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Mycorrhizal type dictates soil microbial diversity and function and the integrated root-microbial response to water stress in temperate forests

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Mycorrhizal type dictates soil microbial diversity and function and the integrated root-microbial response to water stress in temperate forests

Nanette C Raczka

**Dissertation submitted
to the Eberly College of Arts and Sciences
at West Virginia University**

in partial fulfillment of the requirements for the degree of

**Doctor of Philosophy in
Biology**

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**Morgantown, West Virginia
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**Keywords: temperate forest, water stress, carbon, soil, arbuscular mycorrhizae,
ectomycorrhizae**

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Abstract

Mycorrhizal type dictates soil microbial diversity and function and the integrated root-microbial response to water stress in temperate forests

Nanette Christine Raczka

Our understanding of the mechanisms that control the magnitude of the temperate forest carbon (C) sink and its response to global change remain uncertain. Much of this uncertainty lies in the extent to which differences between tree species in their mycorrhizal symbionts and corresponding nutrient acquisition strategies control the activity of soil microbes that mobilize nutrients and decompose soil organic matter. ECM trees allocate substantial amounts of C to ECM fungi and rhizosphere microbes to mine soil organic matter for nutrients. By contrast, AM trees invest less C belowground and rely on AM fungi to scavenge for nutrients. While these strategies have been shown to lead to differences in microbial function at the plot scale, there has been limited research that has investigated how these strategies shape microbial diversity or how the resulting differences in diversity impact function at the microbial scale. Moreover, the ability of these nutrient acquisition strategies to shape microbial communities likely controls ecosystem responses to global change. Thus, my research questions are: (1) Does microbial diversity drive function and the resulting products of decomposition in temperate forest soils? (2) To what extent do temperate forest trees shift their investment of C above vs. belowground under water stress? (3) How do plant-microbial interactions impact decomposition in temperate forests under water stress? For question 1, I examined the extent to which differences between AM and ECM trees in their nutrient acquisition strategies alter microbial diversity and function in a ~120-year-old forest in Tom's Run Natural Area, West Virginia. I sampled soils in plots dominated by either AM or ECM trees and assayed microbial diversity and function through quantitative stable isotope probing and metabolomic analysis. I found that AM soils had greater microbial diversity than ECM soils. This difference in diversity led to more flexible decomposition pathways and more products that could form more stable soil C in AM than ECM soils. For question 2, I built a throughfall exclusion experiment at Tom's Run in AM and ECM dominated plots and measured the effect of water stress on C allocation to above- vs. belowground processes. In response to the treatment, I found that ECM trees maintained root biomass and mycorrhizal colonization, while AM trees increased investment in roots and mycorrhizae. This reflects the ability of ECM trees to leverage their already extensive nutrient acquisition infrastructure to enhance water uptake. By contrast, it was necessary for AM trees to upregulate investment belowground to ensure access to water. For question 3, I measured the response of microbial activity to the water stress treatment at Tom's Run. I show that the treatment led to declines in soil respiration, nitrogen mineralization and oxidative enzyme activity in AM soil, which may be due to AM trees reducing root C transfers to the soil. In ECM soils, the treatment enhanced soil respiration, as well as rates of N mineralization and peroxidase activity in the rhizosphere soils, suggesting ECM roots provided optimal conditions to prime microbial activity. Collectively, these results provide evidence that differences between AM and ECM nutrient acquisition strategies led to divergent microbial diversity and function that can impact soil C storage and ecosystem responses to global change.

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“We have the world to live in on the condition that we will take good care of it. And to take good care of it, we have to know it. And to know it and be willing to take care of it, we have to love it.” – Wendell Berry

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Chapter 1. Introduction: The role plant-microbial interactions and plant functional traits drive forest response to global change

1.1 The extent to which plant-microbial interactions and plant functional traits drive forest responses to global change

Temperate forests are an important C sink and our understanding of the mechanisms that control the magnitude of this sink and its response to global change remain uncertain (Schlesinger & Bernhardt, 2020). Much of this uncertainty lies in belowground processes and, in the extent to which plant-microbial interactions enhance access to resources to fuel plant growth, alter soil C dynamics, and influence microbial communities and function. Recent research has shown that trees are active engineers belowground; using a diversity of strategies to acquire nutrients and water including root C exudation, mycorrhizal symbionts, and rhizosphere processes. At ecosystem to global scales, the strategies plants used to acquire nutrients drive variability in C storage as well as control the magnitude of global change responses (Averill et al., 2019; Carrara et al., 2018; Sulman et al., 2019; Terrer et al., 2017). At finer scales, plant nutrient acquisition strategies have been shown to shape the composition, traits and function of soil microbes that are the proximate control on soil C and nutrient cycling (Chapman et al., 2006; Freschet et al., 2013; Hobbie et al., 2006).

Mycorrhizal association has been a successful framework for classifying forest ecosystems on the strength of their plant-microbial interactions. Most temperate forest trees associate with either arbuscular mycorrhizae (AM) or ectomycorrhizae (ECM), and these associations lead to known differences in how plants and microbes feedback on ecosystem function (Frey, 2019; Phillips et al., 2013). At the global scale, research suggests that forests dominated by ECM trees may store more C in the soil than AM trees (Averill et al., 2014). At the ecosystem level, temperate forest ECM trees differ from AM in their above- and belowground traits that influence biogeochemical cycling. ECM trees produce low-quality, high C: N (Carbon: Nitrogen) litter that promotes slow decomposition. This slowed decomposition

can result in lower nitrification rates and greater accumulation of soil organic matter (SOM) (Averill et al., 2014). Oppositely, AM trees produce high-quality, low C: N litter that promotes rapid nutrient cycling and may lead to reduced stable C storage. While there is some evidence that these plant traits also feedback on microbial communities, there still lies significant uncertainty in how differences in nutrient acquisition strategies impact microbial diversity, function, and traits that control SOM cycling (Cheeke et al., 2017a).

Efforts to link microbial diversity, traits and function across AM and ECM systems have been inferred through coarse measures such as phospholipid fatty acid analyses and ergosterol concentrations of the fungal group, Dikarya (Cheeke et al., 2017b, 2020). These studies have been invaluable in understanding differences between AM and ECM microbial communities and have documented greater fungal to bacterial ratios and hyphal production in ECM soils than AM soils (Cheeke et al., 2017b, 2020). Moreover, research using potential gene functions show that AM and ECM soils can differ in microbial composition and diversity, whereby ECM soils can harbor greater fungal to bacterial ratios but lower diversity than AM soils (Bahram et al., 2020). While these methods are useful in inferring differences between microbial community composition and soil processes, a key unknown lies in directly linking active microbial taxa to their resulting function in AM and ECM soils.

Quantitative stable isotope probing (qSIP) is a cutting edge technique that links microbial diversity to function (Hungate et al., 2015). qSIP can directly quantify the assimilation of an isotopically labeled substrate into microbes at the taxon-level. When soils are incubated with a stable isotopically labeled substrate, the assimilation of the substrate into active taxa is reflected in the incorporation of the heavy stable isotope into their DNA (Hungate et al., 2015). Data generated from extracting, density fractionating, and sequencing the DNA is then ingested into

the qSIP data pipeline which estimates substrate assimilation at the taxon level (Hungate et al., 2015). Using qSIP, research has shown differences between bacteria taxa in N assimilation, whereby a few orders rather than all taxa were the dominant assimilators of ammonium (Morrissey et al., 2018). Additionally, researchers used qSIP to understand taxon-specific bacterial responses to rewetting after drought and found growth across the community after wet-up was highly correlated to total soil respiration and dominated by a few select taxa (Blazewicz et al., 2020). Thus, qSIP has the potential to unlock differences between AM and ECM soils in how their nutrient acquisition strategies shape microbial diversity and function.

Moving beyond research in how AM and ECM microbial communities may function at ambient conditions, research has shown that this mycorrhizal framework can advance our predictive understanding of ecosystem responses to global change. However, most of this research has focused on ecosystem responses to atmospheric nitrogen deposition and elevated CO₂. Across the US, nitrogen deposition has been found to increase the growth of AM trees more than ECM (Thomas et al., 2010). Elevated CO₂ has been shown to increase forest productivity, and over time ECM trees sustain growth through mining for needed N while AM trees do not (Luo et al., 2004; Terrer et al., 2017). While these results show distinct mycorrhizal responses to global change drivers that remove limitations to forest growth, much less is known about whether ECM and AM trees respond differently to drivers that induce stress and act to limit growth.

An increase in the frequency and severity of drought events is predicted to occur globally and could induce more intense water stress on forest ecosystems leading to potential declines in tree productivity and C sequestration (Brzostek et al., 2014). In temperate forests, ECM trees appear to be more drought tolerant than AM trees (Roman et al., 2015). As such, drought has

been shown to reduce AM tree growth and C storage to a greater extent than ECM trees (Au et al., 2020). While these responses to drought have been observed aboveground, belowground responses are less understood. Evidence from observational, theoretical and greenhouse experiments have found a wide range of belowground nutrient strategies such as enhanced root growth and mycorrhizal colonization to aid trees in water uptake, but field experiments to test the variability in these responses remain elusive (Phillips et al., 2016).

Differences in belowground strategies used by plants have the potential to feedback on the response of microbial activity and function to water stress. Independent of plants, during times of water stress, microbes in the soil may reduce their activity, which can lower decomposition rates and nutrient cycling. Drought may also shift microbial community composition to favor drought resistant microbes, funneling the C assimilated away from growth to survival strategies such as making osmolytes and strengthening cell walls (Manzoni et al., 2012). However, integrating tree and microbial interactions is crucial since shifts in microbial function may also alter soil C and nutrient dynamics. Emerging research has shown differences between microbial adaptations to drought may depend on the chemical composition of plant litter present (Malik et al., 2020). Given that AM and ECM trees differ in litter quality, belowground C allocation and mycorrhizal fungi, it is likely that their belowground strategies may cascade through the soil system to drive ecosystem responses to water stress.

In this dissertation, I have utilized quantitative stable isotope probing and metabolomic analysis to examine the extent to which differences between AM and ECM trees in their nutrient acquisition strategies alter microbial diversity and function in a ~120-year-old forest. Then, through a multi-year water-exclusion experiment in AM and ECM dominated plots, I measured the effect of water stress on C allocation to above- and belowground processes. Further, I

measured the response of microbial activity to the water stress treatment and the subsequent impacts on C and nutrient cycling. The overall aim of this research was to examine how differences between AM and ECM nutrient acquisition strategies lead to divergent microbial diversity and function that can impact soil C storage and ecosystem responses to global change.

Tom's Run Natural Area

Elizabeth Woods is located in Tom's Run Natural Area, a 34-ha forest operated by the West Virginia Land Trust, located in Monongahela County, West Virginia, approximately 10 km south of Morgantown, WV (39°32'50.6"N, -80°00'00.4"W). Mean annual temperature is approximately 11.62 °C, and mean annual precipitation is 1063 mm measured nearby at the Morgantown Hart Fields Airport (NOAA station # USW00013736). These sites have been characterized previously as silt loam soils (Soil Survey Staff, 2018).

Vegetation is mixed temperate broadleaved deciduous forests, consisting of a diverse mixture of ECM and AM tree species. The dominant species here are *Acer saccharum* Marshall (sugar maple), *Acer rubrum* L. (red maple), *Quercus velutina* Lam. (black oak), *Quercus rubra* L. (red oak), *Quercus alba* L. (white oak), *Liriodendron tulipifera* L. (tulip poplar), *Carya ovata*. (shagbark hickory), *Fagus grandifolia* Ehrh. (American beech), *Cornus florida* L. (flowering dogwood), *Platanus occidentalis* L. (sycamore).

