX Sample Prep 1600 MiniG tissue homogenizer (SPEX SamplePrep, Metuchen, NJ, USA). DNA extractions were prepared using the Omega E-Z 96 Plant DNA Kit (Omega Bio-Tek, Norcross, GA, USA) and the ITS2 region was amplified through polymerase chain reaction (PCR) using the ITS7 (Tedersoo et al. 2015) and ITS4 (White et al. 1990) primers. An additional round of PCR attached Illumina adapters and

unique 12-base barcodes to each sample, which were then sequenced on one run using Illumina Miseq PE 2x300 chemistry. Sequences were denoised and clustered into exact amplicon sequence variants using the DADA2 program (Callahan et al. 2016) as implemented in the QIIME2 pipeline. Amplicon Sequence Variants were identified to the lowest possible taxonomic level using the RDP Naïve Bayesian classifier trained on the UNITE database (Abarenkov et al. 2010). Fungal species were assigned to guilds based on the Fungal Traits database (Põlme et al. 2020). Fungi were assigned to one of six functional guilds based on genus identity (Arbuscular Mycorrhizal, Ectomycorrhizal, Plant Pathogen, Endophyte, Saprotroph, "Other", and Unassigned). For genera with multiple lifestyles, we assigned guild with the following priority: Ectomycorrhizal > Plant Pathogen > Endophyte > Saprotroph > Other.

Analysis

Mycorrhizal Colonization

Although treatment pots were intended to have mycorrhizal inoculum treatments of EM-only, AM-only, and no-fungal controls (see Methods), clearing and staining procedures revealed that all pots had EM colonization and that the degree of colonization did not differ between the *L. bicolor* inoculation treatment and no-fungal controls (P value = 0.99). There was high degree of variation in EM colonization between pots, with percentages of intersection scored containing EM mantles ranging from 1% to 89%. Additionally, the variation was rather

normally distributed (Figure S3.1). Mantles ranged from rather well developed to very thin, with most being relatively thin to intermediate. Sequencing of both plant roots and litter tissue from hyphal only bags identified one major EM fungus, *Geopora* sp. This genus accounted for 99.6% of the fungal sequence reads assigned to EM in roots and 66.9% of EM assigned sequences in the litter bags. Although sequences of some other putative EM genera were detected in litter material (including *Sphaerosporella* sp., *Sebacina* sp., and *Inocybe* sp.), since these genera were not detected in root tissue, the sequences likely do not reflect functional EM relationships. Thus, while we analyze and report on the total %EM relative abundance, it should be noted that this reflects almost entirely the abundance of the *Geopora* genus. To account for the non-independence of samples that received the same original inoculum type, we have including inoculum source as a random effect in our model. There was not enough AM colonization on roots to properly test for any meaningful correlations with AM fungi; however, the variable was included in models to account to any differences in AM colonization between seedlings that may influence our interpretation of C and N transfer.

Excluded Samples

Samples 132 and 133 both showed values suggestive of experimental error, such as contamination or improper chemical measurement, and were therefore removed from analysis. See the Supplement (including Figures S3.2 and S3.3) for more information.

^{13}C and ^{15}N values

^{13}C transferred to mycorrhizal fungi was defined as the atm% of ^{13}C measured in the Time Point 1 (Day 5) nutrient bag. Whether this value should be relativized to the mass of the litter in the hyphal bags is under consideration (see Figure S3.4). We focus our analysis on the Time Point 1 bag, since the Time Point 2 (Day 10) bag showed a slightly less variable N:C exchange rate for those receiving a lower quality nutrient source (roots), though qualitative and quantitative patterns were similar to the Time Point 1 data (Figures S3.5 and S3.6). Furthermore, once plant total ^{15}N and atm% ^{13}C were relativized (N:C), the data between hyphal bags 1 and 2 become virtually indistinguishable (Figure S3.7).

Total plant ^{15}N accumulation was calculated in a multistep sequence. First, total N per unit sample mass was calculated by dividing the total N in the analyzed sample (determined by SIF, UC-Davis) by the submitted sample weight; this was done for each tissue type within each sample (root, stem, leaves). This value was then multiplied by the total respective tissue mass to give total N per tissue type per seedling. (Example: root sample N per unit mass * total root dry weight for each seedling = total seedling root N). Total tissue N was multiplied by the atm% ^{15}N value (determined by SIF) to calculate the total ^{15}N mass for each tissue type for each seedling. The total ^{15}N values for roots, stems, and leaves were then summed to calculate total plant ^{15}N. (See Supplement for equation.) Overall, for this analysis, the plant nitrogen return rate per unit carbon invested is measured

as the ratio of total plant ^{15}N to atm% ^{13}C in the hyphal-only Time Point 1 nutrient bag.

Statistical Analysis

To test our predictions for how the plant nitrogen return rate per unit carbon invested into (mycorrhizal) fungi varied with the quality of the nutrient source (Hypothesis 1) and degree of EM investment (Hypothesis 2), we used a linear mixed effects model and ANOVA test (R package lmerTest; Table S3.1). The ratio of total plant ^{15}N to atm% ^{13}C in the hyphal-only nutrient bag (see notes on both above) was included as the response variable and nitrogen source, %EM colonization, and %AM colonization were included as explanatory variables. Both %EM and %AM colonization were included as fixed effects to account for differential levels of mycorrhizal association and inoculum source was included as a random effect to account for non-independence of samples sharing the sample initial inoculum source.

To test Hypothesis 1, the difference in the economic exchange rate between the two litter types was assessed using the R package emmeans (Table S3.2). A statistically significantly higher rate of nitrogen return per unit carbon invested for the clover leaf litter treatment compared to the rye root litter treatment would support Hypothesis 1. The C:N ratio was used to define litter quality in this study.

To test Hypothesis 2, that the nutrient acquisition efficiency (or rate of plant nitrogen acquired per unit carbon invested to fungi) increases with increasing degree of EM colonization, we assessed the direction, magnitude, and statistical significance of the %EM colonization term. A statistically significant positive slope for this term would indicate support for Hypothesis 2.

To test Hypothesis 3, that the N:C exchange rate would increase as the relative abundance of the extraradical EM fungal hyphae increased in the nutrient patch, we used a similar linear model, but replaced the %EM colonization in roots with the proportion of EM fungal sequence reads (out of the total number of fungal sequence reads) detected in the hyphal only litter bag. To test whether this relationship was nonlinear, we compared the AIC of the model described above to one where the relative abundance of EM fungal reads was log transformed.

RESULTS

Hypothesis 1: Economic exchange rate vs. nutrient quality

Overall, nitrogen source was strongly and positively correlated with the plant nitrogen return rate per unit carbon invested (ratio of total plant ^{15}N to atm% ^{13}C in hyphal only bags) (P = < .0001, Table S3.1; Figure 2). The exchange rate value was higher for the higher quality nutrient source, clover leaves (marginal mean = 8.86; Table S3.2), than the lower quality nutrient source, ryegrass roots (marginal

mean = 2.21; Table S3.2). Therefore, litter quality was significantly and positively correlated with the plant nitrogen return rate per unit carbon invested.

Hypothesis 2: Economic exchange rate vs. EM Investment in roots

EM colonization had a significant and positive relationship with the plant nitrogen return rate per unit carbon invested (p = 0.000181, Table S3.1; Figure 2). As the degree of EM colonization increased, plants received more N per unit C transferred to the hyphal-only nutrient bags.

Hypothesis 3: Economic exchange rate vs. EM abundance in nutrient patches

%EM abundance in the hyphal-only nutrient bag was positively correlated with the plant nitrogen return rate per unit carbon within each litter type. EM relative abundance was calculated as the proportion of fungal sequences assigned to putative EM genera among the total fungal sequences. Across both litter types, %EM relative abundance was significantly and positively correlated with higher plant nitrogen exchange rates (Estimate = 90.51, P = 0.0168; Table S3.5). EM relative abundance was also positively correlated with the nitrogen return rate separately within the *Trifolium* litter type (*Trifolium* leaves, Estimate = 101.2, P = 0.056; *Lolium* roots, Estimate= -167, P = 0.065).

Because the relationship between EM relative abundance in the litter material and plant nitrogen return rate appeared non-linear, we also modeled this using log-transformed values of EM relative abundance. This non-linear model had a lower

AIC compared to the linear model described above (87.84 vs. 94.18), suggesting a better fit to the data. The log-transformed EM relative abundance was a highly significant predictor of the plant nitrogen return rate across both litter types (Estimate = 4.21, P = 0.00089; Table S3.6), and separately within each litter type (*Trifolium* leaves, Estimate = 4.675, P = 0.0073; *Lolium* roots, Estimate= -3.23, P = 0.047).

Several putative EM fungal species were detected in the litter material, but only one was also detected in plant roots (*Geopora* sp.). Therefore, to provide a more conservative test of functional EM abundance, we repeated the statistical models using only the relative abundance of *Geopora* as the predictor, rather than all potential EM fungi. This analysis showed very similar results: the relative abundance of *Geopora* (log-transformed) was a significant predictor of the plant nitrogen return rate across all samples (Estimate = 134.7, P = 0.013) as well as within the *Trifolium* leaf litter type separately (*Trifolium* leaves, estimate = 4.48, P = 0.006; *Lolium* roots, 1.78, P = 0.224; Figure 3). The relative abundance of the non-*Geopora* genera assigned as putative EM fungi was not significantly related to the plant nitrogen return rate (Estimate = -606.7, P = 0.23).

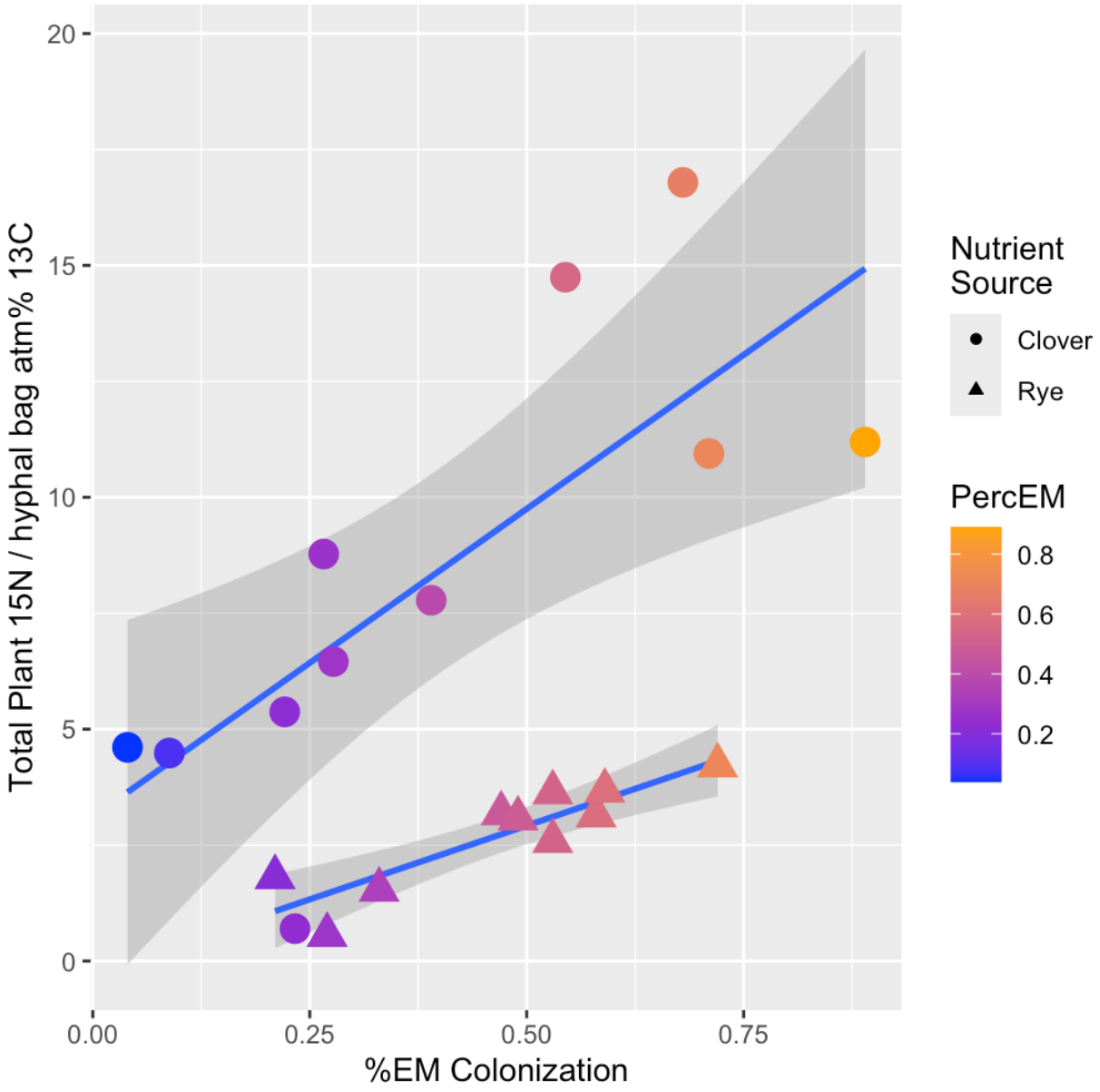

Figure 2. Relationship between EM colonization and the plant nitrogen rate of return per unit carbon invested for different quality nutrient sources. The relationship was positive for both nutrient sources.