1.2 Major questions of this study

The research undertaken in this dissertation has three broad questions:

- 1.** (*Chapter 2*) – Does microbial diversity drive function and the resulting products of decomposition in temperate forest soils?
- 2.** (*Chapter 3*) – To what extent do temperate forest trees shift their investment of C above vs. belowground under water stress?
- 3.** (*Chapter 4*) – How do plant-microbial interactions impact decomposition in temperate forests under water stress?

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**Chapter 2. Interactions between microbial diversity and substrate chemistry determine
the fate of carbon in soil**

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2.1 Abstract

Microbial decomposition drives the transformation of plant-derived substrates into microbial products that form stable soil organic matter (SOM). Recent theories have posited that decomposition depends on an interaction between SOM chemistry with microbial diversity and resulting function (e.g., enzymatic capabilities, growth rates). Here, we explicitly test these theories by coupling quantitative stable isotope probing and metabolomics to track the fate of ^{13}C enriched substrates that vary in chemical composition as they are assimilated by microbes and transformed into new metabolic products in soil. We found that differences in forest nutrient economies (e.g., nutrient cycling, microbial competition) led to arbuscular mycorrhizal (AM) soils harboring greater diversity of fungi and bacteria than ectomycorrhizal (ECM) soils. When incubated with ^{13}C enriched substrates, substrate type drove shifts in which species were active decomposers and the abundance of metabolic products that were reduced or saturated in the highly diverse AM soils. The decomposition pathways were more static in the less diverse, ECM soil. Importantly, the majority of these shifts were driven by taxa only present in the AM soil suggesting a strong link between microbial identity and their ability to decompose and assimilate substrates. Collectively, these results highlight an important interaction between ecosystem-level processes and microbial diversity; whereby the identity and function of active decomposers impacts the composition of decomposition products that can form stable SOM.

2.2 Introduction

Microbial decomposition is the foundation for carbon (C) and nutrient cycling in terrestrial ecosystems and the primary step in the transformation of plant-derived substrates into stable soil organic matter (SOM, Austin et al., 2014; Cotrufo et al., 2015). There is a long and rich history of research that has linked differences in the chemical composition of substrates with their subsequent decomposition rates and residence time in SOM (Aerts, 1997; Hobbie, 2015). Recent theoretical and empirical studies, however, have posited that the rate at which substrates are transformed and the degree to which decomposition products form stable SOM are influenced by the composition and functional traits of soil microbial communities (Keiser & Bradford, 2017; Strickland et al., 2009). While these studies have been successful in explaining empirical patterns in SOM decomposition and stabilization, the role of microbes in driving these patterns has been inferred from aggregate metrics describing composition (e.g., diversity, and/or richness). This focus on aggregate measures reflects methodological limitations in quantifying taxon-specific metabolic rates in natural communities and hinders our fundamental understanding of the contribution of individual taxa to community-level decomposition processes (Glassman, Wang, & Bruns, 2017; García-Palacios et al., 2016). Thus, linking microbial identity with function continues to represent a grand challenge in microbial ecology (Isobe et al., 2020). Meeting this challenge also has the potential to reduce uncertainty in Earth System Models that explicitly model the impacts of microbial function and traits on soil C cycling.

As our conceptual understanding of decomposition has evolved from theories centered on substrate chemistry to those accounting for microbial community composition and function, important interactions between these two drivers have largely been ignored. For instance, substrate and nutrient availability influence microbial biodiversity via habitat filtering and also by

promoting competitive interactions between microbial guilds (Glassman, Wang, & Bruns, 2017; Song et al., 2019). On the other hand, microbial biodiversity can increase soil organic matter formation through faster transformation of plant inputs into decomposition products, which can be associated to a larger number of syntrophic/facilitative biotic interactions (Amelung et al., 2008; Wang et al., 2017; Goldfarb et al., 2011; Koeck et al., 2014; Andlar et al., 2018; Marsland et al., 2019). We developed a new conceptual model that integrates interactions between substrate chemistry, microbial biodiversity, and the resulting decomposition products (Fig. 1). In this conceptual model, we use known differences between ecosystems dominated by trees that associate with arbuscular mycorrhizal (AM) fungi vs. those dominated by ectomycorrhizal (ECM) fungal association in their nutrient economies as our model system. ECM substrates that are chemically complex and require more energy investment to decompose (e.g., rich in lignocellulose) may foster a low diversity saprotrophic community since comparatively few species will have the metabolic capability to degrade the substrate (Goldfarb et al., 2011; Koeck et al., 2014; Andlar et al., 2018). In contrast, AM substrates that are less chemically complex (e.g., sugars and amino acids) can be easily decomposed by most microorganisms (Morrissey et al., 2011; Morrissey et al., 2017; Chapman & Newman, 2010; Maron et al., 2018) and consequently may foster more diverse saprotrophic communities. Microbial diversity can, in turn, influence the functional potential of the community to decompose a given substrate. More diverse communities in AM soils likely harbor more metabolic pathways, exhibit greater metabolic flexibility, and produce a wider variety of decomposition products that are potentially sorbed onto mineral surfaces forming stable organic matter (Schmidt et al., 2011). While AM soils have been shown to have greater stable SOM pools than ECM soils across the soil profile, the cascading linkages between substrate complexity, microbial diversity, decomposition products, and stable SOM

formation have yet to be investigated (Craig et al., 2018). Moreover, our conceptual model relies on the assumption that substrates with greater chemical complexity foster less diverse microbial communities. However, there is the potential for a competing hypothesis; whereby, a chemically complex substrate heightens microbial diversity due to the need for microbes to possess a larger number of metabolic pathways to drive decomposition (Freschet, Aerts, & Cornelissen, 2012). Thus, understanding the linkages between substrate chemistry, microbial biodiversity and the chemistry of decomposition products they produce is a critical unknown, since their characteristics may impact the rates of stable SOM formation through physio-chemical interactions with mineral surfaces (Mikutta et al., 2019).

Here, we explicitly test our conceptual model (Fig. 1) using a novel approach that couples quantitative Stable Isotope Probing (qSIP, Hungate et al., 2015), to quantify the amount of litter C that is assimilated by active bacterial and fungal taxa, with Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FTICR-MS) to track changes in metabolite composition during microbial degradation. We used known differences in plant traits and microbial communities between ecosystems influenced by ECM fungi vs. AM fungi as a model system to test our conceptual framework (Midgley, Brzostek, & Phillips, 2015). ECM trees generally have leaf litter that has greater lignin content and less nitrogen than AM trees. Further, ECM trees have more reliance on rhizosphere processes to access nutrients from organic sources than AM trees (Cheeke et al., 2017). ECM microbial communities have higher fungal to bacterial ratios and stronger competitive interactions between mycorrhizal symbionts and free-living microbes than AM microbial communities (Malik et al., 2016; Averill, Turner, & Finzi, 2014). To test our conceptual model, we incubated isotopically labeled ^{13}C AM (tulip poplar, *Liriodendron tulipifera*) and ECM

(English oak, *Quercus rubra*) substrates in soils from AM and ECM dominated plots in a fully-factorial mesocosm experiment.

2.3 Methods

Soil and litter sampling.

Mineral soil (0-15 cm) was collected at the Elizabeth Woods site, a 120-year-old deciduous forest in West Virginia, US (39°32'50.6" N, -80°00'00.4" W). Soils were collected from four 20x20 m plots dominated by either AM-associated trees (i.e. *Liriodendron tulipifera* and *Acer saccharum*), or ECM-associated trees (i.e. *Quercus rubra*, *Quercus velutina* and *Carya ovata*). These sites have been characterized previously as Culleoka-Westmoreland silt loam soils at the AM sites and Dormont and Guernsey silt loams at the ECM sites⁴⁰. Soils were also characterized by C:N ratios 11.7 and 14.1 for the AM and ECM soils respectively, with a pH of 6.8 for both soils. Soils with the same mycorrhizal status were pooled and homogenized, air-dried at room temperature for ~24 h and sieved through 2.0 mm mesh before the initiation of the experiment. Uniformly ¹³C labeled litter (>97 atom % ¹³C) from *Quercus robur* (i.e., ECM substrate) and *Liriodendron tulipifera* (i.e., AM substrate) leaves (Isolife BV, Wageningen, NL) were incubated in soil mesocosms in a factorial design with five replicates for each treatment combination (2 soil types x 2 substrate types), along with five replicate controls (no ¹³C substrate addition) for each soil type. The ¹³C enriched substrates were dried and ground to a powder and added in a suspension of 0.5ml sterile water to 20 g of soil at a concentration of 400 ug ¹³C g⁻¹ soil. The control soils received 0.5ml sterile water additions. These incubations were well mixed and kept at 60% water-holding capacity for the 21-day period at room-temperature. Chemical characteristics of soils and plant substrates are provided in table S1.

DNA processing and qSIP

For quantitative stable isotope probing, DNA was extracted, quantified, ultracentrifuged, fractionated, and sequenced as described in Morrissey et al. (2019) and Hungate et al. (2015).

DNA was extracted using a MoBio PowerSoil HTP Kit following the manufacturer's instructions. For stable isotope probing, 5 ug of DNA was loaded into a 5-ml ultracentrifuge tube with ~3.5ml of a saturated cesium chloride (CsCl) solution and ~900ml gradient buffer (200mM Tris, 200mM KCl, 2mM EDTA). DNA was separated via ultracentrifugation at 127000 g for 72 h using a TLN-100 rotor in an Optima Max bench top ultracentrifuge (Beckman Coulter, Fullerton, CA, USA). Tubes were fractionated into ~25 fractions of 150 µl each, and the density of each fraction was measured with a Raichart AR200 digital refractometer. DNA was purified using an isopropanol precipitation method. The 16S rRNA gene was subsequently quantified and sequenced in samples containing DNA, within the density range 1.660–1.735 gml⁻¹ (~10 fractions per sample). To quantify the 16S rRNA gene, quantitative PCR was performed in triplicate using a QuantStudio 5 applied biosystems (Thermo Fisher Scientific) and primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACVSGGGTATCTAAT-3') (Liu et al., 2012). The PCR program used was as follows: 95 °C for 2min followed by 45 cycles of 95 °C for 30 s, 64.5 °C for 30 s and 72 °C for 1 min. Libraries were sequenced on an Illumina MiSeq instrument (Illumina, Inc., San Diego, CA, USA) using a 300-cycle v2 reagent kit. Fungal 18S rRNA gene copies in each fraction were also quantified using primers 1380F (5'-CCCTGCCHTTTGTACACAC -3') and 1510R (5'-CCTTCYGCAGGTTCACCTAC -3'). The PCR program used was as follows: 98 °C for 3min followed by 40 cycles of 98 °C for 45 s, 60 °C for 45 s and 72 °C for 30 s. DNA fractions were amplified for fungal ITS rRNA genes using primers ITS4F (5'-AGCCTCCGCTTATTGATATGCTTAART-3') and 5.8SF (5'-AACTTTYRRCAAYGGATCWCT-3') (Liu et al., 2012) and 300-bp paired-end read chemistry on an IlluminaMiSeq (Illumina, Inc., San Diego, CA, USA). The PCR program used was as

follows: 95 °C for 6 min followed by 35 cycles of 95 °C for 15s, 55 °C for 30s, and 72 °C for 1 min. DNA fractions were then sequenced using a 500 cycle v2 reagent kit.

Files came pre-split and joined multiple paired ends that we combined to pick operational taxonomic units (OTU). Open reference OTUs were picked at 97% identity using SILVA 128 release database for Bacteria and RDP database for Fungi. Taxa were analyzed at the ‘OTU’ level from the QIIME L7 table. Calculation of ^{13}C excess atom fraction (EAF) was performed for each taxon as described previously by Morrissey et al. (2019). Briefly, using the CsCl density gradient data, a weighted average density (WAD) was computed for each taxon’s DNA extracted from control soils that did not receive an isotopically enriched substrate. This natural abundance WAD was then compared to the taxon’s WAD following incubation with the ^{13}C enriched material. The change in WAD can be used to quantify the amount of isotope incorporated into the DNA (Morrissey et al., 2019). Preliminary data analysis revealed an effect of ultracentrifuge tube on estimation of phylotype weighted average density, probably a consequence of slight differences in CsCl density gradients between tubes. This technical error was corrected as previously described in Morrissey et al. (2019). In addition to the samples subjected to qSIP analysis we also extracted and analyzed fungal and bacterial OTU’s from control soils where the DNA was extracted prior to incubation.

FTICR-MS and lipidomic analyses

Soil from substrate-incubated and controls mesocosms were processed and analyzed with Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS), using a 12 T Bruker Solarix FTICR mass spectrometer at the Environmental Molecular Sciences Laboratory in Richland, WA, as described in Fudyma et al. (2019). Briefly, 100 mg of dried soil or litter substrate was extracted using an adjusted Folch extraction (Folch, Lees, & Stanley, 1957).

Extraction was performed on each sample by sequentially adding 2 ml MeOH, followed by a 5 s vortex; 4 ml CHCl₃, followed by a 5 s vortex; sonication at 25°C for 1 hr (CPX3800 Ultrasonic Bath, Fisherbrand); addition of 1.25 ml of H₂O, followed by a slight mix to achieve bi-layer separation; and incubated at 4°C overnight. The top, aqueous layer (metabolite—polar) was pipetted off into 1 ml glass vials and stored at –80°C until FTICR-MS. The bottom, chloroform layer was dried down and stored in 50:50 methanol:chloroform until lipidomics analysis.

A standard Bruker electrospray ionization (ESI) source was used to generate negatively charged molecular ions in the metabolite fraction. Samples were then introduced directly to the ESI source. The instrument settings were optimized by tuning on a Suwannee River fulvic acid (SRFA) standard, purchased from International Humic Substances Society (IHCC). Blanks (HPLC grade methanol) were analyzed at the beginning and end of the day to monitor potential carry over from one sample to another. The instrument was flushed between samples using a mixture of water and methanol. One hundred and forty-four individual scans were averaged for each sample and internally calibrated using an organic matter homologous series separated by 14 Da (CH₂ groups). The mass measurement accuracy was less than 1 ppm for singly charged ions across a broad m/z range (m/z 300– 800). Data analysis software (Bruker Daltonik version 4.2) was used to convert raw spectra to a list of m/z values, applying the FTMS peak picker module with a signal-to noise ratio (S/N) threshold set to 7 and absolute intensity threshold set to the default value of 100. Chemical formulae were then assigned using in-house software following the compound identification algorithm that was described in Tolić *et al.* (2017). Peaks below 200 and above 800 were dropped to select only for calibrated and assigned peaks. Chemical formulae were assigned based on the following criteria: $S/N > 7$ and mass measurement error < 0.5 ppm, taking into consideration the presence of C, H, O, N, S, and P and excluding other elements. Detected peaks

and the associated molecular formula were uploaded to the in-house pipeline FTICR R Exploratory Data Analysis (FREDA) to obtain abundance of compound classes (carbohydrate-, lipid-, protein-, amino-sugar-, lignin-, tannin-, condensed hydrocarbon-, and unsaturated hydrocarbon-like) based on molar H : C and O : C ratios of the compounds (Bailey et al., 2017). For further analysis, we only consider those masses that meet the above criteria and were detected in more than five samples. Mass-to-charge ratios with assigned molecular formulae meeting the criteria (1546 different m/z values) were normalized to the sum of intensities. Ions with $m/z > 800$ were not detected in our samples. The m/z values represent the molecular mass (in Dalton) of the detected ions since all detected ions were singly charged ions. While our results do not represent a quantitative characterization of OM, the values presented are relative differences and should be representative of the samples. Finally, we would like to acknowledge that we were not able to see any clear evidence of ^{13}C label in our FTICR-MS analysis of the soil samples. The lack of ^{13}C label in our FTICR-MS analysis of the soil samples even though they received labeled substrate could be either due to the fact that most of the labeled substrates produced by microbial activities were of low molecular weight, which cannot be detected by FTICR-MS and/or the leftover labeled substrate was of low abundance compared to the organic compounds previously present in the soil matrix. As such, we used the FTICR-MS data to identify shifts in the overall composition of the chemical compounds in each soil.

Lipids in the chloroform fraction were analyzed by LC-MS/MS in both positive and negative ESI modes using a linear trap quadrupole (LTQ) Orbitrap Velos mass spectrometer (Thermo Fisher Scientific), as described in detail previously in Kyle et al. (2017). Lipid species were identified using the LIQUID tool followed by manual data inspection. Confidently identified lipid species were quantified using MZmine 2 (Pluskal et al., 2010) and the peak intensities were normalized

by linear regression and central tendency (i.e., identifying a central or typical value for a probability distribution) using InfernoRDN.

Statistical analysis

All data analyses were performed using R 3.2.0 (R Core Team, 2019). To examine the effects of soil type, substrate type and their interaction in the bacterial, fungal and chemical composition of DOM and the lipid pool; Bray-Curtis distance matrices were compared with permutational multivariate analysis of variance (PerMANOVA) and visualized with Principle Coordinate Analysis (PCoA) using *vegan* package (Oksanen et al., 2019). PerMANOVA analysis were run on the relative abundance and on the ^{13}C EAF of individual microbial taxa, separately for both bacterial and fungal communities.

The analyses for FTICR-MS were performed separately for control and incubated soils using all assigned molecular formulae remaining after quality filtering (Roth et al., 2019). In all cases, we applied a Z-score standardization before calculating Bray-Curtis distance matrices (Oksanen et al., 2019). We analyzed the results from FTICR-MS as resulting from the decomposition of the added substrates for two reasons. First, this is a fully factorial design where individual soil samples were split to either receive AM poplar or ECM oak litter substrate. Thus, each soil sample starts with the same characteristics and the changes at the end of the incubation period should reflect the processing of litter. Second, we excluded molecular formulae present in the litters and thus, the differences we report in each soil type are derived from this processing (or the lack of it).

We calculated aggregated indices that characterize both the composition and the physicochemical properties of the microbial (both bacteria and fungi) and the SOM and lipid pool (Eichorst & Kuske, 2012; Koch & Dittmar, 2006). For bacterial and fungal communities, we quantified Shannon-Weaver diversity index for each sample $H' = -\sum_{i=1}^S p_i \ln(p_i)$ (where p_i is

the proportion of species *I*) using the relative abundance of individual microbial taxa (Hill, 1973). To find the percent of substrate assimilation by individual taxa, we calculated the proportion of C assimilated by each group as previously described (Hungate et al., 2015) as a percent. For SOM and lipid molecular formulae, we separately calculated weighted means of formula-based characteristics (i.e. m/z , Aromaticity Index -AI; H/C, O/C, and Nominal Oxidation State of Carbon -NOSC) as the sum of the product of the single-formula information (i.e. m/z_i , AI_i , H/C_i and $NOSC_i$) and the relative intensity (I_i) divided by the sum of all intensities (e.g., $m/z_{\text{sample1}} = \sum_{i=1}^S (m/z_i \cdot I_i) / \sum(I_i)$). With these metrics we obtained sample-level information related to the molecular size (i.e. m/z), the molecular bioavailability (i.e. higher H/C ratio), the molecular reactivity (i.e. lower AI) and the energetic rewards from molecular oxidative degradation (i.e. higher NOSC) of the SOM, which allows to infer the potential of decomposition products to form stable SOM (Amelung et al., 2008; Roth et al., 2019; Roth et al., 2015). Detailed information of the calculated indices can be found in the literature (Roth et al., 2019; Roth et al., 2015; Koch & Dittmar, 2006).

We further tested the effects of soil type, substrate type and their interaction on each index using the “lm” function from the “stats” package. In these analyses, P-values were approximated by an F test using Type II ANOVA tests with Kenward-Roger Degrees of Freedom (Kenward & Roger, 1997). When interactions between soil and substrate type were found at $P < 0.1$, we examined differences for each level of a given factor by pairwise comparisons using the “lsmeans” package. All analyses were checked for the assumptions of residual normality and variance homogeneity.

2.4 Results & Discussion

Here we show the extent to which differing routes of decomposition and metabolic byproducts hinge on an interaction between microbial community biodiversity with substrate chemistry. Our results support our conceptual model in that the diversity of microbes is greater in AM than ECM soils (Fig. 1) and that microbial diversity is tightly coupled to the breadth of functional capabilities and the resulting shift in SOM chemistry with microbial degradation (Fig. 2). This outcome links community ecology theory with ecosystem processes and produces a transformative framework to advance our ability to incorporate these fundamental pathways into ecosystem models.

Differences in habitat characteristics between adjacent AM and ECM-dominated tree stands over nearly 120 years of ecosystem development appear to have led to divergent microbial communities, soil chemical properties, and SOM chemical composition (Table S1, Fig. S1, S2). Fungal and bacterial communities found in AM soils were more diverse than those in ECM soils (p -value < 0.01 and 0.01 , respectively; Fig. 2). The greater microbial diversity in AM soils than ECM likely reflects both habitat filtering and competitive interactions. The long-term inputs of higher quality litter to AM soil likely reduced microbial stress and enhanced access to resources that fuel microbial growth and release competition (Glassman, Wang, & Bruns, 2017; Song et al., 2019; Mikutta et al., 2019). By contrast, in ECM soils, the lower quality litter may have reduced diversity by selecting a lower number of taxa with the metabolic ability to degrade more complex substrates (Goldfarb et al., 2011; Koeck et al., 2014), or by indirectly promoting greater competition for limiting resources between microbial guilds (Averill, Turner, & Finzi, 2014). The soils also differed in C: N stoichiometry with AM soils having a lower C:N ratio (-24% , p -value < 0.05 ; Table S1) relative to ECM soils, a pattern commonly observed in forest ecosystems (Averill, Turner, & Finzi, 2014). Further, microbial community composition appeared to be coupled with

SOM chemical composition as determined using FTICR-MS, with AM soils possessing a different profile than ECM soils (PerMANOVA p -value < 0.01, Fig. S2, Table S1). ECM soils had lower percentages of lignin- and protein-like compounds (-43%, p -value < 0.05; -92%, p -value < 0.05, respectively), but relatively more amino sugar- (+85%, p -value < 0.05), unsaturated hydrocarbon- (+127%, p -value < 0.05) and lipid-like compounds (+92%, p -value < 0.05) than AM soils. This lower lignin content in ECM soils may reflect a greater capacity of the saprotrophic communities in ECM soils to process lignin-rich substrates and the ability of plant-C subsidies to ECM fungi to stimulate lignin degradation (Talbot & Treseder, 2012). Overall, these results suggest a tight coupling between differences in the nutrient economies of ECM and AM ecosystems and microbial diversity that regulate decomposition pathways to alter the chemical composition of soils.