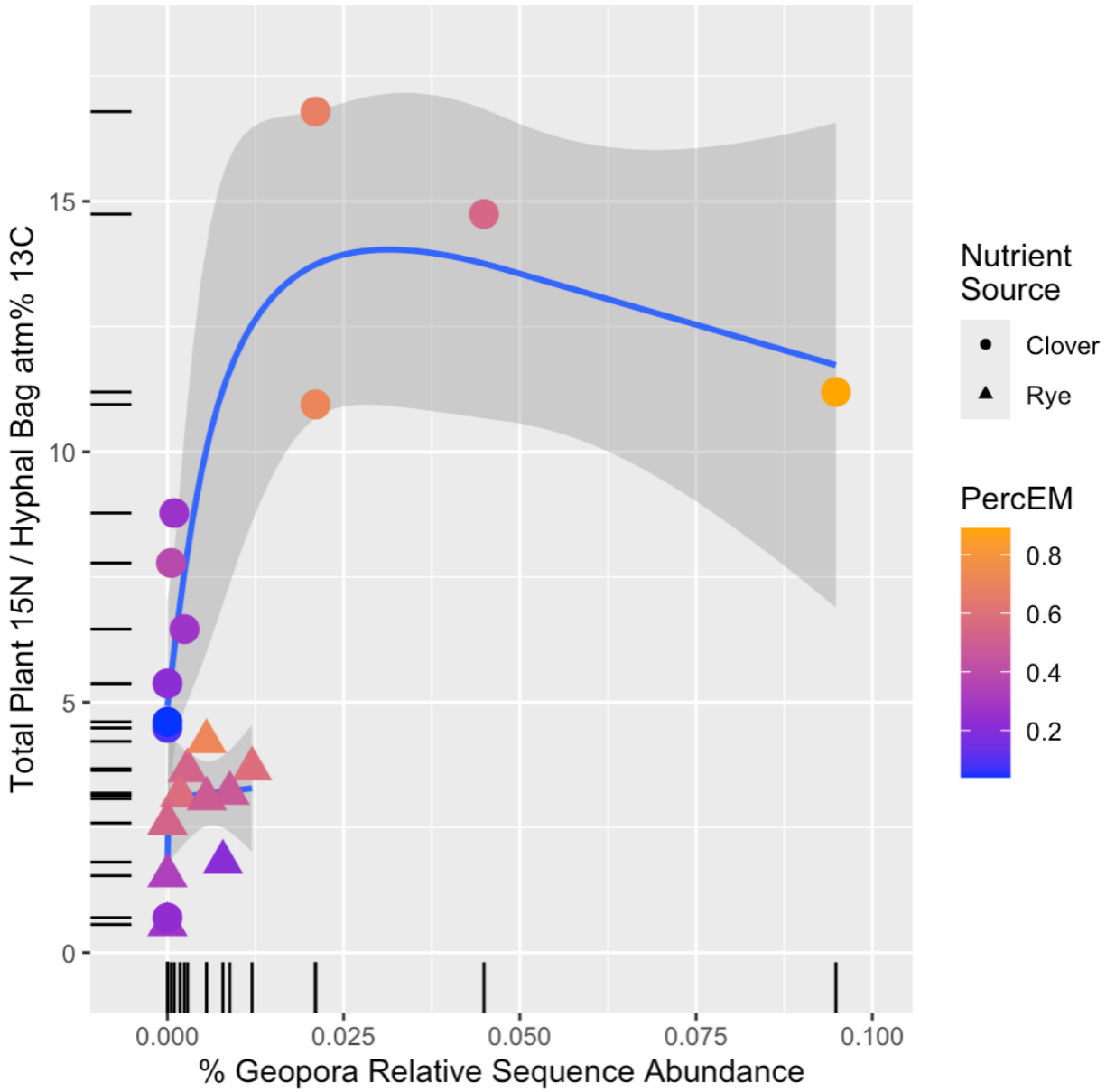

Figure 3. Relationship between relative abundance of Geopora sequences in hyphal-only nutrient bags and the plant nitrogen rate of return per unit carbon invested. Both nutrient sources displayed a logarithmic growth curve. Margins include a rug plot, where marks show the distribution of data along both axes.

DISCUSSION

This study aimed to assess the plant nitrogen return rate per unit carbon invested into ectomycorrhizal symbiosis (N:C exchange rate) as well as how that rate varied with organic nutrient quality and degree of EM investment. Additionally, it aimed to assess how the exchange rate was related to EM fungal relative abundance in hyphal-only bags and pair that with the assessment of EM colonization to better understand how morphological infrastructure "investment" between tree roots and EM fungi might affect the N:C exchange rate. This study found evidence that nutrient quality, particularly litter quality, was significantly and positively correlated the N:C exchange rate: higher quality litter sources (lower C:N) resulted in a higher N:C exchange rate. This is reasonable given that access to nutrients from more recalcitrant sources would likely require increased production of carbon-rich lignin-modifying enzymes (Hobbie et al. 2013).

This study also demonstrated that as the degree of EM colonization increases, the N:C exchange rate increases, suggesting that the efficiency with which plants are able to acquire nitrogen is a function of their degree of EM investment. This challenges the idea that N:C exchange rates are fixed ratios based purely on the "cost" to access organically bound nitrogen (enzymatic costs). This, instead, implies a more complex, economics-oriented framework, where rates change based on how invested a particular plant is. Moreover, including the relative EM

abundance data in this consideration strengthens the idea. The relationship between the relative abundance of EM fungi in the hyphal-only nutrient bags displayed a positive but nonlinear relationship with N:C exchange rate. The pattern was monotonically increasing but saturating, where hyphal bags with low EM relative abundance had low to moderate values of N:C exchange rate, followed by a dramatic increase in the N:C exchange rate as EM fungi transitioned from <3% of fungal sequences to >5%. However, above this threshold the N:C exchange rate seemed to stabilize.

An additional interesting aspect is that this logarithmic relationship was also positively correlated with EM colonization. Therefore, once colonization (and N:C exchange rate) reached relatively intermediate values, the logarithmic nature of the relationship between N:C and EM abundance became apparent. Overall, this suggests that there may be a period of morphological infrastructure investment between plants and EM fungi before maximum rates of N:C can be reached. EM fungi are defined by their unique morphology, which includes extensive intraradical Hartig nets and thick (though variable) hyphal mantles. When plants, particularly young saplings, are first becoming colonized with EM fungi, it is likely that most of their invested carbon is being allocated to the morphological structures involved in symbiosis and nutrient exchange. This would explain the low EM abundances in hyphal-only nutrient bags when colonization was low and when there was a low rate of N:C exchange. Once the symbiosis infrastructure has been established, however, invested carbon can be used to produce nutrient-

acquisitive hyphae and enzymes. This would explain why our data shows a large "jump" in the relationship between N:C and EM abundance in nutrient bags (the logarithmic curve) at intermediate values of EM colonization. Overall, this emphasizes that plant nitrogen return per unit carbon invested in EM fungi is not a fixed rate, but instead a result of complex economic interactions between a plant and its associated fungi.

P. deltoides is considered to be a dually-colonized species due to its ability to associate with both EM and AM fungi, often simultaneously, and throughout its lifespan (Teste et al. 2020). The temporal nature of dual colonization is important, as many non-dual EM species are known to support AM association when they are young (Egerton-Warburton and Allen 2001, Chen et al. 2000, Teste et al. 2020). However, these associations are generally lost as the seedling ages. Given the results of this study, it is possible that the prevalence of, and perhaps retained ability to support, AM associations in the roots of "non-dual" EM plants is particularly to assist young saplings during a nutritionally vulnerable period of their lifespan. AM interactions are considered to be less carbon-costly than EM interactions as a result of their reduced morphology and lack of nutrient-acquisitive enzymes (see Chapter 1, Lu and Hedin 2019). If achieving high levels of N:C exchange rates requires a period of colonization investment, it is possible that young seedlings are optimizing their carbon resources by investing in lower cost symbionts and slowly increase investment in the EM strategy as resources become available. This framework also explains why most AM-to-EM mycorrhizal

evolutionary transitions have resulted in taxa that are non-dually colonized and instead fully invested in the EM strategy (Tedersoo et al. 2018): the more invested you become, the greater the return on investment – suggestive of an alternative stable state model in AM-to-EM transition (Werner et al. 2018).

However, this raises the question: what are dually colonized species doing? Perhaps they are mid-transition from AM to EM (Teste et al. 2020) and we are just catching a glimpse of that transition. Or, perhaps there are other selective forces that keep them in this liminal state. For species that are commonly subjected to increased levels of disturbance, such as flooding or fire, perhaps it is best to host intermediate values of investment in both guilds. Lodge (1989) showed that Populus and Salix both showed increased AM colonization when moisture habitats were extreme. *Quercus* spp. have been documented as dually colonized (Egerton-Warburton and Allen 2001) and are considered to be fire adapted; however, reports of dual colonization seem to be most prevalent in red oaks versus white oaks, which are thought be less fire tolerant (Lorimer 1985, Abrams 2006). Regardless, dually-colonized plants are considered to benefit from the increased nutrient acquisition flexibility despite the increased carbon costs (see Chapter 1, Tedersoo et al. 2018).

At this point in time, the relative N:C rates have all been considered within the framework of the study, and the absolute value of the N:C measured in this study have not been compared to other studies quantitatively. One reason is that we

investigated nitrogen acquisition and carbon deposition in our litter bags on different timescales. The ^{15}N transferred to our seedlings represented an integrated measure of nitrogen acquisition over the course of the seven-week experiment, with nitrogen transfer likely occurring at different instantaneous rates as the seedling and mycorrhizal symbiosis developed. However, ^{13}C deposition occurred during a short pulse-chase period (five days post pulse), and so represents a snapshot of carbon exchange. Additionally, based on the current analysis, comparing the final N and C units is not straightforward (mg ^{15}N /atm% ^{13}C). As mentioned, relativizing the atm% ^{13}C to the total mass of the hyphal-only nutrient bag is under consideration. Relativization would be appropriate if the ^{13}C in the sample submitted to SIF for analysis was representative of the whole bag. However, given the low N value of the rye roots, the amount of mass in the bag was larger than that of clover (Table S1), which yielded less surface area per unit volume of the nutrient mass. The surface is where most of the N-acquisition via EM fungi would have taken place, and there was a discernable outer layer in the rye nutrient bags at harvest that was not visible in the clover treatment. If we were to relativize to the bag weight, it would significantly change the total ^{13}C values and the relationship magnitude between N:C and EM colonization would diminish (Figure S4).

However, given that the N:C ratio showed significant variation across multiple ecological axes, interpreting this value outside of the context of this experiment may not be useful; or, more specifically, may not consider other important axes

that influence *in situ* rates of N:C, leading to an oversimplified understanding of plant-fungal N and C dynamics. Due to constraints of parameterization, a single, static rate of the N:C exchange is often what is incorporated into global models. However, the results of this study suggest that a fixed exchange rate is not accurate, as the rate varied over nutrient contexts and degree of colonization. Additionally, our results suggest that a significant portion of the carbon costs associated with EM symbiosis is incurred during the initial morphological infrastructure investment period. Therefore, accounting for variables such as tree age, disturbance, and – perhaps most importantly – access to appropriate symbionts need to be considered. Common mycorrhizal networks have recently garnished considerable attention in the scientific literature and popular media (Egerton-Warburton et al. 2007, McGuire 2007, van der Heijden and Horton 2009, Booth and Hoeksema 2010, Courty et al. 2010, Bingham and Simard 2012), particularly for their role in C and N cycling to young trees. Given the results of this study, it is possible that common mycorrhizal networks may serve as a way for young saplings to avoid (or at least overcome) the initial high carbon-cost investment period associated with the morphological infrastructure of the EM symbiosis. Instead, saplings would be able to plug into an already established network of hyphae that has access to nutrient pockets, perhaps securing a higher N:C exchange during their vulnerable and costly morphological investment period. This would also corroborate why seedlings have increased survival and performance in stands dominated by the same mycorrhizal guild (Allsup et al. 2023).

Regardless, even if oversimplified, including mycorrhizal relationships into global models of nutrient cycling and predicted change is a step in the right direction. As we continue to unravel the ecological and contextual complexity of mycorrhizal associations on the small scale, our models will become more refined and lead us to an increased understanding of large-scale ecological and chemical processes.