When we followed the fate of the ^{13}C AM poplar and ECM oak substrates into microbial taxa (i.e., ^{13}C excess atom fraction (EAF)), we found distinctive responses to substrate type between AM and ECM microbial communities (Fig. 2, Table S2.). Many, bacterial and fungal taxa readily assimilated the added substrates in both soil types with the average ^{13}C EAF ranging from ~0.04 to 0.14 for bacteria and 0.04 to 0.36 for fungi (Fig. S3). However, bacterial ^{13}C assimilation was significantly different between substrates in both AM and ECM soils (substrate p -value < 0.05, Table S2); whereas, fungal ^{13}C assimilation was only altered by substrate type in AM soils (soil X substrate interaction p -value < 0.01, Fig. 2 C and D). These differences can be visualized on the principal coordinate analysis of community ^{13}C assimilation (Fig. 2) which shows distinct clustering due to substrate type in the AM soil but not in the ECM soil for fungi. Importantly, AM and ECM microbial communities responded differently to substrate type even though there were similar rates of ^{13}C loss from the soils that was derived from the added substrate (Fig. S4). These results suggest that the greater fungal diversity in AM than ECM soils lead to communities that

can rapidly shift the identity of active decomposers to metabolize plant-derived inputs of contrasting characteristics. In addition, abundance weighted ^{13}C assimilation was greater in fungi than bacteria when analyzed across soils and substrate treatments (Fig. S3), supporting previous research showing that the decomposition of plant-derived substrates is driven to a greater extent by fungi than bacteria in aerobic forest soils (Malik et al., 2016; Eichorst & Kuske, 2012).

By leveraging the taxonomic resolution of our qSIP analysis, we were able to identify the fungal and bacterial families that were the dominant active decomposers out of all the taxa that assimilated the substrates across soil and substrate types (Fig. 3, S5, S6). Within fungal families, an unclassified group of *Helotiales* was an important assimilator of the substrate at both sites. Some bacterial families also appeared to be generalists assimilating both litter types in both sites (e.g., *Bradyrhizobiaceae*, *Xanthomonadaceae*, *Comamonadaceae*, and *Soilbacteraceae*). However, we found that the fungal and bacterial families which assimilated most ^{13}C were only present in one soil type (either AM or ECM) (Fig. S5, S6), and often showed a preference based on substrate type. For example, *Chthoniobacteraceae* were only active in the AM soil where there was more ECM oak than AM poplar substrate (Fig. S5). For fungi, in AM soil, *Boletaceae* was a key assimilator of the AM poplar litter; whereas, in ECM soils, a family within *Russulales* accounted for rough 5% of C assimilation from both litter types (Fig. S6). These results highlight that decomposition of plant-derived substrates relies upon the identity of actively decomposing microbes, and that in many cases the dominant decomposing families in one system can be absent from another (Eichorst & Kuske, 2012).

We used FTICR-MS analysis to examine the extent to which differences in the composition of active saprotrophic communities between AM and ECM soils affect SOM chemical composition. In the AM soil, there were ~3 fold more unique compounds that appear as decomposition products

of either AM poplar or ECM oak substrates than in the ECM soil where soils receiving AM poplar or ECM oak substrates shared a greater number of compounds (Fig. 4 a and d). These pools of molecules unique to each substrate type likely played an important role in driving the observed differences in the chemical characteristics of the broader SOM pool in AM soils. In particular, weighted mean indices of the broader SOM pool show that AM soils receiving the AM poplar substrate had greater O:C ratio (p-value < 0.05), NOSC (p-value < 0.001), and aromaticity values (p-value < 0.05), but had lower average H:C ratio (p-value < 0.05) when compared to the ECM oak substrate (Fig. 4). The ability of decomposition products to form stable SOM can be inferred through their molecular properties such as, bioavailability (i.e., higher H/C ratio), reactivity (i.e. lower A_i) and the energetic rewards from oxidative degradation (i.e. higher NOSC, Roth et al., 2019; Roth et al., 2015; Koch & Dittmar, 2006; Tanentzap et al., 2019). Overall, these results indicate a lower abundance of reduced and saturated C-containing compounds (i.e., the C atoms are linked by double and single bonds, Amelung et al., 2008; Bailey et al., 2017; Roth et al., 2019) and a greater abundance of compounds that require a higher energy investment to breakdown and provide a lower energy reward for microbes in the AM soils that received the AM poplar substrate when compared to ECM oak substrate. These differences are important because both metrics indicate a greater potential for the AM poplar substrate to enter the microbial stabilization loop than the oak substrate in the AM soil. Higher O:C suggests more microbial processing of the AM poplar substrate in the AM soil – and because microbially processed C is more stable via organo-mineral interactions this could lead to greater stabilization interactions (Fig. 4, Amelung et al., 2008; Schmidt et al., 2011; Shao et al., 2017). In contrast, the ECM oak substrate has lower A_i and NOSC which should make it less susceptible to microbial decomposition due to greater activation energy.

We used lipidomics analysis to further explore the potential of decomposition products to form stable SOM, as widespread evidence suggests a dominance of microbial-originated lipids in newly-formed SOM (Kallenbach, Frey, & Grandy, 2016), as well as in C associated with soil minerals. Mirroring the results from the qSIP analysis, the lipidomic profiles varied with substrate type in AM soils and were unresponsive to substrate in the ECM soil (Fig. 5, Table S3). For instance, in AM soils, AM poplar substrate led to greater relative intensity of lipids of the class *Glycerophosphoethanolamines* (+7%, p -value < 0.05), but lower intensity of *Diacylglycerols* (-3%, p -value < 0.05 Fig. S7). In addition, the trends observed on the chemical characteristics of lipids are in line with the SOM results; substrate chemistry led to significant differences in weighted mean indices of O:C and H:C ratios in AM soil but not in ECM soil (p -value < 0.01; Fig. 5). Thus, our results suggest substrate chemistry drove divergent responses in the community of active decomposers as well as their decomposition products in AM soil, leading to a distinct chemical signature that can be indicative of the microbial communities involved in the decomposition process. These results are consequential because they suggest a novel mechanistic cascade whereby the composition of active decomposers can shift in response to substrate chemistry in highly diverse microbial communities and alter the resulting decomposition products that have the potential to form stable SOM. By contrast, it appears that the active decomposers and decomposition products in soils with less diverse microbial communities are more narrowly constrained.

Collectively, these results support our conceptual model by showing a novel dynamic interplay between substrate chemistry and microbial diversity that advances our mechanistic understanding of linkages between microbial community composition and function (Fig. 1). Differences between mycorrhizal associations in their nutrient economies over the course of ecosystem development

led to AM soil harboring greater microbial diversity than ECM soil (Fig. S1). This coupling of nutrient economies and microbial diversity also appears to have led to divergent functional capabilities of the resulting microbial communities. In the AM soil, greater diversity led to flexibility in the decomposition pathways with the identity of the active decomposers and the composition of their decomposition products shifting dynamically in response to variability in substrate type (Figs. 2-3). By contrast, in the less diverse ECM soil, the decomposition pathway was comparatively static (Figs. 2-3). The more diverse AM community generated a greater proportion of highly-processed decomposition products supporting our conceptual model that higher diversity saprotrophic communities have the potential to generate more stable soil organic matter. Moving forward, more research is needed to identify whether the patterns we observed here between AM and ECM systems operate at longer timescales (i.e., greater than 21 days) that capture slower processes that can destabilize or stabilize soil organic matter. Conceptually, these results transform our understanding of stable SOM formation by showing that diversity in microbial identity and function is coupled directly to the chemical diversity of decomposition products that are the foundation of SOM.

2.5 Tables and Figures

Figure 2-1. Conceptual framework of plant-microbial-soil feedbacks within ECM and AM nutrient economies. We used known differences in ECM and AM nutrient economies as the foundation for our conceptual model. ECM trees have leaf substrates that are chemically complex, which promote a low diversity microbial community that has constrained function. This constrained functionality provides only minimal metabolic pathways in which few metabolic products are produced to be sorbed onto SOM. The end result is less stable soil organic matter. In contrast, AM trees have less chemically complex substrates that can be easily degraded by most microbes. This ease of degradation may lead to a microbial community with higher diversity in both species composition and function. This diversity, in turn, generates diverse metabolic pathways that produce a wider variety of microbially processed compounds and more stable soil organic matter.

Figure 2-2. Variation in microbial diversity and composition between soils. Bar chart depicting fungal (a) and bacterial (b) Shannon diversity index (mean, standard error) in AM and ECM soils prior to incubation. Asterisks denote differences between soils at $p < 0.05$. Principal Coordinate Analysis showing variation in ^{13}C substrate assimilation for fungal (c) and bacterial communities (d) as determined via quantitative stable isotope probing. For bacterial communities, there were significant differences in composition between substrates and soil type at $p < 0.05$. For fungal communities, there was a significant substrate x soil interaction at $p < 0.05$.

Figure 2-3. Example taxa illustrative of variation in substrate assimilation by soil and substrate type. Substrate assimilated is shown with sample bacterial and fungal families.

Symbols represent averages and error bars show standard errors.

Figure 2-4. Impacts of substrate incubations on soil organic matter chemistry. Van Krevelen diagrams showing molecular formulae of compounds that are unique to each substrate addition and soil combination in (a) AM soil and (d) ECM soil. Bar plots depicting mean $\pm$ standard error of aggregated indices showing differences in the physicochemical properties, (b) H:C ratio, (c) O:C ratio, (e) aromaticity index (Ai), and (f) Nominal Oxidation State of C (NOSC), of the SOM pool. Error bars indicate standard error (n=5). Asterisks denote differences between substrate types in each soil at $p < 0.05$.

Figure 2-5. Impacts of substrate incubation on lipid pools. Principal coordinates analysis (PCoA) of the lipid pool composition in incubated soils (a). Bar plots depicting mean $\pm$ standard error of aggregated indices showing physicochemical properties (b) H:C ratio and (c) O:C ratio of the lipid pool. Error bars indicate standard error (n=5). Asterisks denote differences between substrate types in each soil at $p < 0.05$.

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**Chapter 3. Arbuscular mycorrhizal and ectomycorrhizal trees have divergent responses
of belowground carbon investment to water stress**

Under review and formatted for New Phytologist

3.1 Abstract

With global change already enhancing drought frequency and severity, there is a critical need to determine if temperate forests will continue to act as carbon (C) sinks. While aboveground responses to water stress are relatively well understood, the degree to which belowground strategies sustain tree growth under water stress remains uncertain. We investigated the extent to which belowground strategies to mitigate water stress differed between trees that associate with ectomycorrhizae (ECM) vs. arbuscular mycorrhizae (AM) via throughfall exclusion in the field. We tested competing hypotheses that belowground C investment in response to water stress in AM and ECM trees may be either parallel or inverse to their known aboveground strategies. We found support for the inverse hypothesis. ECM trees maintained belowground C investment at the same level, reflecting their ability to leverage a higher baseline level of investment to enhance water uptake. By contrast, AM trees upregulated belowground C investment with water stress to ensure access to water. Our findings indicate that mycorrhizal association controls the response of belowground C investment to water stress. Ongoing increases in AM dominance in temperate forests coupled with water stress may alter the balance of C investment to above vs. belowground pools.

3.2 Introduction

Most climate models predict that the Eastern US will continue to experience drought at a greater frequency and severity (Dai 2013, Ficklin and Novick 2017, Xu et al. 2019). However, there remains uncertainty in the degree to which temperate forests can maintain their carbon (C) sink strength in the face of increasing drought stress, particularly during the growing season. Water stress during the growing season restricts photosynthesis leading to declines in tree growth, wood production, and hydraulic failure (Allen et al. 2010, Anderegg et al. 2012, Choat et al. 2012). This response appears to vary by tree species with ‘drought-tolerant’ species continuing to grow while ‘drought-sensitive’ species downregulate their growth with water stress (Brzostek et al. 2014, Au et al. 2020). While prior research shows distinct strategies between tree species to mitigate water stress, much less is known about how these species operate belowground to aid in this aboveground response. Understanding this above- and belowground linkage is critical to improving model predictions, because it can lay a foundation to parameterize new functional groups that capture differences between tree species in their integrated, whole tree response to drought stress.