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CONCLUSIONS

Through the use of a dually-colonized *Populus* system, which can associate with both ecto- (EM) and arbuscular mycorrhizal (AM) fungi simultaneously, this research program has investigated the role of environmental abiotic factors and genetics to determine which factors favor one mycorrhizal guild over the other. In Chapter 1, we found evidence that dually colonized *Populus deltoides* is able to shift its mycorrhizal investment strategies in adaptive ways across environments to maximize carbon costs and nutrient acquisition benefits. The conditions in which *P. deltoides* invests more heavily in EM and AM fungi correlates with global latitudinal patterns of soil nutrient cycling and mycorrhizal guild dominance, suggesting that mechanisms driving the investment strategy in dually-colonized plants may also be driving the current ranges and distributions of non-dual plants at the global scale. This finding emphasizes the influence of environmentally dependent costs and benefits in shaping plant mycorrhizal strategies and also highlights the unique flexibility of *P. deltoides*, and potentially all dually-colonized species, in navigating cost:benefit tradeoffs.

In additional to abiotic variables, genetics was also found to influence mycorrhizal associations. Chapter 2 demonstrated that the degree of EM colonization in *P. tremuloides* is a heritable trait, and one that is genetically and functionally linked with abscised leaf litter chemistry, specifically the C:N ratio.

Abscised leaf C:N was the only trait that correlated with EM colonization at both the phenotypic and genotypic level, indicating co-selection between the alleles for these traits. This result suggests that clonal stands of *P. tremuloides* may have experienced nutrient conservation/acquisition feedbacks throughout evolutionary history, leading to genetic fixation of traits related to foliar nutrient conservation and symbiotic nutrient acquisition via EM fungi. Additionally, abscised leaf litter C:N was also correlated with EM functional traits involved in nutrient acquisition, such as enzymatic capabilities and exploration type. Overall, this suggest that tree litter chemistry can promote and reinforce closed nutrient feedback loops, with the potential to establish adaptive trait syndromes. These results indicate that both nutrient use and nutrient acquisition strategies, specifically involving mycorrhizal symbionts, can be coordinated at the whole plant level even among genotypes of single species. Such genetic variation in individual traits and in the correlation between traits provides the raw materials for the evolution of the plant lifestyle strategies that shape the functioning of ecosystems globally.

This research also investigated the nutrient economics of mycorrhizal interactions in a more mechanistic manner. Chapter 3 found that the that the plant nitrogen return rate per unit carbon invested in EM fungi is a function of nutrient quality and degree of symbiotic investment. Nutrient quality, particularly litter quality, was significantly and positively correlated the N:C exchange rate: higher quality litter sources (lower C:N) resulted in a higher N:C

exchange rate. This aligns with our current understanding of high carbon-cost involved in enzymatic production by EM fungi. However, this study also found that as the degree of EM colonization increases, the N:C exchange rate increases, suggesting that the efficiency with which plants are able to acquire nitrogen is also a function of their degree of EM investment. Furthermore, the relationship between the N:C exchange rate and EM relative abundance was positive but saturating in growth, suggesting that there may be a period of morphological infrastructure investment between plants and EM fungi before maximum rates of N:C can be achieved. Taken together, this challenges the idea that N:C exchange rates are fixed ratios based purely on the "cost" to access organically bound nitrogen (enzymatic costs). This, instead, implies a more complex, economics-oriented framework, where rates change based on how invested a particular plant is. This becomes increasingly interesting when we consider the implications for forest structure (including patterns of guild dominance) and forest recruitment dynamics (such as young saplings establishing their own mycorrhizal connections) as well as the general status of dually-colonized tree species. It also becomes increasing relevant as we continue to incorporate mycorrhizal associations into predictive models of biogeochemical cycling and global change.

Due to constraints of parameterization, a single, static rate of N:C exchange is often what is incorporated into global models. However, the results of this work suggest that a fixed exchange rate is not accurate. Instead, the rate varied over nutrient contexts and degree of investment and may be influenced by other

ecological variables related to symbiotic association, such as tree age and access to symbionts. Regardless, even if oversimplified, including mycorrhizal relationships into global models of nutrient cycling and predicted change is a step in the right direction. As we continue to unravel the ecological and contextual complexity of mycorrhizal associations on the small scale, our models will become more refined and lead us to an increased understanding of large-scale ecological and chemical processes. Overall, the results of this work contribute to our understanding of influences dictating the current ranges and geographic distributions of tree species – including non-dual species. Understanding factors controlling the current compositional structure and biogeochemical cycling of our forested systems is central to predicting future forest states and functions.

SUPPLEMENTAL INFORMATION – CHAPTER ONE

TABLE S1.1 – Soil Moisture gradient

> mod_smgradient <- lmer(Soil.Moisture ~ Moisture + Light + (1|Plot..), data=fulldataset)							
> anova(mod_smgradient)							
	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)	
Moisture	0.117232	0.058616	2	105.07	7.8412	0.0006699	***
Light	0.019348	0.009674	2	3.863	1.2941	0.3713847	

TABLE S1.2 – Soil Moisture gradient, comparison of means

> emmeans(mod_smgradient, list(pairwise ~ Moisture), adjust = "tukey", type = "response")

$`emmeans of Moisture`

Moisture	emmean	SE	df	lower.CL	upper.CL
Low	0.0882	0.023	7.08	0.0338	0.143
Medium	0.1147	0.023	7.03	0.0604	0.169
High	0.1659	0.023	7.05	0.1115	0.22

Results are averaged over the levels of: Light

Degrees-of-freedom method: kenward-roger

Confidence level used: 0.95

$`pairwise differences of Moisture`

1	estimate	SE	df	t.ratio	p.value
Low - Medium	-0.0266	0.0199	105	-1.336	0.3785
Low - High	-0.0777	0.02	105	-3.891	0.0005
Medium - High	-0.0511	0.0199	105	-2.573	0.0306

Results are averaged over the levels of: Light

Degrees-of-freedom method: kenward-roger

P value adjustment: tukey method for comparing a family of 3 estimates

TABLE S1.3 – Total Colonization

mod_totcol = lmer(EMAMsum~Nitrogen*Light*Moisture + Tree.ID + Width..cm. + (1|Plot..), data=fulldataset)

> anova(mod_totcol)

Type III Analysis of Variance Table with Satterthwaite's method

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)	
Nitrogen	0.161	0.08	2	108.6	2.503	0.08654	.
Light	0.089	0.045	2	6.238	1.389	0.31701	
Moisture	0.805	0.403	2	108.8	12.52	1.28E-05	***
Tree.ID	0.364	0.364	1	109.6	11.33	0.00106	**
Width..cm.	0.012	0.012	1	110.5	0.366	0.54621	
Nitrogen:Light	0.123	0.031	4	108.7	0.953	0.43633	
Nitrogen:Moisture	0.242	0.061	4	108.9	1.883	0.11858	
Light:Moisture	0.151	0.038	4	109	1.176	0.32567	
Nitrogen:Light:Moisture	0.068	0.008	8	108.8	0.264	0.976	

TABLE S1.4 – Total colonization, comparison of means

> emmeans(mod_totcol, list(pairwise ~ Moisture), adjust = "tukey")					
NOTE: Results may be misleading due to involvement in interactions					
$`emmeans of Moisture`					
Moisture	emmean	SE	df	lower.CL	upper.CL
Low	0.477	0.0396	18.6	0.394	0.56
Medium	0.587	0.0362	13.1	0.509	0.665
High	0.672	0.0394	18.2	0.59	0.755
Results are averaged over the levels of: Nitrogen, Light, Tree.ID					
Degrees-of-freedom method: kenward-roger					
Confidence level used: 0.95					
$`pairwise differences of Moisture`					
1	estimate	SE	df	t.ratio	p.value
Low - Medium	-0.1096	0.04	109	-2.743	0.0194
Low - High	-0.1954	0.0392	109	-4.989	<.0001
Medium - High	-0.0857	0.0397	108	-2.161	0.0826
Results are averaged over the levels of: Nitrogen, Light, Tree.ID					
Degrees-of-freedom method: kenward-roger					
P value adjustment: tukey method for comparing a family of 3 estimates					

TABLE S1.5 – Plant growth

> mod_growth = lmer(sqrt(Total_NewMass) ~ Nitrogen*Light*Moisture + Original.Cutting.Stem..g. + (1|Plot..), data=fulldataset)							
> anova(mod_growth)							
Type III Analysis of Variance Table with Satterthwaite's method							
	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)	
Nitrogen	16.137	8.0686	2	111.668	55.8228	< 2e-16	***
Light	1.991	0.9953	2	5.907	6.886	0.0286	*
Moisture	38.591	19.2953	2	112.051	133.4947	< 2e-16	***
Original.Cutting.Stem..g.	0.104	0.1038	1	116.975	0.7181	0.39849	
Nitrogen:Light	1.038	0.2595	4	111.76	1.7957	0.13468	
Nitrogen:Moisture	17.255	4.3136	4	112.471	29.844	< 2e-16	***
Light:Moisture	1.402	0.3505	4	112.229	2.4247	0.05218	.
Nitrogen:Light:Moisture	2.212	0.2765	8	112.33	1.9131	0.06473	.

TABLE S1.6 – Plant growth, comparison of means

> emmeans(mod_growth, list(pairwise ~Nitrogen|Light|Moisture), adjust = "tukey", type = "response")

Note: Use 'contrast(regrid(object), ...)' to obtain contrasts of back-transformed estimates

$`emmeans of Nitrogen | Light, Moisture`

Light = Low, Moisture = Low:

Nitrogen	response	SE	df	lower.CL	upper.CL
NPK	1.97	0.508	75.5	1.088	3.11
Leaves	1.81	0.541	88.1	0.89	3.04
Control	2.42	0.569	73.1	1.415	3.68

Light = Medium, Moisture = Low:

Nitrogen	emmean	SE	df	lower.CL	upper.CL
NPK	2.06	0.532	75.1	1.133	3.25
Leaves	1.99	0.645	103.1	0.914	3.47
Control	2.25	0.5	65.2	1.362	3.36

Light = High, Moisture = Low:

Nitrogen	emmean	SE	df	lower.CL	upper.CL
NPK	1.89	0.431	55.6	1.127	2.85
Leaves	2.79	0.605	75.5	1.714	4.12
Control	2.67	0.549	63.7	1.682	3.88

Light = Low, Moisture = Medium:

Nitrogen	emmean	SE	df	lower.CL	upper.CL
NPK	3.59	0.637	63.4	2.427	4.97
Leaves	6.24	1.001	88.1	4.406	8.38
Control	3.12	0.557	53.1	2.107	4.34

Light = Medium, Moisture = Medium:

Nitrogen	emmean	SE	df	lower.CL	upper.CL
NPK	7.59	0.919	65.2	5.865	9.53
Leaves	7.52	1.099	88.3	5.498	9.87
Control	3.76	0.617	56.5	2.624	5.1

Light = High, Moisture = Medium:					
Nitrogen	emmean	SE	df	lower.CL	upper.CL
NPK	9.37	1.036	63.9	7.412	11.55
Leaves	6.42	1.014	88.5	4.561	8.59
Control	4.6	0.672	55.6	3.347	6.04
Light = Low, Moisture = High:					
Nitrogen	emmean	SE	df	lower.CL	upper.CL
NPK	10.55	1.19	75.4	8.316	13.06
Leaves	6.22	1	88.6	4.388	8.36
Control	3.08	0.59	63.1	2.015	4.37
Light = Medium, Moisture = High:					
Nitrogen	emmean	SE	df	lower.CL	upper.CL
NPK	12.26	1.099	55.2	10.159	14.56
Leaves	9.19	1.408	96.4	6.604	12.19
Control	3.42	0.621	63	2.29	4.77
Light = High, Moisture = High:					
Nitrogen	emmean	SE	df	lower.CL	upper.CL
NPK	17.14	1.293	55.1	14.642	19.83
Leaves	11.38	1.361	88.9	8.841	14.25
Control	3.7	0.646	66	2.52	5.1
Degrees-of-freedom method: kenward-roger					
Confidence level used: 0.95					
Intervals are back-transformed from the sqrt scale					
$`pairwise differences of Nitrogen | Light, Moisture`					
Light = Low, Moisture = Low:					
3	estimate	SE	df	t.ratio	p.value
NPK - Leaves	0.0601	0.259	114	0.233	0.9706
NPK - Control	-0.1506	0.242	113	-0.621	0.8088
Leaves - Control	-0.2108	0.259	114	-0.815	0.6944
Light = Medium, Moisture = Low:					

3	estimate	SE	df	t.ratio	p.value
NPK - Leaves	0.0245	0.279	112	0.088	0.9958
NPK - Control	-0.0658	0.233	113	-0.282	0.9571
Leaves - Control	-0.0903	0.269	111	-0.335	0.9399
Light = High, Moisture = Low:					
3	estimate	SE	df	t.ratio	p.value
NPK - Leaves	-0.2944	0.224	112	-1.316	0.3891
NPK - Control	-0.2572	0.212	112	-1.213	0.4478
Leaves - Control	0.0372	0.231	111	0.161	0.9858
Light = Low, Moisture = Medium:					
3	estimate	SE	df	t.ratio	p.value
NPK - Leaves	-0.603	0.248	112	-2.436	0.0431
NPK - Control	0.127	0.212	111	0.599	0.8212
Leaves - Control	0.7299	0.239	112	3.051	0.0079
Light = Medium, Moisture = Medium:					
3	estimate	SE	df	t.ratio	p.value
NPK - Leaves	0.0119	0.247	112	0.048	0.9987
NPK - Control	0.8161	0.216	112	3.787	0.0007
Leaves - Control	0.8042	0.241	112	3.341	0.0032
Light = High, Moisture = Medium:					
3	estimate	SE	df	t.ratio	p.value
NPK - Leaves	0.5271	0.246	111	2.14	0.0865
NPK - Control	0.9169	0.213	112	4.307	0.0001
Leaves - Control	0.3898	0.24	112	1.626	0.2389
Light = Low, Moisture = High:					
3	estimate	SE	df	t.ratio	p.value
NPK - Leaves	0.7552	0.258	112	2.923	0.0116
NPK - Control	1.4931	0.233	111	6.413	<.0001
Leaves - Control	0.7379	0.247	113	2.987	0.0096
Light = Medium, Moisture = High:					
3	estimate	SE	df	t.ratio	p.value

NPK - Leaves	0.4709	0.265	113	1.779	0.1812
NPK - Control	1.6529	0.213	112	7.744	<.0001
Leaves - Control	1.182	0.272	113	4.349	0.0001
Light = High, Moisture = High:					
3	estimate	SE	df	t.ratio	p.value
NPK - Leaves	0.7655	0.239	111	3.2	0.005
NPK - Control	2.2167	0.214	112	10.382	<.0001
Leaves - Control	1.4512	0.25	112	5.795	<.0001
Note: contrasts are still on the sqrt scale					
Degrees-of-freedom method: kenward-roger					
P value adjustment: tukey method for comparing a family of 3 estimates					

TABLE S1.7 – Total Colonization vs Plant Growth

mod_totcol_Mass = lmer(EMAMsum~Total_NewMass + Tree.ID + Width..cm. + (1|Plot..), data=fulldataset)							
> anova(mod_totcol_Mass)							
Type III Analysis of Variance Table with Satterthwaite's method							
	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)	
Total_NewMass	0.533	0.533	1	137.34	15.1627	0.0001533	***
Tree.ID	0.42008	0.42008	1	133.32	11.9503	0.0007336	***
Width..cm.	0.00598	0.00598	1	134.42	0.1703	0.6805382	

TABLE S1.8 – Mediation Analysis

Model 1: lmer(EMAMsum~**Total_NewMass** + Tree.ID + Width..cm. + (1|Plot..), data=fulldataset)

Model 2: lmer(EMAMsum~Nitrogen*Light*Moisture + Tree.ID + Width..cm. + (1|Plot..), data=fulldataset)

Model 3: lmer(EMAMsum~ Nitrogen*Light*Moisture + **Total_NewMass** + Tree.ID + Width..cm. + (1|Plot..), data=fulldataset)

Model	Variable	P-value	Estimate: low vs high	Low-High P-Value
1	Total New Mass	0.0001533		
2	Moisture	1.28E-05	-0.1954	<.0001
2	Nutrients	0.086540		
3	Total New Mass	0.294163		
3	Moisture	0.0889225	-0.142	0.0737
3	Nutrients	0.0680983		

TABLE S1.9 – AM Colonization

> mod_AM = lmer(asin(sqrt(PercAMF))~Nitrogen*Light*Moisture + Tree.ID + Width..cm. + (1|Plot..), data=fulldataset)

> anova(mod_AM)

Type III Analysis of Variance Table with Satterthwaite's method

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)	
Nitrogen	0.34886	0.174432	2	108.659	6.7414	0.001738	**
Light	0.08124	0.040621	2	5.732	1.5699	0.285957	
Moisture	0.1439	0.071951	2	109.111	2.7807	0.066392	.
Tree.ID	0.0258	0.0258	1	110.963	0.9971	0.320186	
Width..cm.	0.19764	0.197642	1	112.008	7.6384	0.006682	**
Nitrogen:Light	0.37917	0.094792	4	108.827	3.6635	0.007714	**
Nitrogen:Moisture	0.12928	0.032321	4	109.292	1.2491	0.294564	
Light:Moisture	0.173	0.04325	4	109.382	1.6715	0.161767	
Nitrogen:Light:Moisture	0.16788	0.020986	8	109.179	0.811	0.594304	

TABLE S1.10 – AM colonization, comparison of means: moisture

emmeans(mod_AM, ~Moisture, adjust = "tukey", type ="response")					
NOTE: Results may be misleading due to involvement in interactions					
Note: adjust = "tukey" was changed to "sidak" because "tukey" is only appropriate for one set of pairwise comparisons					
Moisture	response	SE	df	lower.CL	upper.CL
Low	0.197	0.0238	36.7	0.141	0.26
Medium	0.203	0.021	22.8	0.152	0.26
High	0.26	0.0261	35.5	0.198	0.328

TABLE S1.11 – AM Colonization, comparison of means: three-way interactions

> emmeans(mod_AM, ~Light|Nitrogen|Moisture, adjust = "tukey", type ="response")					
Note: adjust = "tukey" was changed to "sidak" because "tukey" is only appropriate for one set of pairwise comparisons					
$`emmeans of Nitrogen | Light, Moisture`					
Nitrogen = NPK, Moisture = Low:					
Light	response	SE	df	lower.CL	upper.CL
Low	0.2915	0.0686	85.4	0.1421	0.469
Medium	0.1135	0.0498	85.3	0.0231	0.26
High	0.2129	0.0539	69.5	0.098	0.357
Nitrogen = Leaves, Moisture = Low:					
Light	response	SE	df	lower.CL	upper.CL
Low	0.3062	0.0882	107.1	0.1203	0.533
Medium	0.2059	0.0787	107.8	0.0541	0.422
High	0.3357	0.0718	86.5	0.1759	0.518
Nitrogen = Control, Moisture = Low:					
Light	response	SE	df	lower.CL	upper.CL
Low	0.159	0.0554	81.4	0.05	0.314
Medium	0.1345	0.0483	79.5	0.0405	0.272

High	0.0797	0.0382	76.1	0.013	0.196
Nitrogen = NPK, Moisture = Medium:					
Light	response	SE	df	lower.CL	upper.CL
Low	0.3007	0.0639	72.4	0.159	0.465
Medium	0.2029	0.0554	76.1	0.0868	0.352
High	0.2569	0.0608	73.6	0.1251	0.417
Nitrogen = Leaves, Moisture = Medium:					
Light	response	SE	df	lower.CL	upper.CL
Low	0.1432	0.0589	95.9	0.0334	0.312
Medium	0.2756	0.0748	96.3	0.1171	0.471
High	0.1748	0.0633	95.9	0.0514	0.351
Nitrogen = Control, Moisture = Medium:					
Light	response	SE	df	lower.CL	upper.CL
Low	0.1786	0.0501	63.4	0.0741	0.316
Medium	0.196	0.0515	66.1	0.0872	0.335
High	0.1263	0.0428	65.5	0.0417	0.248
Nitrogen = NPK, Moisture = High:					
Light	response	SE	df	lower.CL	upper.CL
Low	0.4062	0.084	98.3	0.2159	0.612
Medium	0.1977	0.0522	68.3	0.0875	0.339
High	0.3216	0.0604	66.7	0.185	0.476
Nitrogen = Leaves, Moisture = High:					
Light	response	SE	df	lower.CL	upper.CL
Low	0.241	0.0716	96.4	0.0929	0.431
Medium	0.1285	0.0652	101.9	0.0169	0.322
High	0.4498	0.083	96.2	0.2576	0.65
Nitrogen = Control, Moisture = High:					
Light	response	SE	df	lower.CL	upper.CL
Low	0.2705	0.0624	73.8	0.1343	0.433
Medium	0.2341	0.0605	75.8	0.1053	0.395
High	0.1482	0.0511	81.1	0.0475	0.292

Results are averaged over the levels of: Tree.ID
Degrees-of-freedom method: kenward-roger
Confidence level used: 0.95
Conf-level adjustment: sidak method for 3 estimates
Intervals are back-transformed from the asin(sqrt(mu)) scale

TABLE S1.12 – EM Colonization

> mod_EM = lmer(asin(sqrt(PercEM_new))~Nitrogen*Light*Moisture + Tree.ID + Width..cm. + (1|Plot..), data=fulldataset)

> anova(mod_EM)

Type III Analysis of Variance Table with Satterthwaite's method

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)	
Nitrogen	1.0936	0.5468	2	108.674	13.2756	6.95E-06	***
Light	0.2267	0.11335	2	6.052	2.752	0.1412503	
Moisture	0.63472	0.31736	2	109.026	7.7051	0.0007414	***
Tree.ID	0.29798	0.29798	1	110.295	7.2345	0.0082627	**
Width..cm.	0.05627	0.05627	1	111.369	1.3661	0.244972	
Nitrogen:Light	0.247	0.06175	4	108.806	1.4992	0.2074747	
Nitrogen:Moisture	0.45696	0.11424	4	109.117	2.7736	0.0306437	*
Light:Moisture	0.02696	0.00674	4	109.231	0.1637	0.9563319	
Nitrogen:Light:Moisture	0.1856	0.0232	8	109.037	0.5633	0.8058462	

TABLE S1.13 – EM colonization, comparison of means

emmeans(mod_EM, list(pairwise ~ Nitrogen|Moisture), adjust = "tukey", type = "response")					
$`pairwise differences of Nitrogen | Moisture`					
Moisture = Low:					
2	estimate	SE	df	t.ratio	p.value
NPK - Leaves	0.051	0.0806	109	0.633	0.8022
NPK - Control	-0.2632	0.0706	109	-3.728	0.0009
Leaves - Control	-0.3142	0.0803	109	-3.914	0.0005
Moisture = Medium:					
3	estimate	SE	df	t.ratio	p.value
NPK - Leaves	-0.0694	0.0759	109	-0.913	0.633
NPK - Control	-0.2476	0.0655	109	-3.781	0.0007
Leaves - Control	-0.1783	0.074	109	-2.411	0.046
Moisture = High:					
3	estimate	SE	df	t.ratio	p.value
NPK - Leaves	-0.1195	0.08	109	-1.493	0.298
NPK - Control	-0.0789	0.0694	109	-1.137	0.4935
Leaves - Control	0.0406	0.0799	110	0.509	0.8673

TABLE S1.14 – EM vs. AM Tradeoff

mod_AMbyEM2 = lmer(asin(sqrt(PercAMF))~PercEM_new + Nitrogen*Light*Moisture + Tree.ID + Width..cm. + (1|Plot..), data=fulldataset)							
> anova(mod_AMbyEM2)							
Type III Analysis of Variance Table with Satterthwaite's method							
	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)	
PercEM_new	0.281	0.281	1	112.9	12.1399	0.0007035	***
Nitrogen	0.097	0.048	2	108	2.0863	0.1291259	
Light	0.043	0.022	2	6.137	0.9361	0.4417597	
Moisture	0.258	0.129	2	108.2	5.5776	0.0049499	**
Tree.ID	0.088	0.088	1	110.2	3.7991	0.0538228	.
Width..cm.	0.14	0.14	1	110.1	6.0574	0.0154004	*
Nitrogen:Light	0.291	0.073	4	107.6	3.1398	0.0174423	*
Nitrogen:Moisture	0.094	0.023	4	108.4	1.0132	0.4039198	
Light:Moisture	0.185	0.046	4	108.1	1.9937	0.1005823	
Nitrogen:Light:Moisture	0.112	0.014	8	108	0.6016	0.7747439	

TABLE S1.15 – EM vs. AM Tradeoff, comparison of means: Moisture

> emmeans(mod_AMbyEM2, list(pairwise~Moisture), adjust = "tukey", type = "response")

NOTE: Results may be misleading due to involvement in interactions

Note: Use 'contrast(regrid(object), ...)' to obtain contrasts of back-transformed estimates

$`emmeans of Moisture`

Moisture	response	SE	df	lower.CL	upper.CL
Low	0.172	0.0243	28.6	0.125	0.224
Medium	0.202	0.0221	16.4	0.157	0.25
High	0.265	0.027	24.4	0.212	0.323