Mycorrhizal association may be key to capturing the divergent strategies that tree species use to respond to drought above- and belowground. Most trees associate with either arbuscular mycorrhizae (AM) or ectomycorrhizae (ECM), and grouping trees by mycorrhizal type has been successful in advancing our predictive understanding of temperate forest response to atmospheric nitrogen deposition and elevated CO₂ (Luo et al. 2004, Thomas et al. 2010b, Averill et al. 2018, Terrer et al. 2021). There is also increasing evidence that mycorrhizal association may control drought responses as well. For example, leaf-level measurements during the severe 2012 Midwest drought show that ECM trees maintained greater rates of photosynthesis than AM trees

(Roman et al. 2015). Further, regional analysis of tree core and FIA (Forest Inventory and Analysis) data from across the eastern US shows that drought reduces AM tree growth and C storage to a greater extent than ECM trees (Brzostek et al. 2014, Au et al. 2020). However, belowground differences between AM and ECM trees in their drought response are less straightforward. Observational work in the field documented that ECM associated trees can access deeper water than AM trees, leading to hydraulic lift of water to shallower soils, and thus enabling continued transpiration during drought (Matheny et al. 2017). This result is supported by a greenhouse study that found that AM associated trees reduced fine root biomass and colonization in response to drought, while drought had no significant effect on ECM roots and mycorrhizae (Liese et al., 2019). Oppositely, in another greenhouse study, AM trees were shown to increase coarse root non-structural carbohydrates to prevent whole plant declines in these reserves, while ECM trees maintained the same concentration of carbohydrates (Kannenberg et al., 2018). This variability in belowground responses raises a critical question: When faced with water stress, to what extent are differences between AM and ECM trees in their strategies to maintain belowground resource acquisition linked to their aboveground strategies to maintain C assimilation?

To answer this question, we examined differences between AM and ECM trees in their strategies to maintain belowground resource acquisition under water stress over the course of three growing seasons. We developed two hypotheses of how water stress may impact tree species differently belowground, which link back to influence tree responses aboveground (Fig 1). The foundation for these hypotheses is that, under ambient conditions, ECM trees send higher amounts of C belowground than AM trees in order to obtain belowground resources (Fig 1a; Hobbie, 2006), potentially leading to a greater investment in root biomass and mycorrhizal

colonization. The ‘Parallel hypothesis’, predicts that under water stress, drought-tolerant, ECM trees ramp up their belowground C investment in response to water stress, leveraging the C fixed to continue to gain access to water and nutrients through increasing root biomass, morphology and mycorrhizal colonization (Fig 1b; Brzostek et al., 2014; McDowell et al., 2008). By contrast, AM trees, known to be more drought-sensitive aboveground, will maintain or reduce their belowground C investment since less C will be available due to water stress restricting photosynthesis (Querejeta et al. 2007). The second hypothesis, the ‘Inverse hypothesis’, predicts that with water stress, drought sensitive, AM trees upregulate belowground C investment despite reduced photosynthesis to maintain access to water through increasing root growth, changing root morphology and enhancing mycorrhizal colonization (Fig 1c; Fuchs et al., 2020). ECM trees may maintain belowground C investment, since their ambient strategy already enables them to maintain resource acquisition (Liese et al., 2019). However, ECM trees may also downregulate belowground C investment as a consequence of the need to invest C to build drought-resistant xylem or repair embolisms that result from sustaining photosynthesis (Bréda et al., 2006; Meier & Leuschner, 2008; Querejeta et al., 2009). Given that these two hypotheses have opposing outcomes for species response to water stress and C storage, understanding these belowground links is critical to improving our ability to predict the strength of the temperate forest C sink under future drought extremes.

To test these hypotheses, we built a multi-year water-exclusion experiment in a 120-year-old temperate forest outside of Morgantown, West Virginia, USA. We established two 20 x 20m water exclusion plots dominated by either AM or ECM associated trees, each paired with a nearby control plot of similar species composition. By using this experimental design, we were able to link belowground responses to water stress to known divergent AM and ECM tree

strategies aboveground over three growing seasons with water stress (McDowell et al. 2008). To investigate these belowground responses, we measured several indices of belowground C investment. Specifically, we measured the impacts of drought on fine root biomass, mycorrhizal colonization, and root-derived C in soils. Additionally, to link our measurements to aboveground responses to water stress, we also measured aboveground growth.

3.3 Methods

Study Site

This research was conducted at the Tom's Run Natural Area, an 84-acre deciduous forest that is nearly 120-years old, located 8 miles south of Morgantown, West Virginia, US (39°32'50.6"N, -80°00'00.4"W). Mean annual temperature is approximately 11.62 °C, and mean annual precipitation is 1063 mm as measured nearby at the Morgantown Hart Fields Airport (NOAA station # USW00013736). These sites have been characterized previously as silt loam soils (Soil Survey Staff, 2018).

Elevation at this site ranges from 336 m to 438 m and slopes range from 3-25% (Soil Survey Staff, 2018). Vegetation is mixed temperate broadleaved deciduous forests, consisting of a diverse mixture of ECM and AM tree species. The dominant AM species here are *Acer saccharum* Marshall (sugar maple), *Acer rubrum* L. (red maple), and *Liriodendron tulipifera* L. (tulip poplar). The dominant ECM species are *Quercus velutina* Lam. (black oak), *Quercus rubra* L. (red oak), *Quercus alba* L. (white oak), *Carya ovata* (shagbark hickory), and *Fagus grandifolia* Ehrh. (American beech) (Table S1).

Throughfall exclusion experiment

To determine the extent to which differences in belowground responses between ECM and AM trees are linked to their strategies aboveground during times of water stress, we built water exclusion shelters to remove throughfall during the growing seasons of 2017, 2018, and 2019 (Tom's Run, Fig S1). We established a total of four 20 x 20m plots consisting of an AM treatment and AM control plot located on a hillside 40 m apart and an ECM treatment and

control plot located on a nearby slope west of the AM plots and 20 m apart. Each control plot received ambient rainfall and were located in stands with similar species composition (Table S1). The structures, built in 2016 (Fig S1), consisted of four rows of V-shaped panels at a 75° angle connected to a gutter to allow for drainage. At the end of each gutter a drainage hose was installed to divert the rainfall away from the plot edge by approximately 10 m. For each year, the treatment started in March and ran for the duration of the growing season. To divert rain, reinforced, clear polyethylene sheeting plastic was stretched over the panels and connected to the gutter. Each October, the plastic was removed in order to manipulate throughfall only during the growing season and also to alleviate any structural stress from snowfall. Over the course of our three-year experiment, we used treatments of increasing intensity each consecutive growing season. In 2017, 50% of the rainfall was excluded over the entire growing season. In 2018, we again began in March with a 50% reduction in rainfall, increased to a 90% exclusion in July to enhance the effect of water stress on the trees, and returned the treatment back to 50% for the remainder of the growing season. To maximize water stress in the final year, 90% of throughfall was excluded beginning in March and lasted throughout the growing season.

As with any large-scale global change experiment, we had to balance experimental artifacts with logistical constraints. We acknowledge that there may be an artifact of increasing the percentage of the throughfall removed over the course of the experiment. However, we decided to increase drought intensity over time in order to ensure that the treatment plots were experiencing a prolonged state of water stress. We also address this artifact statistically by including soil moisture as a covariate in our analysis to address these differences. One other potential limitation is that we did not trench the treatment plots. We did this to limit belowground disturbance since we were interested in observing fine root dynamics in the top 15

cm of the soil profile. To minimize this limitation, we did not sample soils or roots within 2m of the plot's edge.

Soil moisture

We measured gravimetric soil moisture in six locations in each plot year-round every two weeks starting in August of 2016 and until the cessation of the experiment at the end of the growing season in 2019. Approximately 88 sampling dates occurred over the three years of the experiment. Mean annual precipitation (MAP) for the Tom's Run site was obtained from the National Climate Data Center (Table 1). To examine how climate variability impacted the treatment effect, we also calculated soil moisture deficit for the treatment and control plots as the difference between precipitation (P) and potential evapotranspiration (PET) (i.e., P-PET; Brzostek et al., 2012). We used monthly mean temperatures and latitude to calculate PET during the treatment duration using the Thornthwaite method (Thornthwaite 1948). We used the approximate percent of throughfall removal to calculate treatment specific P-PET (Table 1).

Aboveground biomass and growth

To determine the impact of drought on tree growth, we examined differences in wood production over the course of the three-year experiment by measuring changes in the diameter at breast height using dendrometer bands every month on each tree within the experiment. We used published allometric equations to determine total aboveground biomass accumulation (Brzostek et al., 2014; Ehman et al., 2002). In order to standardize by biomass relative to the first year, we calculated the relative growth rates of the trees from 2017 for both 2018 and 2019. To examine

differences between species in growth response under drought, for each tree we calculated annual relative growth rates (RGR) as:

$$\text{RGR} = (\Delta\text{BA}/\text{BA}_i) * 100$$

Where ΔBA is the annual change in basal area ($\text{m}^2 \text{yr}^{-1}$) and BA_i is the initial basal area (Brzostek et al, 2014). For our plots, BA_i represents the first basal area measurement in 2017 and ΔBA was calculated as the change in basal area at the end of the growing season in 2018 and in 2019.

Fine root biomass

To determine the extent to which trees alter belowground C allocation under drought, we used a suite of measurements to analyze belowground C allocation including fine root biomass, mycorrhizal colonization and root-derived C. Across the three years, we sampled soils during the growing season, with the number of sampling dates varying each year due to logistical constraints (7 in 2017, 4 in 2018, 3 in 2019). For each sampling date, we extracted six mineral soil cores to a depth of 0-15 cm in treatment and control plots. We then removed all roots from the soil sample by carefully sieving the soil and extracting fine roots with forceps.

Mycorrhizal colonization and root-derived C

To determine if water stress decreased plant subsidies to symbiotic fungi, we examined AM and ECM colonization in July, the height of the growing season, of each year. For AM colonization, the roots were cleared of pigment and then stained with trypan blue (Comas et al 2014). We then used the grid intersect method to calculate percent AM colonization (Giovannetti

& Mosse 1980; McGonigle et al 1990). Briefly, a sample was placed on a 1 x 1 cm gridded Petri dish and examined under a stereoscopic microscope. At each root-gridline intersect, the presence of hyphae and/or arbuscules indicated colonization and percent AM colonization was then calculated as the percent of total intersects examined that had visible AM structures (Carrara et al 2018). To identify percent ECM colonization, the grid intersect method was additionally used, only without staining the roots. Instead, a sample was spread on a gridded Petri dish and the presence of fungal sheaths and branching on the roots determined ECM colonization.

To further investigate plant-derived belowground C inputs, we installed six rigid mesh root ingrowth cores into 15 cm deep holes at random areas in each plot. These cores were 15 cm height by 5 cm diameter. Root ingrowth cores were installed at the beginning of the 2017 and 2018 growing season and were collected at the end of each season. Roots were removed from each core, washed and then dried and weighed for biomass. To measure root-derived C, once cores were placed in the field, they were filled with 50% sand and 50% C₄ agricultural soils. Through the growth of C₃ tree roots, the release of C₃ rhizodeposits, and symbiont growth, shifts in the ¹³C signature of the C₄ soil coupled with a two-end member mixing model allowed us to assess root-derived C (Keller et al 2021). This was calculated by the fraction of soil C derived from root inputs (RI) from individual ingrowth cores collected in 2017 and 2018, as:

$$RI = (\delta^{13}C_{\text{ingrowth}} - \delta^{13}C_{\text{control}}) / (\delta^{13}C_{\text{root}} - \delta^{13}C_{\text{control}})$$

For each plot, $\delta^{13}C$ of the root was calculated as the mean $\delta^{13}C$ for the plot and final RI was calculated from the mean across the two years the cores were installed.

Statistical analysis

We assessed differences in growing season soil moisture across years between AM and ECM plots by performing an analysis of variance (ANOVA) in R (R Core Team, 2017), using the day of year 150-250 as our growing season cut-off dates. We chose day of year 150, which is the end of May to be a conservative start to the growing season and day of year 250, which is the beginning of September to be a conservative end to the growing season. To determine the extent to which chronic water stress altered relative growth rates and root biomass, we performed an analysis of covariance (ANCOVA) in which growing season soil moisture from day 150 to 250 was used as a covariate to compare plot and date as factors. We used growing season soil moisture as a covariate to test the effects of water stress on our measurements to be conservative in our analysis. Post hoc multiple comparisons were also made among plots and dates using the Tukey-Kramer HSD test. Across all measurements of root biomass, we first analyzed the data with time as a random effect, with month nested within year, to answer if the effect of treatment on root biomass depended on mycorrhizal type. To examine differences between years, since all groups were measured in the same months, a repeated measures design was used for our ANCOVA, with year as a fixed effect. For root colonization and root-derived C, an ANCOVA was also performed with growing season soil moisture as a covariate. Given the limited replication of the experiment, we present $P < 0.05$ as strong statistical evidence and $P < 0.1$ as marginal statistical evidence.

3.4 Results

Soil moisture and soil moisture deficit

Experimental drought led to a decline in soil moisture over the growing season for AM and ECM plots (Fig 2). Both ECM and AM treatment plots had a decline in soil moisture across all three years. However, in 2018, while treatment intensity was increased, precipitation during the growing season was also greater than the year before leading to a similar treatment effect as 2017 (Table 1). The largest reduction in soil moisture for both treatment plots was seen in 2019, when the plots were covered approximately 90% for the duration of the growing season. For each year the treatment ran, the AM treatment plot had significantly reduced soil moisture than the control plot (Fig 2a, b), with 2019 showing a 66% decline in soil moisture. The ECM treatment plot had significant declines in soil moisture for 2017 and 2019, but not for 2018. The largest decline in 2019 showed a 21% reduction in soil moisture in the ECM treatment plot, compared to the control.

There was interannual variability in the effect of the exclusion treatment on the soil moisture deficit (P-PET; Table 1). In 2017 and 2018, the soil moisture deficits were nearly identical (14mm in 2017 vs. -47mm in 2018). In 2019, there was a large increase to -415mm in the effect of the treatment on soil moisture deficit (Table 1).

Fine root biomass

Across all root biomass measurements, drought led to a significant increase in root biomass in AM plots ($p < 0.05$, Fig 3a, b) but led to no change in root biomass in ECM plots (Fig 3c, d). When looking at individual years, in the first year of treatment there was no difference

between ECM and AM plots in root biomass, or between the treatment and control plots. The second year of treatment show that AM root biomass significantly increased in the treatment plots compared to the control ($p < 0.05$), while ECM root growth did not significantly differ between control and treatment. In the last year of treatment, AM trees continued their trend and grew more roots in response to water stress. ECM trees continued to show no change in root biomass between the plots.

Mycorrhizal colonization and root-derived C

Water stress led to significant changes in mycorrhizal colonization in both AM and ECM plots, though the magnitude of these changes varied over time (Fig 4). In the first year of treatment, AM plots had significantly greater percent colonization than ECM plots but there was no difference between treatment and controls ($p < 0.05$). During the second year, mycorrhizal colonization was marginally higher in the treatment plots than the control plots ($p = 0.08$) for both mycorrhizal types. In the third year, when 100% of throughfall was excluded, mycorrhizal colonization in the AM treatment plot was 58% lower than the AM control plot ($p < 0.05$), but there were no differences between the ECM treatment and control plot.

Root-derived Carbon

There were no significant differences in root-derived C and ingrowth core root biomass between control and treatment plots for AM or ECM trees (Fig 5). However, there was marginally significant evidence for a main effect of mycorrhizal association on the amount of C sent belowground. ECM trees sent more C belowground than AM trees over the 2017 and 2018

growing seasons (Fig 5a, $p=0.06$). Additionally, there was a marginally significant interaction between treatment and mycorrhizal type in ingrowth core root biomass. AM trees grew more roots in the treatment plot over 2017 and 2018, where ECM trees did not (Fig 5b, $p=0.08$).

Aboveground relative growth rates

Water stress had no significant effects on the relative growth rates of AM and ECM trees when soil moisture was analyzed as a covariate (Fig S2). In the first two years of treatment, AM treatment plots trended towards lower relative growth rates than the control plots, while ECM trees showed no difference or a trend towards increasing relative growth rates. However, in the third year of experimental water stress, ECM trees showed a trend in which they grew 63% more in the treatment plot compared to control, while AM trees in the treatment plot had grown 13% compared to the control.

3.5 Discussion

The Eastern US is predicted to experience more frequent and extreme droughts (Dai 2013). Thus, understanding the degree to which tree species alter their belowground resource acquisition strategies to aid in aboveground C assimilation is critical to predicting how temperate forests will respond to future water stress. Here, we show that AM and ECM trees have divergent responses of belowground C investment to water stress. Specifically, our results provide support for the ‘Inverse hypothesis’. That is, drought-tolerant, ECM trees which have been shown to continue to grow with water stress (Au et al. 2020), maintained their belowground C investment at the same level in both the treatment and control plots (Fig 3c, d , Fig 4b). This lack of belowground response likely reflects their ability to leverage their higher baseline level of belowground C investment to enhance water uptake (Fig 5). By contrast, AM trees, which have been shown to reduce C assimilation with water stress (McDowell et al. 2008), paradoxically upregulated C belowground (Fig 3a, b, Fig 4a). When faced with water stress, it seems AM trees which function on a lower baseline level of belowground C investment, need to invest more C to ensure access to water (Fig 5). Collectively, our support of the inverse hypothesis suggests that mycorrhizal association controls the extent to which drought alters the balance of C sequestration in above vs. belowground pools.

Differences in the responses of AM and ECM trees to water stress may be due to the inherent strategies that each use belowground to gain nutrients. For AM trees, rapid rates of decomposition and their preference for mesic sites allow them to operate using a thin margin of belowground C investment to gain access to nutrients and water (Phillips et al. 2013, Midgley et al. 2015, Anderegg et al. 2018). As such, their constitutive belowground C investment and the resulting water uptake capacity of the root-mycorrhizal system may not be optimized for

maintaining the water balance of the tree when water stress occurs. In response to water stress, our results suggest that AM trees enhanced their water uptake capacity by producing more roots, maintaining a larger root biomass, and sustaining roots that were initially more heavily colonized with AM fungi (Fig 3b, 4a). This finding raises the question: Why do drought sensitive, AM trees that have reduced photosynthesis increase their belowground C investment to mitigate water stress? Greater investment belowground could offset declines aboveground, ensuring access to water for limited photosynthesis to occur in order to avoid C starvation (Kannenberg and Phillips 2017, Kannenberg et al. 2018). Moreover, AM roots and mycorrhizae are less C costly than ECM roots (Brzostek et al. 2013, Yin et al. 2014). Thus, enhancing the water foraging capacity in this manner is an inexpensive tactic for AM trees to mitigate water stress while at the same time balancing lower C supplies from photosynthesis (Smith and Smith 2011). Given the potential for this greater belowground C investment by AM trees to feedback on C and nutrient cycling in the soil, future research should investigate the extent to which this C could sustain microbial activity and N uptake by trees during times of water stress.

For ECM trees, multiple mechanisms may explain the nearly equivalent levels of belowground C investment between the ambient and treatment plots. First, because ECM trees often grow on xeric sites with slow rates of decomposition, they have higher constitutive levels of belowground C investment to access nutrients and water (Högberg et al. 2001, Hobbie 2006, Anderegg et al. 2018). As a result, ECM trees can leverage this resource acquisition strategy to enhance root-mycorrhizal water uptake capacity during water stress (Liese et al. 2019). Second, these results may indicate an opportunistic belowground strategy by ECM trees to maximize water absorption through increased root turnover rather than increased root biomass (Liese, Leuschner, and Meier, 2019). Though we did not directly measure turnover in this study, the frequency of

sampling over each growing season and ingrowth core data likely indicates that enhanced root turnover did not occur (Fig 3a, c). Lastly, ECM trees have been shown to have greater capacity than AM trees to hydraulically lift water (Lanning et al., 2020; Matheny et al., 2016). In our study, the lower soil moisture declines in the ECM than the AM treatment plots provide some evidence that hydraulic lift of water by ECM roots from depth may have occurred (Fig 2c, d). As a result, ECM trees may not have needed to increase investment in fine roots to increase access to water in the treatment plots. The same may be true for mycorrhizal fungi. Hydraulic lift has been shown to sustain mycorrhizal mediated uptake of nutrients and water (Querejeta et al., 2007). Regardless of the exact mechanism, our experimental results suggest that ECM trees respond differently to resource limitation than resource abundance. In contrast to experiments that enhanced N, CO₂, or temperature, our results did not show a dynamic increase in belowground investment, but instead indicate that ECM trees are constitutively primed to maintain resource acquisition when faced with resource limitation (Carrara et al., 2018; Midgley & Phillips, 2016; Terrer et al., 2016).

The cumulative effects of the throughfall exclusion treatment coupled with interannual variability in climate likely led to the temporal shifts in AM and ECM belowground C investment that we observed (Fig 3, Fig 4). In 2017, the 50% reduction in throughfall did not alter root growth or mycorrhizal colonization rates by AM or ECM trees, indicating that temperate forest trees can tolerate year-to-year variability in the water balance. However, in 2018, where the treatment led to a similar soil moisture deficit as 2017 (P-PET; Table 1), it appears that there was a cumulative, legacy effect on belowground C investment in both AM and ECM trees. While these cumulative or legacy effects have been extensively documented aboveground (Bréda et al. 2006, Kannenberg et al. 2019, Au et al. 2020), our results suggest that these cumulative effects also cascade

belowground. In 2019, the treatment shifted from a press to a large pulse increase in the soil moisture deficit (Table 1) that led to sharp declines in mycorrhizal colonization in AM plots but only a return to baseline levels in ECM plots. As such, this pulse disturbance likely exacerbated the cumulative C deficit to a greater extent for AM trees than ECM trees. Given the potential for future increases in the intensity and frequency of water stress during the growing season in the Eastern US (Dai 2013), these results suggest that the press along with the pulse of water stress can lead to persistent and additive impacts on belowground processes in temperate forests.

Despite water stress leading to increases in the other metrics of belowground C investment in the AM treatment relative to the control plots (Fig 3, 4), we did not observe a shift in root-derived C (Fig 5). These findings can only result from a decrease in inputs from root turnover and rhizodeposition or an increase in decomposition losses (Keller et al. 2021). We can likely rule out major shifts in root turnover due to our high sampling frequency of root biomass. Similarly, decomposition increases are unlikely given that water stress has predominantly been shown to decrease decomposition (Stark and Firestone 1995). As such, static or declining rhizodeposition must have occurred in the AM treatment cores (Fig 5). This proposed mechanism is consequential because it suggests that the increases in belowground C investment we observed in the AM treatment plots does not lead to priming of soil C or increased mobilization of nutrients.