$`pairwise differences of Moisture`

1	estimate	SE	df	t.ratio	p.value
Low - Medium	-0.0383	0.035	108	-1.095	0.5195
Low - High	-0.1139	0.0351	109	-3.243	0.0044
Medium - High	-0.0756	0.0338	108	-2.237	0.0696

Results are averaged over the levels of: Nitrogen, Light, Tree.ID

Note: contrasts are still on the asin.sqrt scale

Degrees-of-freedom method: kenward-roger

P value adjustment: tukey method for comparing a family of 3 estimates

TABLE S1.16 – EM vs. AM Tradeoff, comparison of means: Nitrogen*Light

> emmeans(mod_AMbyEM2, list(pairwise~Nitrogen|Light), adjust = "tukey", type = "response")					
NOTE: Results may be misleading due to involvement in interactions					
Note: Use 'contrast(regrid(object), ...)' to obtain contrasts of back-transformed estimates					
$`emmeans of Nitrogen | Light`					
Light = Low:					
Nitrogen	response	SE	df	lower.CL	upper.CL
NPK	0.27	0.0474	24.1	0.1786	0.373
Leaves	0.203	0.0449	29	0.12	0.302
Control	0.208	0.0383	15.3	0.133	0.295
Light = Medium:					
Nitrogen	response	SE	df	lower.CL	upper.CL
NPK	0.158	0.0347	16.6	0.0917	0.237
Leaves	0.187	0.0447	31.6	0.1051	0.286
Control	0.194	0.0367	15.1	0.1224	0.278
Light = High:					
Nitrogen	response	SE	df	lower.CL	upper.CL
NPK	0.259	0.0397	13.7	0.1789	0.348
Leaves	0.304	0.0474	22	0.2113	0.407
Control	0.14	0.0331	16.4	0.0782	0.217
Results are averaged over the levels of: Moisture, Tree.ID					
Degrees-of-freedom method: kenward-roger					
Confidence level used: 0.95					
Intervals are back-transformed from the asin(sqrt(mu)) scale					
$`pairwise differences of Nitrogen | Light`					
Light = Low:					
2	estimate	SE	df	t.ratio	p.value
NPK - Leaves	0.07872	0.0627	108	1.255	0.4238
NPK - Control	0.07274	0.0585	108	1.244	0.4299

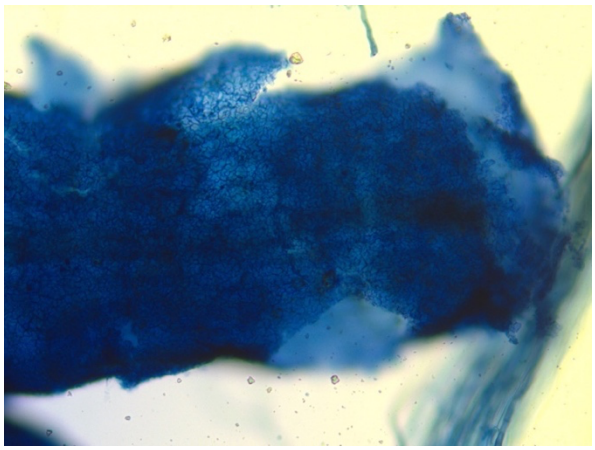
Leaves - Control	-0.00598	0.06	108	-0.1	0.9945
Light = Medium:					
2	estimate	SE	df	t.ratio	p.value
NPK - Leaves	-0.03875	0.061	108	-0.636	0.8009
NPK - Control	-0.04794	0.0518	108	-0.926	0.6251
Leaves - Control	-0.00919	0.0614	107	-0.15	0.9877
Light = High:					
2	estimate	SE	df	t.ratio	p.value
NPK - Leaves	-0.05047	0.0549	107	-0.92	0.6288
NPK - Control	0.14996	0.0506	108	2.964	0.0104
Leaves - Control	0.20044	0.0577	108	3.471	0.0021
Results are averaged over the levels of: Moisture, Tree.ID					
Note: contrasts are still on the asin.sqrt scale					
Degrees-of-freedom method: kenward-roger					
P value adjustment: tukey method for comparing a family of 3 estimates					

TABLE S1.17 - Chemical analysis of pot constituents

Sample ID	Total P * %	Total Nitrogen %	Total Carbon %
Background soil	0.03	0.07	3.05
Inoculum soil	0.03	0.48	12.71
Leaves	0.20	1.23	44.37
Inoculum roots	0.16	1.03	33.69

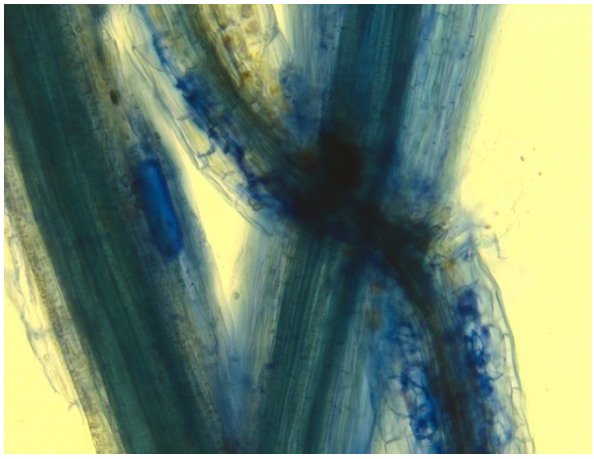
FIGURE S1.1. Compilation of photos of mycorrhizal structructures from *P. deltoides* roots

A. EMF: thin mantle

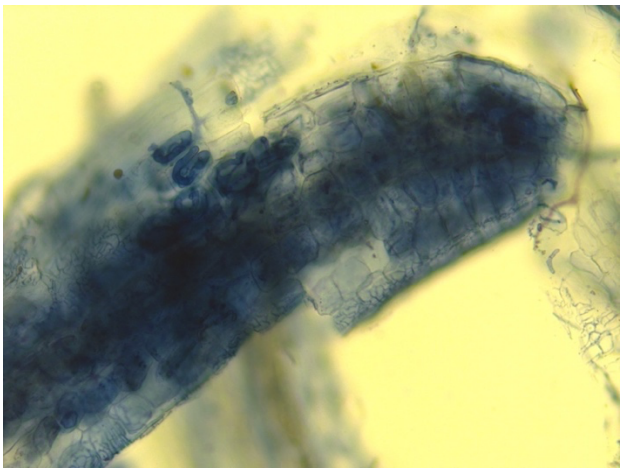


B. EMF: Thick mantle

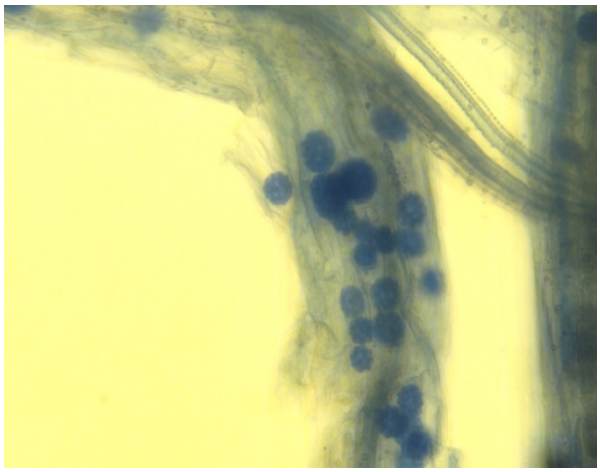
C. AMF: Coils and arbuscules



D. AMF: Vesicle and coils



E. Dual colonization: coils, arbuscules, thin mantle



F. Non-mycorrhizal endophytes

SUPPLEMENTAL INFORMATION – CHAPTER TWO

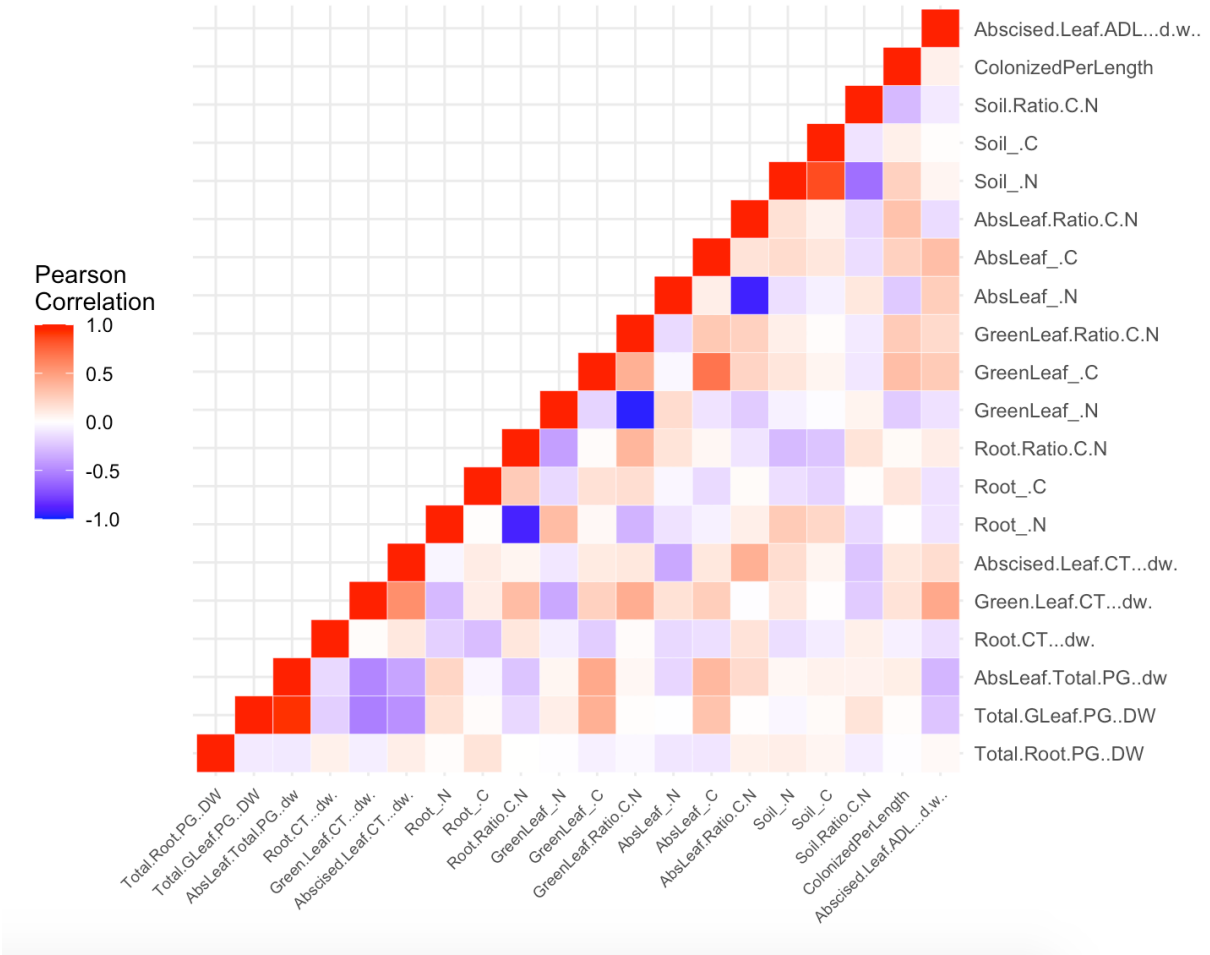


Figure S2.1. Pearson correlations values between *Populus tremuloides* plant chemical traits.