Throughfall exclusion experiments are a common way to impose water stress in grassland ecosystems but can be challenging to conduct in temperate forest sites like the one studied here. In grassland experiments, trenching to restrict subsurface flow and complete coverage of the above- and belowground portions of the plants is possible due to the smaller, low growing species found in these ecosystems. At our site, trenching down to bedrock was prohibitive owing to the rocky soils and the disturbance that cutting large coarse roots would have incurred. Even though

we took statistical and methodological measures to reduce this treatment limitation, we cannot rule out lateral flow of water or root access to water outside the treatment area. Additionally, we built two shelters that covered 2 to 6 times more area than typical grassland shelters cover (Luo et al. 2019, Carroll et al. 2021). However, we still did not have nearly the individual plant replication that grasslands have and thus, were unable to capture significant aboveground responses (Fig S2). Finally, our experiment could be considered pseudo-replicated given that we only had one treatment and one control plot for each mycorrhizal type. We minimized this artifact by using each individual tree as a replicated subplot. This approach of sectioning global change experiments into replicated subplots has been successful in large scale warming experiments, watershed manipulations, and forest drought experiments (Nepstad 2002, Melillo et al. 2011, Carrara et al. 2018). Despite these experimental limitations, we were still able to identify important mechanistic differences between ECM and AM trees in how they respond to the press and pulse of water stress.

Much of the research on water stress in temperate forests has focused on the aboveground responses such as variability in photosynthesis and wood productivity to understand the degree to which forests can maintain their C sink with increasing stress. (McDowell et al. 2008, Allen et al. 2010, Choat et al. 2012, Anderegg et al. 2018). Our results indicate that differences between AM and ECM trees in their strategies to maintain belowground resource acquisition during water stress is inversely linked to their aboveground strategies. Importantly, we show that drought-tolerant, ECM trees maintain belowground C investment to enhance water uptake capacity, while drought-sensitive, AM trees must increase belowground C investment to ensure access to water. The degree to which differences in these belowground responses feedback on C and nutrient cycling in the soil is an important area for future research. However, as temperate forest composition

continues to shift from ECM to AM dominance (Knott et al. 2019), our results suggest that mycorrhizal association controls the response of belowground C investment to water stress. As such, there is the potential for ongoing increases in AM dominance in temperate forests coupled with predicted increases in water stress to alter the balance of C allocation to above vs. belowground pools.

3.6 Tables and Figures

Table 3-1. Ambient and experimentally altered growing season precipitation (P) and soil moisture deficits. P-PET, precipitation (mm) minus potential evapotranspiration (mm).

Year	Ambient P	Treatment P	PET	Ambient P-PET	Treatment P-PET
2017	895.60	447.80	433.50	462.10	14.30
2018	974.60	435.28	483.25	491.35	-47.97
2019	674.12	67.41	482.71	191.41	-415.30

(a) Ambient conditions

(b) Parallel hypothesis

(c) Inverse hypothesis

Figure 3-1. Under ambient conditions (a), ECM trees have a greater belowground C investment than AM trees without water stress. With water stress, the ‘Parallel hypothesis’ (b), predicts that ECM trees increase belowground C investment through increasing root biomass and mycorrhizal colonization while AM trees maintain or reduce belowground C investment. The ‘Inverse hypothesis’ (c) predicts that AM trees will upregulate C investment belowground in order to gain access to water, while ECM trees maintain or downregulate belowground C investment.

Figure 3-2. Mean sampling date soil moisture for AM (a) and ECM plots (c) for each year. Grey shaded areas indicate the duration of the treatment for the growing season each year, with the pink shaded area indicating when the treatment was 90% for 2018. Growing season averages were taken for each year from DOY 150-250 for AM (b) and ECM plots (d). Asterisk indicates $p < 0.05$. Error bars represent SE.

Figure 3-3. Root biomass significantly increased for AM trees under drought stress, but not ECM trees. The average root biomass for each sampling date for (a) AM plots and (c) ECM plots ($n=6$) and average taken for each growing season shown as bar plots for (b) AM and (d) ECM plots. For a. and c., time was analyzed as a random effect in which water stress caused root biomass to increase in AM treatment but led to no change in ECM plots. For b. and c., root biomass was analyzed in which time was a fixed effect. NS means no significance. Error bars represent SE.

Figure 3-4. Percent mycorrhizal colonization was altered by drought for AM and ECM trees over three years. Bar plots are the average percent colonization for July of each year (n=6) in (a) AM and (b) ECM plots. Error bars represent SE. Asterisks represent $p < 0.05$ while crosses represent $p < 0.10$.

Figure 3-5. (a) Root-derived C does not change with drought for AM and ECM trees, while (b) ingrowth core roots increase for AM trees. Cross indicates marginally significant differences between (a) mycorrhizal type and (b) an interaction between treatment and mycorrhizal type ($p < 0.10$). Error bars represent SE.

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**Chapter 4. Plant-microbial responses to experimental water stress depend on tree
mycorrhizal association in a temperate forest**

4.1 Abstract

Given that global change is predicted to increase the frequency and severity of drought in temperate forests, it is critical to understand the degree to which plant belowground responses cascade through the soil system to drive ecosystem responses to water stress. While most research has focused on plant and microbial responses independently of each other, a gap in our understanding lies in the integrated response of plant-microbial interactions to water stress. We investigated the extent to which the divergent belowground responses to water stress between trees that associate with ectomycorrhizae (ECM) vs. arbuscular mycorrhizae (AM) may influence microbial activity via throughfall exclusion in the field. We built upon previous research at this site that found greater belowground C investment by AM trees in response to the water stress treatment, while ECM trees maintained belowground C investment. We tested the hypothesis that microbial activity would be mirror the root responses to water stress, whereby microbes would be stimulated in the AM treatment and would maintain activity in the ECM treatment compared to their respective controls. In contrast to our hypothesis, we found that the water stress treatment led to declines in N mineralization, soil respiration, and oxidative enzyme activity in the AM treatment plot. These declines may be due to AM trees reducing root C transfers to the soil. By contrast, the water stress treatment enhanced soil respiration, as well as rates of N mineralization and peroxidase activity in the ECM rhizosphere. This enhanced activity suggests that ECM roots provided optimal rhizosphere conditions during water stress to prime microbial activity to support net primary production. With future changes in precipitation predicted for forests in the Eastern US, we show that the degree to which water stress impacts soil C and nutrient cycling depends on the strength of plant-microbial interactions.

4.2 Introduction

Temperate forest ecosystems are predicted to experience more periods of water stress during the growing season, with current and predicted global change leading to more frequent and intense droughts (IPCC 2021; Brzostek et al., 2014; Roman et al., 2015; Xu et al., 2019). Despite the potential for water stress to alter ecosystem function, the ability of temperate forests to mitigate water stress and sustain their carbon (C) sink strength remains uncertain. A large part of this uncertainty lies in the degree to which water stress alters plant-microbial interactions. On the microbial side, rates of decomposition and nutrient cycling typically decline with water stress due to stress responses and reduced mobility of enzymes and substrates (Manzoni et al. 2012, Deng et al. 2021). On the plant side, temperate forest trees have been shown to differ in the belowground response of roots and mycorrhizae to water stress (Brunner et al., 2015; Liese et al., 2019; Raczka et al., In review). However, there has been limited research that has investigated the integrated response of plants and microbes to water stress. As such, connecting plant and microbial responses to water stress is crucial because root-induced feedbacks on microbial function can alter the amount of C stored in soils and the amount of nitrogen (N) available to sustain net primary production (Carrara et al. 2018, Eastman et al. 2021).

Microbial processes have been shown to be sensitive to variation in precipitation, not only from physical decreases in soil water and nutrient mobility, but from changes in plant inputs as well (Schimel et al., 2007). Greenhouse studies and meta-analyses have found reductions in root exudation, soil respiration, and N mineralization in forest soils with drought (Brzostek et al. 2012, Homyak et al. 2017, Liese et al. 2018, Ficken and Warren 2019). However, more recently, research has found that plants may continue to allocate labile C belowground, as well as change the chemical composition of the exudates, causing greater soil respiration (Liese et al. 2018, de

Vries et al. 2019). Drought can also reduce plant-microbial interactions with research showing that bacterial uptake of exudate C was reduced by drought even though there were no declines in root exudation rates (Fuchslueger et al. 2014). Collectively, this research highlights that there remains uncertainty when water stress enhances or decreases the strength of plant-microbial interactions.

Mycorrhizal association may control the magnitude and direction of plant-microbial responses to water stress (Phillips et al. 2013). Evidence that AM and ECM trees differ in how their roots and mycorrhizae respond to water stress (Liese et al., 2019, 2018; Raczka et al., In review) suggests there is the potential for corresponding impacts on microbial activity. Indeed, results from the same throughfall exclusion experiment studied here by Raczka et al. (In review) found that AM trees increased root biomass and mycorrhizal colonization to a greater degree than ECM trees in response to water stress. These differential impacts of water stress on belowground C investment by ECM and AM trees must impact the availability of C to fuel microbial activity suggesting that there are also corresponding feedbacks on C and N cycling (Yin et al. 2014, Cheeke et al. 2017). Thus, we hypothesized that soil microbial activity during water stress will be greater in AM soils than ECM soils. We expect that greater belowground C investment by AM trees will lead to a stimulation of microbial activity and fuel increases in decomposition (Phillips and Fahey 2006, Brzostek et al. 2013). By contrast, in ECM plots, we expect that the limited response of belowground C investment will lead to microbes maintaining their activity with water stress and have little to no impact on decomposition (Lin et al., 2017). Given that decomposer activity, nutrient availability, and plant growth are all linked (Kaisermann et al. 2017), understanding the strength of these interactions is crucial for improving predictions of the C consequences of drought.

To test these hypotheses, we built a multi-year water-exclusion experiment in a 120-year-old temperate forest outside of Morgantown, West Virginia, USA. We established two 20 x 20m water exclusion plots dominated by either AM or ECM associated trees, each paired with a nearby control plot of similar species composition. This effort builds upon previous work at the site where we examined the response of belowground C investment to water stress (Raczka et al. In review). To investigate how shifts in belowground C investment with water stress will impact microbial activity, we coupled our previous results with new measurements of decomposition processes including soil respiration, N mineralization, and potential soil extracellular enzyme activity over three growing seasons in the treatment and control plots.

4.3 Methods

Study Site

This research was conducted at the Tom's Run Natural Area, an 84-acre deciduous forest that is nearly 120-years old, located 8 miles south of Morgantown, West Virginia, US (39°32'50.6"N, -80°00'00.4"W). Mean annual temperature is approximately 11.62 °C, and mean annual precipitation is 1063 mm as measured nearby at the Morgantown Hart Fields Airport (NOAA station # USW00013736). These sites have been characterized previously as silt loam soils (Soil Survey Staff, 2018).

Elevation at this site ranges from 336 m to 438 m and slopes range from 3-25% (Soil Survey Staff, 2018). Vegetation is mixed temperate broadleaved deciduous forests, consisting of a diverse mixture of ECM and AM tree species. The dominant AM species here are *Acer saccharum* Marshall (sugar maple), *Acer rubrum* L. (red maple), and *Liriodendron tulipifera* L. (tulip poplar). The dominant ECM species are *Quercus velutina* Lam. (black oak), *Quercus rubra* L. (red oak), *Quercus alba* L. (white oak), *Carya ovata* (shagbark hickory), and *Fagus grandifolia* Ehrh. (American beech) (Table S1).