TABLE S2.1 – Soil C:N

> mod_wa6 = lmer(ColonizedPerLength ~ Soil.Ratio.C.N + AbsLeaf.Ratio.C.N + (1|Genet) + (1|Plot), data=wisasp)

> anova(mod_wa6)

Type III Analysis of Variance Table with Satterthwaite's method

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)	
Soil.Ratio.C.N	0.087369	0.087369	1	85.857	7.9727	0.005902	**
AbsLeaf.Ratio.C.N	0.054365	0.054365	1	74.502	4.9609	0.028946	*

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

TABLE S2.2 – Soil C:N using subset of data

> mod_wa6 = lmer(ColonizedPerLength ~ Soil.Ratio.C.N + AbsLeaf.Ratio.C.N + (1|Genet) + (1|Plot), data=wisasp_subset_soil)

> anova(mod_wa6)

Type III Analysis of Variance Table with Satterthwaite's method

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)	
Soil.Ratio.C.N	0.004043	0.004043	1	82.973	0.3822	0.53814	
AbsLeaf.Ratio.C.N	0.058005	0.058005	1	69.899	5.483	0.02206	*

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

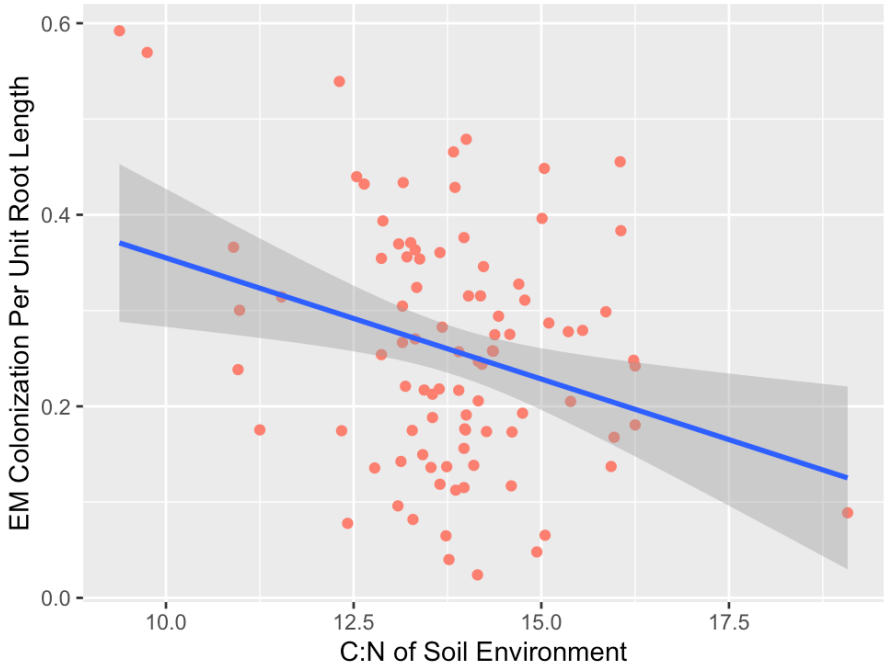


Figure S2.2. Relationship between soil C:N and EM colonization. Notice three influential points.

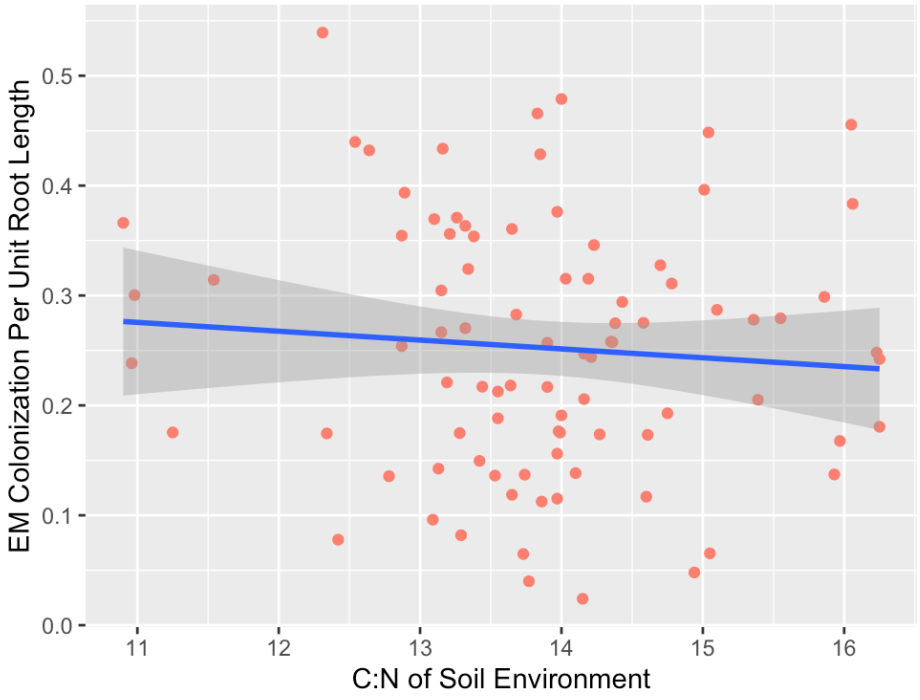


Figure S2.3. Relationship between soil C:N and EM Colonization with subset of data, lacking three influential points shown in Figure S2.2. Notice loss of correlation.

TABLE S2.3 – Enzymatic Capabilities vs Abscised Leaf C:N

> mod_absCN_Enzyme = lm(log2FoldChange ~ Enzymatic_Capabilities, data=function_table)						
> anova(mod_absCN_Enzyme)						
Analysis of Variance Table						
Response: log2FoldChange						
	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Enzymatic_Capabilities	1	52.963	52.963	9.5948	0.007871	**
Residuals	14	77.279	5.52			

TABLE S2.4 – Foraging distance vs Abscised Leaf C:N

> mod_absCN_Distance = lm(log2FoldChange ~ Distance, data=function_table)						
> anova(mod_absCN_Distance)						
Analysis of Variance Table						
Response: log2FoldChange						
	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Enzymatic_Capabilities	3	68.61	22.870	4.4529	0.02536	*
Residuals	12	61.632	5.136			

TABLE S2.5 – Comparison of means between different exploration distances

emmeans(mod_absCN_Distance, list(pairwise ~Distance), adjust = "tukey", type="response")					
$`emmeans of Distance`					
Distance	emmean	SE	df	lower.CL	upper.CL
Contact	4.094	2.266	12	-0.844	9.03
Long	4.838	1.602	12	1.346	8.33
Medium	-0.633	1.014	12	-2.841	1.58
Short	-0.742	0.801	12	-2.488	1
Confidence level used: 0.95					
$`pairwise differences of Distance`					
1	estimate	SE	df	t.ratio	p.value
Contact - Long	-0.744	2.78	12	-0.268	0.9929
Contact - Medium	4.727	2.48	12	1.904	0.2769
Contact - Short	4.836	2.4	12	2.012	0.2370
Long - Medium	5.47	1.9	12	2.885	0.0578
Long - Short	5.580	1.79	12	3.114	0.0389
Medium - Short	0.109	1.29	12	0.085	0.9998
P value adjustment: tukey method for comparing a family of 4 estimates >					

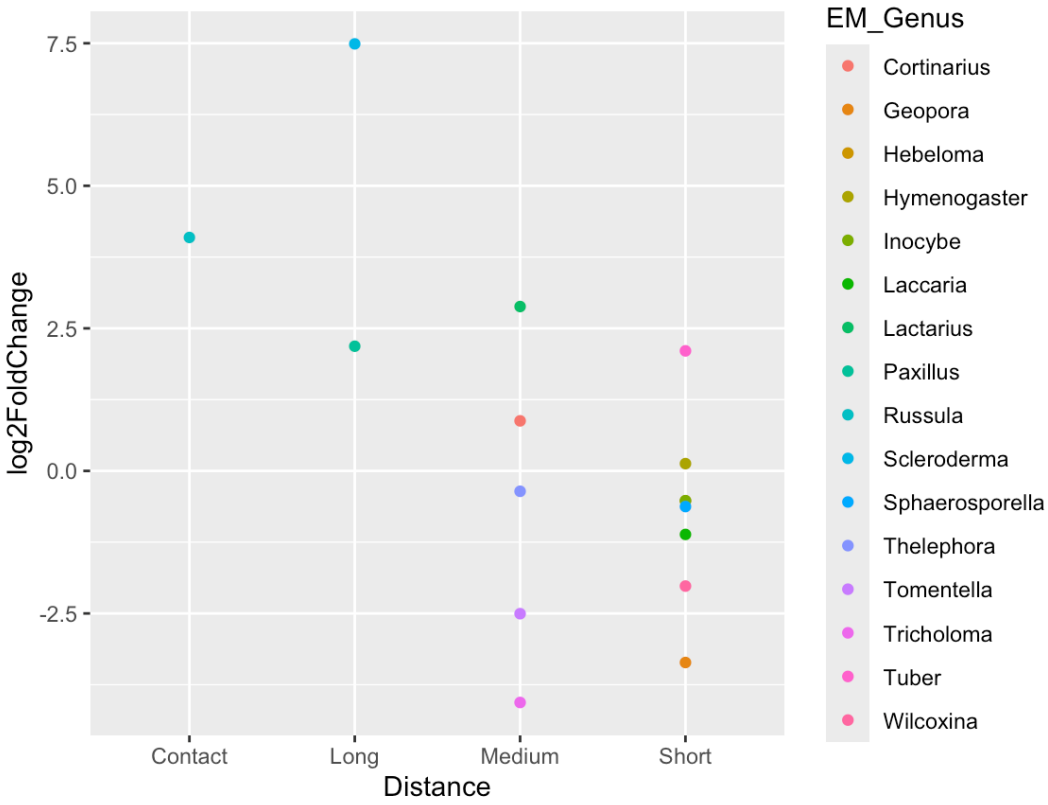


Figure S2.4. Log2 fold change in abscised leaf C:N in relation to EM exploration type distance.

SUPPLEMENTAL INFORMATION – CHAPTER THREE

Table S3.1. Nutrient Bag Calculation Table

Cohort 3						
		(Actual used)		Amount added		
	%N*	% converted	Goal N added (gN/pot)	Substance to add (g/pot)	Substance PER bag (g)	Number of pots trial 3
Ammonium nitrate	34	0.34	0.05	0.15	0.08	13
Trifolium (aboveground)	1.78	0.0178	0.027	1.52	0.76	20
Lolium (belowground)	0.63	0.0063	0.027	4.29	2.14	14

Samples omitted from analysis

Samples 132 and 133 both showed values suggestive of experimental error, such as contamination or improper chemical measurement. Sample 132 has a suspiciously high atm% ^{15}N value for its stem tissue (Figure S1). For a stem to have achieved this level of ^{15}N labelling, there would have needed to be considerable rates of nitrogen mineralization of the organic labelled nutrient source. However, the foliar and root atm% ^{15}N values for this sample are all well within the normal bounds, suggesting that (already unlikely) scenario was likely not the case. Therefore, this stem value has been deemed an error and the sample has been removed from the current analysis. Sample 133 has an atm% ^{15}N value that falls within range of others of the same sample type. However, the total N value measured is very high (Figure S2). When we extrapolate the total amount of ^{15}N that would be in the plant based on tissue mass ((total N in analyzed sample/sample weight) * total root weight * root atm% ^{15}N = total root ^{15}N + total stem ^{15}N + total foliar ^{15}N), this value is amplified, leading to a high projected total ^{15}N value in the roots. Again, other tissue values for this sample fall within the range of others of the same tissue type receiving the same treatment and we have no reason to believe that this sample would allocate its nitrogen resources differently than others, especially those in the same treatment. For this reason, we have decided that the total N measurement for this sample may have been and error and have decided to exclude this point from the current analysis. The low foliar ^{15}N value of sample 131 is more challenging to confidently chalk up to potential error. It's possible that this particular sample had reduced access to the labeled nitrogen fertilizer than others in the same treatment. However, given that the other tissue types from that sample fell within the typical ranges of similar tissue types from other seedlings in the same treatment, it seems unlikely. We have no reason to think this particular seedling would allocate its nitrogen resources differently than others, especially those in the same treatment. Although these samples would have theoretically not been run in subsequent order on the isotopic analysis machine given their order on the submitted plate of rolled tins, the fact that these sample numbers are located next to each other increases suspicion of an experimental error.

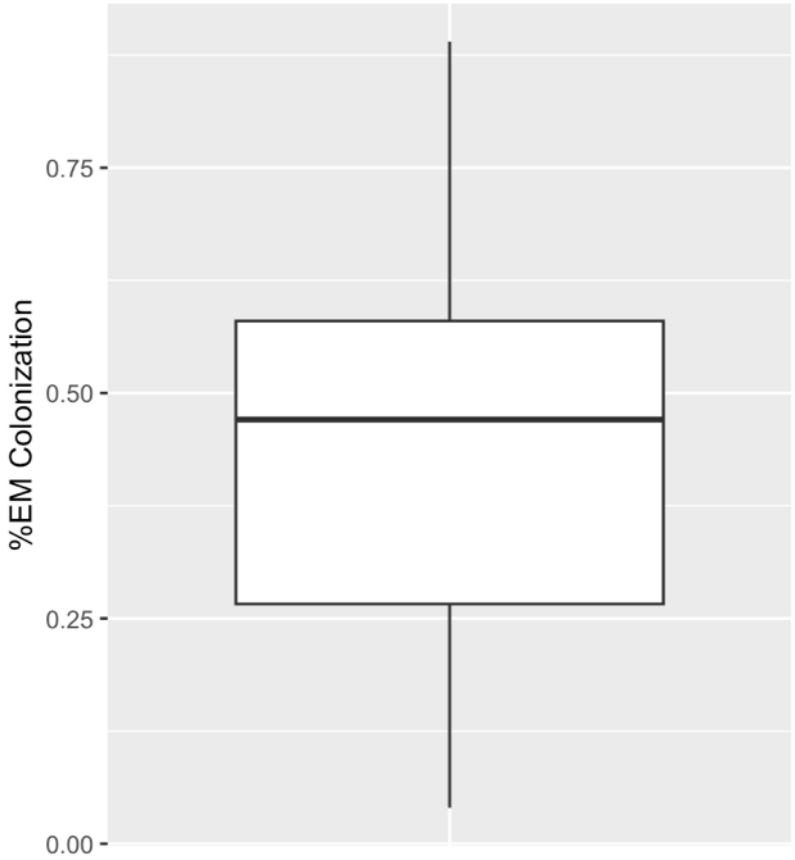


Figure S3.1. Variation in %EM colonization amongst samples in dataset. Data was relatively normally distributed with % EM colonization ranging from 1% to 89%.

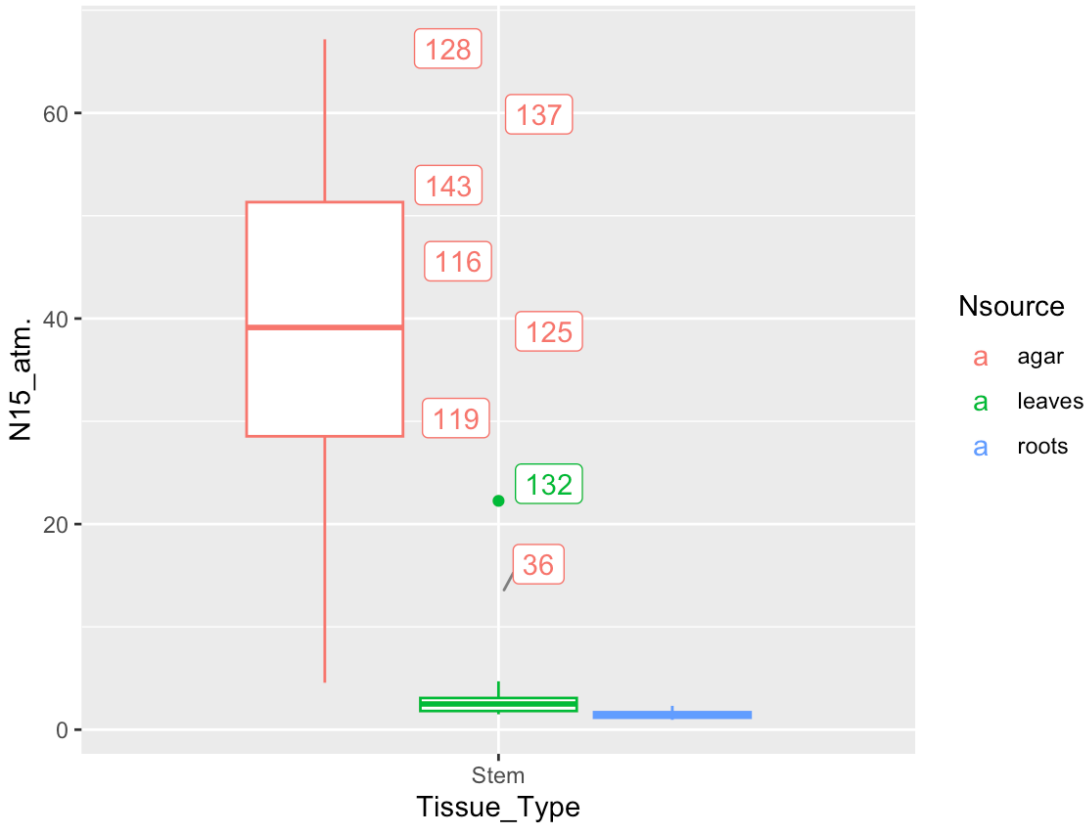


Figure S3.2. Isotopic atm% ^{15}N values for stem tissues of plants subjected to different nutrient source treatments. Notice the high value of sample pot 132 relative to treatment. (Note: agar samples were not included in the dissertation analysis, but they were included here during quality control steps for context and comparison. "Nsource" = nutrient source, "leaves" = clover, "roots" = rye)

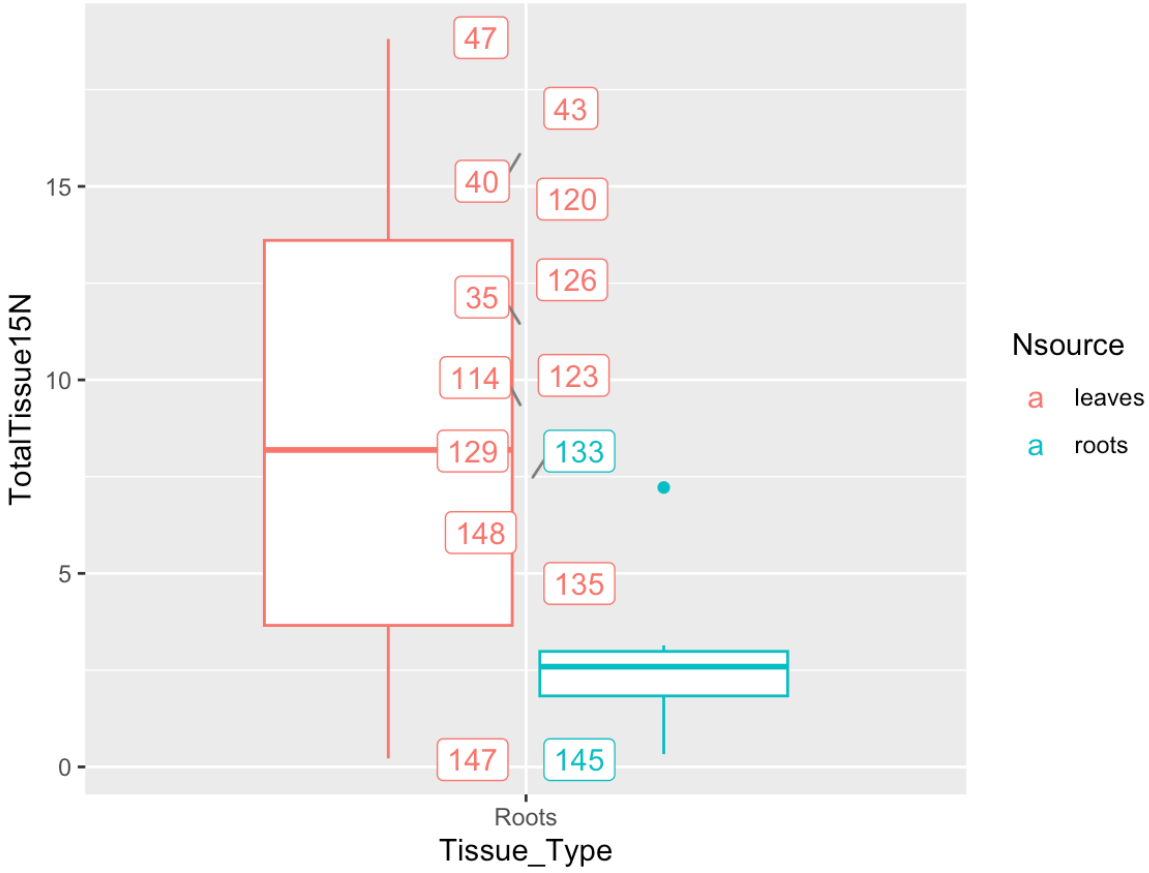


Figure S3.3. Total ^{15}N values for root tissues of plants subjected to different nutrient source treatments. Notice the high value of sample pot 133 relative to others of the same treatment. ("Nsource" = nutrient source, "leaves" = clover, "roots" = rye)

Plant ^{15}N Calculations

(total N in <plant tissue> sample analyzed/analyzed sample weight) * total <plant tissue> weight * <plant tissue> atm% ^{15}N = total <plant tissue> ^{15}N

<plant tissue> = roots, stem, leaves; repeat for each.
Mass units were accounted for at each step of the above calculation

Total root ^{15}N + total stem ^{15}N + total leaves ^{15}N = total plant ^{15}N

Table S3.1 – Plant nitrogen return rate per unit carbon invested as explained by EM colonization and nitrogen source

> model_hypoth1 = lmer(I(Total15NPlant/C13_atm.) ~ PercEM + PercAM + Nitrogen.Source + (1|Inoculum.Type) , data=subset(CNdata_subset, Nsource != "agar" & DNAtissue == "bag1"))							
> anova(model_hypoth1)							
Type III Analysis of Variance Table with Satterthwaite's method							
	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)	
PercEM	138.542	138.542	1	15.669	23.7031	0.000181	***
PercAM	2.097	2.097	1	16.446	0.3587	0.557374	
Nitrogen.Source	220.635	220.635	1	15.9	37.7485	1.45E-05	***

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1							

Table S3.2 – Treatment means of different nutrient (nitrogen) sources

> emmeans(model_hypoth1, list(pairwise~Nitrogen.Source), adjust = "tukey")					
$`emmeans of Nitrogen.Source`					
Nitrogen.Source	emmean	SE	df	lower.CL	upper.CL
Clover	8.86	0.814	4.39	6.678	11.04
Rye_BG	2.21	0.825	5.36	0.132	4.29
Degrees-of-freedom method: kenward-roger					
Results are given on the identity (not the response) scale.					
Confidence level used: 0.95					
$`pairwise differences of Nitrogen.Source`					
1	estimate	SE	df	t.ratio	p.value
Clover - Rye_BG	6.65	1.12	15.8	5.958	<.0001
Note: contrasts are still on the identity scale					
Degrees-of-freedom method: kenward-roger					

TABLE S3.3 – EM colonization vs. relative %EM abundance

> Model_EM = lmer(PercEM ~ perEM + Nitrogen.Source + (1|Inoculum.Type) , data=subset(CNdata_subset, Nsource != "agar" & DNAtissue == "bag1"))

> anova(Model_EM)

Type III Analysis of Variance Table with Satterthwaite's method

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)	
perEM	0.48529	0.48529	1	18	17.7033	0.0005293	***
Nitrogen.Source	0.13661	0.13661	1	18	4.9836	0.0385313	*

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

TABLE S3.5. N:C exchange rate and relative %EM abundance

> model_hypoth3.3 = lmer(I(Total15NPlant/C13_atm.) ~ perEM + PercAM + Nitrogen.Source + (1|Inoculum.Type) , data=subset(CNdata_subset, Nsource != "agar" & DNAtissue == "bag1"))							
boundary (singular) fit: see help('isSingular')							
> summary(model_hypoth3.3)							
Linear mixed model fit by REML. t-tests use Satterthwaite's method [lmerModLmerTest]							
Formula: I(Total15NPlant/C13_atm.) ~ perEM + PercAM + Nitrogen.Source + (1 | Inoculum.Type)							
Data: subset(CNdata_subset, Nsource != "agar" & DNAtissue == "bag1")							
REML criterion at convergence: 82.2							
Scaled residuals:							
Min	1Q	Median	3Q	Max			
-1.94039	-0.48925	0.07457	0.36774	2.54798			
Random effects:							
Groups	Name	Variance	Std.Dev.				
Inoculum.Type	(Intercept)	0	0				
Residual		9.935	3.152				
Number of obs: 21, groups: Inoculum.Type, 3							
Fixed effects:							
	Estimate	Std. Error	df	t value	Pr(>|t|)		
(Intercept)	6.678	1.16	17	5.756	2.33E-05	***	
perEM	90.51	34.126	17	2.652	0.0168	*	
PercAM	10.796	39.334	17	0.274	0.787		
Nitrogen.SourceRye_BG	-4.537	1.439	17	-3.154	0.0058	**	

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1							
Correlation of Fixed Effects:							
	(Intr)	perEM	PercAM				
perEM	-0.546						
PercAM	-0.241	0.121					
Ntrgn.SR_BG	-0.68	0.279	-0.041				
optimizer (nloptwrap) convergence code: 0 (OK)							
boundary (singular) fit: see help('isSingular')							

TABLE S3.6 - N:C exchange rate and log-transformed relative %EM abundance

> model_hypoth3.2 = lmer(I(Total15NPlant/C13_atm.) ~ (log((perEM*100)+1)) + PercAM + Nitrogen.Source + (1|Inoculum.Type) , data=subset(CNdata_subset, Nsource != "agar" & DNAtissue == "bag1"))
> summary(model_hypoth3.2)
Linear mixed model fit by REML. t-tests use Satterthwaite's method [lmerModLmerTest]
Formula: I(Total15NPlant/C13_atm.) ~ (log((perEM * 100) + 1)) + PercAM + Nitrogen.Source + (1 | Inoculum.Type)
Data: subset(CNdata_subset, Nsource != "agar" & DNAtissue == "bag1")
REML criterion at convergence: 83.4
Scaled residuals:

Min	1Q	Median	3Q	Max	
-1.93151	-0.49212	-0.06317	0.61816	2.32522	

Random effects:				
Groups	Name	Variance	Std.Dev.	
Inoculum.Type	(Intercept)	0.1605	0.4006	
Residual		7.0968	2.664	
Number of obs: 21, groups: Inoculum.Type, 3				

Fixed effects:						
	Estimate	Std. Error	df	t value	Pr(>|t|)	
(Intercept)	5.407	1.126	10.966	4.803	5.56E-04	***
log((perEM * 100) + 1)	4.206	1.033	16.006	4.07	0.00089	***
PercAM	19.787	33.933	16.352	0.583	0.567754	
Nitrogen.SourceRye_BG	-4.597	1.196	15.441	-3.844	0.00152	**

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1						
Correlation of Fixed Effects:						

	(Intr)	l(*1+1	PercAM	
l((EM*100+1	-0.649			
PercAM	-0.263	0.162		
Ntrgn.SR_BG	-0.607	0.21	-0.034	

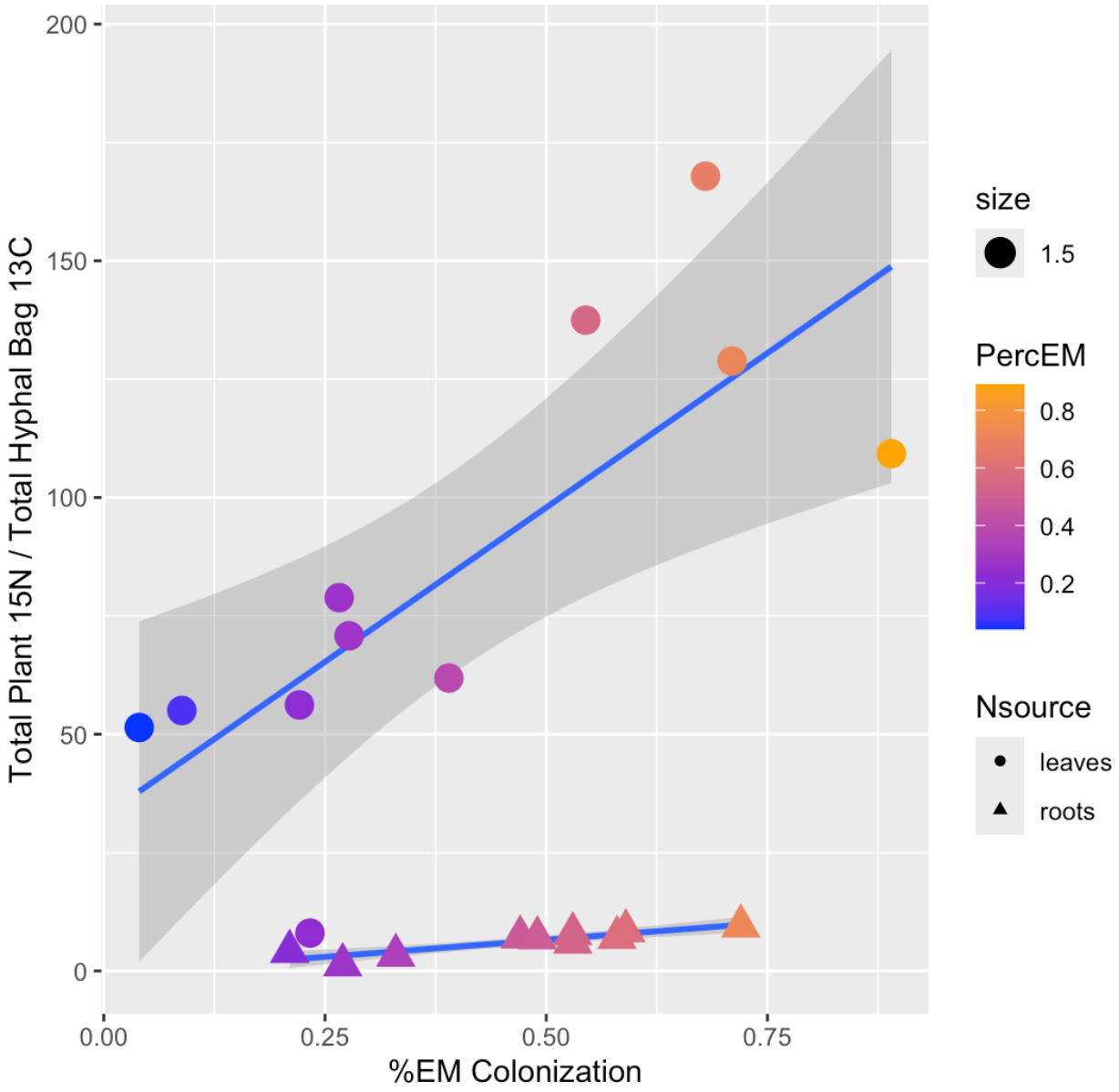
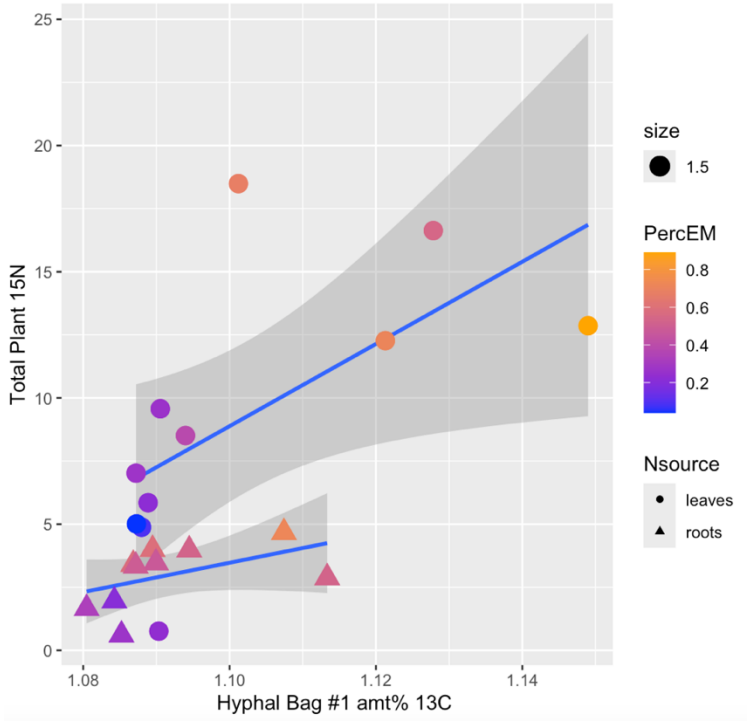
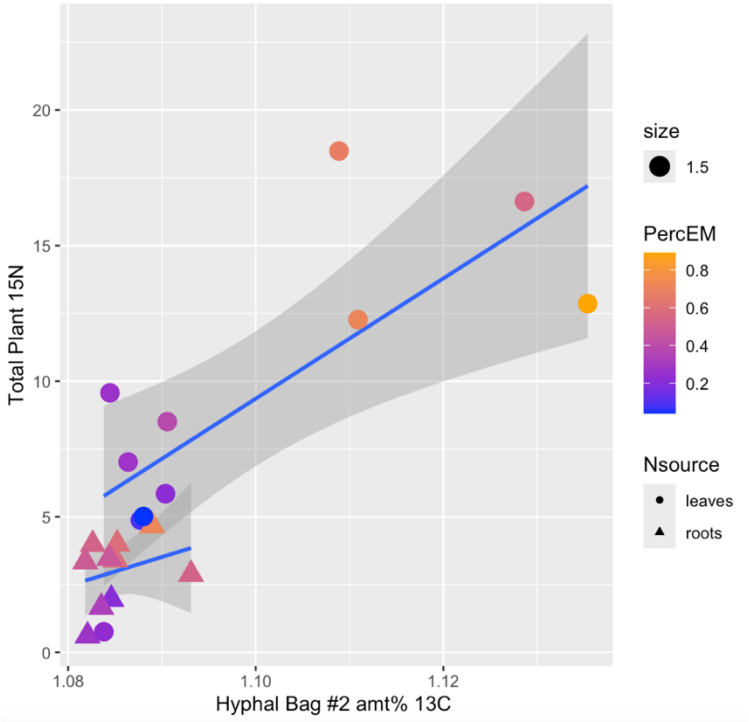


Figure S3.4. %EM Colonization vs. plant rate of N return on C investment, where ^{13}C component is relativized to nutrient bag mass. ("Nsource" = nutrient source, "leaves" = clover, "roots" = rye)





Figures S3.5 and S3.6. Differences in total plant ^{15}N and hyphal bag ^{13}C between nutrient bags 1 and 2. Notice differential axes between graphs. ("Nsource" = nutrient source, "leaves" = clover, "roots" = rye)

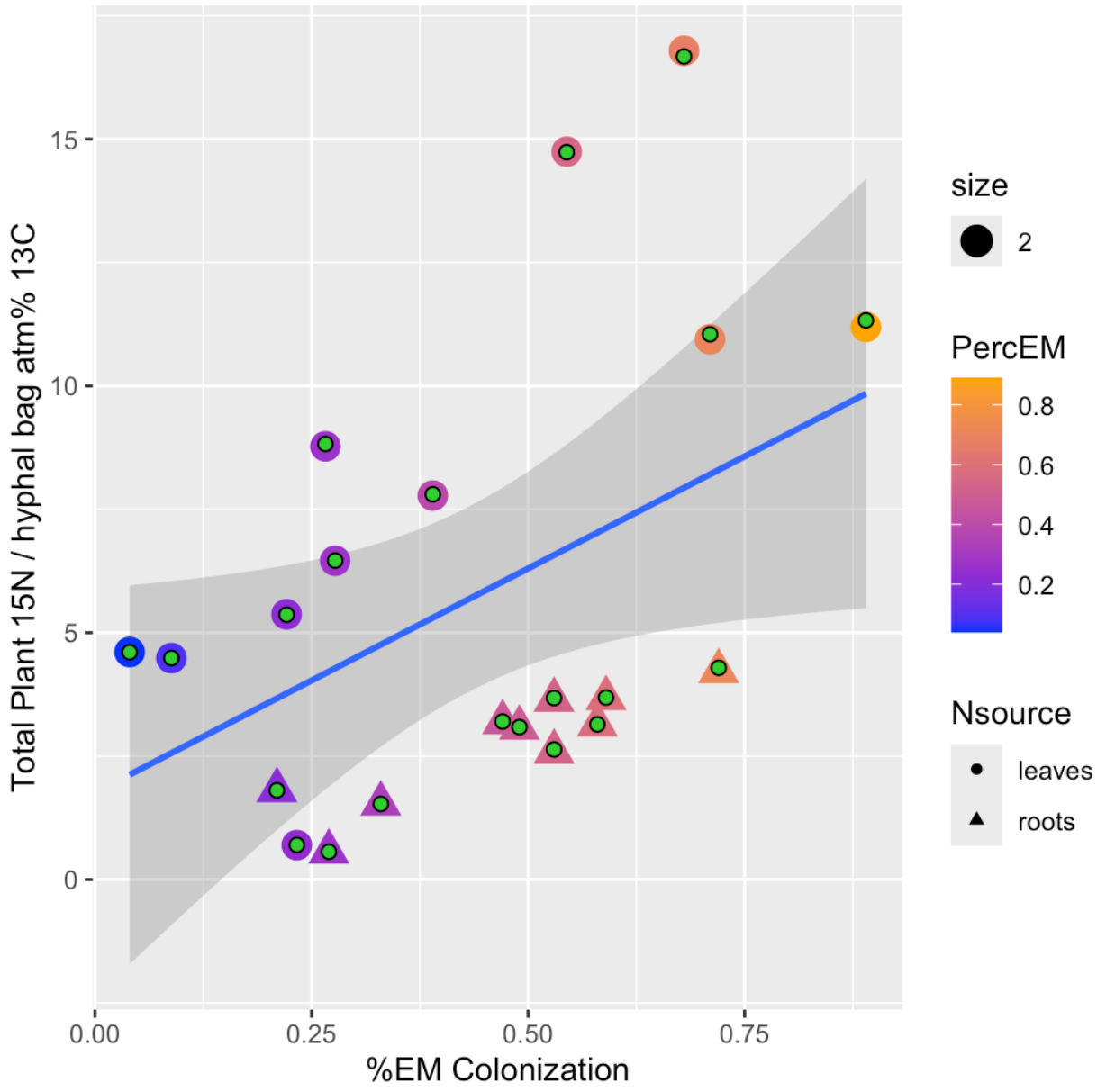


Figure S3.7. Comparison of final graph used in text (Figure 1) using Time Point 1 hyphal bag data (larger points colored by EM colonization gradient) and Time Point 2 hyphal bag data (green overlayed points). Notice the slight differences in upper right quadrant points but how otherwise points are very similar. ("Nsource" = nutrient source, "leaves" = clover, "roots" = rye)