Throughfall exclusion experiment

To determine the extent to which differences in belowground responses between ECM and AM trees are linked to their strategies aboveground during times of water stress, we built water exclusion shelters to remove throughfall during the growing seasons of 2017, 2018, and 2019 (Tom's Run, Fig S1). We established a total of four 20 x 20m plots consisting of an AM treatment and AM control plot located on a hillside 40 m apart and an ECM treatment and

control plot located on a nearby slope west of the AM plots and 20 m apart. Each control plot received ambient rainfall and were located in stands with similar species composition (Table S1). The structures, built in 2016 (Fig S1), consisted of four rows of V-shaped panels at a 75° angle connected to a gutter to allow for drainage. At the end of each gutter a drainage hose was installed to divert the rainfall away from the plot edge by approximately 10 m. For each year, the water stress treatment started in March and ran for the duration of the growing season. To divert rain, reinforced, clear polyethylene sheeting plastic was stretched over the panels and connected to the gutter. Each October, the plastic was removed in order to manipulate throughfall only during the growing season and also to alleviate any structural stress from snowfall. Over the course of our three-year experiment, we used treatments of increasing intensity each consecutive growing season. In 2017, 50% of the rainfall was excluded over the entire growing season. In 2018, we again began in March with a 50% reduction in rainfall, increased to a 90% exclusion in July to enhance the effect of water stress on the trees, and returned the treatment back to 50% for the remainder of the growing season. To maximize water stress in the final year, 90% of throughfall was excluded beginning in March and lasted throughout the growing season.

As with any large-scale global change experiment, we had to balance experimental artifacts with logistical constraints. We acknowledge that there may be an artifact of increasing the percentage of the throughfall removed over the course of the experiment. However, we decided to increase drought intensity over time in order to ensure that the treatment plots were experiencing a prolonged state of water stress. We also address this artifact statistically by including soil moisture as a covariate in our analysis to address these differences. Soil moisture has extensively been measured at this site (Table S1) from Raczka et al. (in review). One other

potential limitation is that we did not trench the treatment plots. We did this to limit belowground disturbance since we were interested in observing fine root dynamics in the top 15 cm of the soil profile. To minimize this limitation, we did not sample soils or roots within 2m of the plot's edge.

Soil Respiration

To investigate soil respiration rates during water stress, we measured CO₂ respiration using a LI-8100 (LI-COR, Lincoln, Nebraska) at four 10 cm diameter PVC collars in each treatment and control plot. We took measurements continuously every two weeks over the year from 2017-2019, resulting in a total of 89 measurements.

Soil Sampling

Across the three years, we sampled soils during the growing season, with the number of sampling dates varying each year due to logistical constraints (7 in 2017, 4 in 2018, 3 in 2019). For each sampling date, we extracted six mineral soil cores to a depth of 0-15 and then 15-30 cm at depth. We then removed all roots from the soil sample by carefully sieving the soil and extracting fine roots with forceps. Further, we separated the mineral soil into bulk and rhizosphere fractions from the 0-15 soil sample by the soil adhesion method (Phillips et al. 2006). Rhizosphere soil was defined as the soil that adhered to roots upon removal from the soil matrix, and carefully subsampled using forceps and homogenized (Phillips et al. 2006, Carrara et al. 2018). Bulk soils were sieved through 2 mm mesh after root and rhizosphere removal and stored at -80 C until further analysis.

Biogeochemical assays

Using July subsamples of soils, we assayed the potential activities of enzymes during water stress. We chose the July sampling date because it is at the height of the growing season and when soil respiration was highest (Fig 1a, c). We measured the potential activities of enzymes that degrade complex C (phenol oxidase and peroxidase), labile C (β -glucosidase (BG)), N (*n*-acetylglucosaminidase (NAG)) and P (Acid phosphatase (AP)) in each soil fraction (Saiya-Cork et al. 2002). All assays were run using 1g subsample of each soil homogenized in a pH 5.0 sodium acetate buffer. NAG, AP, and BG are hydrolytic enzymes, and these activities were determined using a fluorometric microplate assay with methylumbelliferone-linked substrates. Oxidative enzyme activities, phenol oxidase and peroxidase, were determined using a colorimetric microplate assay with L-3,4-dihydroxyphenylalanine as the substrate (Saiya-Cork et al. 2002, Brzostek et al. 2015).

For all soil fractions at each sampling date, we measured the rate of N mineralization within 48 hours of collection. Rates of N mineralization were measured by immediately extracting 5 g initial subsample of each replicate in 2M KCl and then incubating another subsample for 2 weeks prior to extraction. NO_3^- and NH_4^+ concentrations in the KCl extracts were determined colorimetrically in microplates using the salicylate-nitroprusside method and vanadium(III) reduction, respectively (Verdouw et al. 1978, Doane and Horwáth 2003, Waring et al. 2016). N mineralization was calculated as the difference in NO_3^- and NH_4^+ in the incubated and initial samples (Finzi et al. 1998).

Statistical analysis

All data analyses were performed using R 3.2.0 (R Core Team, 2017). To determine the extent to which chronic water stress altered soil respiration and N mineralization, we performed an analysis of covariance (ANCOVA) in which growing season soil moisture from day 150 to 250 was used as a covariate to compare plot and date as factors. We chose day of year 150, which is the end of May to be a conservative start to the growing season and day of year 250, which is the beginning of September to be a conservative end to the growing season. We used growing season soil moisture as a covariate to test the effects of water stress on our measurements in order to be conservative in our analysis. We first ran the full statistical model across all comparisons with mycorrhizal type, treatment and year the main effects in the soils. We then looked across individual years for soil respiration using treatment and mycorrhizal type as the main effects. For N mineralization, after running the full statistical model we then examined differences between soil fractions in which treatment and mycorrhizal type as the main effects in the soils. Post hoc multiple comparisons were also made among plots and dates using the Tukey-Kramer HSD test.

To analyze potential extracellular enzyme activity in AM and ECM control and treatment plots, all enzyme values were used to calculate Bray-Curtis similarity using the `vegdist` function in the `vegan` package in R (Oksanen et al., 2019). Using the `Adonis` function, enzymes were analyzed by PerMANOVA with treatment and mycorrhizal type as main effects between soils.

4.4 Results

Soil respiration

AM and ECM plots had different responses of soil respiration to the water stress treatment.

Across all soil respiration measurements, there was a main effect of the water stress treatment on soil respiration ($p < 0.05$, Fig 1a, c). For AM plots, in the full statistical model, there was no difference between treatment and control (Fig 1a). By contrast, in ECM plots, the water stress treatment led to the treatment plots having higher soil respiration rates than the control plots ($p < 0.01$, Fig 1c). Examining differences between individual years, there were no treatment effects in years 1 and 2 in the AM plots. In year 3, however, there was significantly less soil respiration in the AM treatment than the control plot ($p < 0.01$, Fig 1b). For ECM plots, in the first and second year there was a significant increase in soil respiration in the treatment plots ($p < 0.01$, Fig 1d). In the last year of treatment, this effect faded and there was no effect of the exclusion treatment on soil respiration.

Net N mineralization

The water stress treatment led to an increase in N mineralization in ECM rhizosphere soils but led to a decline in N mineralization across all AM soil fractions (i.e., rhizosphere, 0-15cm and 15-30cm bulk soil). In the rhizosphere, there was a marginally significant interaction between mycorrhizal type and treatment ($p < 0.07$, Fig 2a, b) with the water stress treatment increasing rates in ECM rhizospheres and decreasing rates in AM rhizospheres. For AM rhizospheres, the water stress treatment led to a decrease in N mineralization for all three years of the experiment ($p < 0.01$, Fig 2a). In the bulk soil from 0-15cm, there was a significant effect of mycorrhizal type

on N mineralization ($p < 0.05$, Fig 2c, d), with AM soils having N mineralization rates that were nearly 75% greater than ECM soils. Across individual years, the water stress treatment led to significant declines in N mineralization in 2018 and 2019 in the AM soils, while there was no effect of the treatment in ECM soils (Fig 2c, d). Finally, in the bulk soil from 15-30, there was a significant treatment effect ($p < 0.01$, Fig 2e, f). Similar to the bulk soil from 0-15, the water stress treatment led to declines every year in the AM soils ($p < 0.05$, Fig 2e) and had no significant effect in ECM soils (Fig 2f).

Extracellular enzyme activity

The PerMANOVA showed that water stress led to significant changes in overall enzyme investment between AM and ECM plots (Fig. 3). There was both a main effect of mycorrhizal type on enzyme investment ($p < 0.01$, Fig 3) and a significant interaction between mycorrhizal type and treatment ($p < 0.01$). Using our three-way ANOVA model, we examined which fractions and enzymes contributed to this overall shift in enzyme investment observed in the PerMANOVA analysis. Only in rhizosphere soils was there a significant interaction between treatment and mycorrhizal type in rhizosphere soils for NAG, peroxidase, and phenol oxidase. There were no interactive effects observed for the 0-15 and 15-30 bulk soils or for AP and BG activity (Table S2). For NAG, the water stress treatment led to significant increases in AM rhizospheres ($p < 0.05$, Fig 4a) and little to no change in ECM rhizospheres (Fig 4b). For peroxidase in ECM rhizosphere soil was significantly higher in the treatment and control plot ($P < 0.05$, Fig 4d), while AM rhizosphere Peroxidase activity significantly declined in the treatment compared to control plot (Fig 4c). Lastly, water stress led to a decrease in phenol oxidase activity in the AM treatment rhizosphere than the control rhizosphere ($p < 0.05$, Fig 4f).

4.5 Discussion

Understanding the extent to which water stress enhances or decreases plant-microbial interactions is essential to improving our predictive understanding of the C consequences of increases in the frequency and severity of drought (Xu et al. 2019). Here, we show that in both AM and ECM plots the response of microbial activity to the water stress treatment did not mirror shifts in root biomass and mycorrhizal colonization. In AM soils, the water stress treatment led to increases in root biomass and colonization that were counterbalanced by declines in N mineralization, soil respiration, and oxidative enzyme activity. In ECM soils, the water stress treatment enhanced soil respiration as well as rates of N mineralization and peroxidase activity in the rhizosphere, despite little change in root biomass or mycorrhizal colonization. Collectively, these results suggest that in response to the water stress treatment, ECM trees must have increased root C transfer to a greater extent than AM trees which enhanced soil respiration as well as decomposition in ECM rhizospheres.

Although we hypothesized that increases in belowground C investment by AM trees as a result of the water stress treatment (Raczka et al., In review) would prime soil microbial activity, we found the opposite result with the water stress treatment leading to declines in soil respiration, N mineralization, and oxidative enzyme activity (Fig 1, 2, 4). While reductions in soil moisture likely contributed to these declines, these results also may reflect a reduction in root C transfers to rhizosphere microbes by AM roots. As a consequence, this reduction in root C transfers likely exacerbated microbial C limitation and reduced their ability to synthesize enzymes to fuel decomposition (Ficken & Warren, 2019; Schimel et al., 2007). In support of this mechanism, previous research at the experimental site using root ingrowth cores with C₄ soil found that the treatment led to increased root growth in AM plots but did not alter net root C

transfers to soils (Raczka et al., In review). In addition, the reductions we observed in this study in potential oxidative enzyme activities, a proxy for enzyme pool size, in the AM treatment soils are indicative of resource limitation of microbial enzyme investment (Fig 4c, e). Integrated together, these findings suggest that declining root C transfers to the rhizosphere as a result of the water stress treatment reduced the availability of resources to fuel microbial decomposition in the AM treatment plot.

Similar to the AM hypothesis, we did not find support for our hypothesis that microbial activity in the ECM plot would be maintained in response to the water stress treatment. However, in contrast to the AM results, we found higher microbial activity in ECM treatment rhizospheres without an increase in root biomass and mycorrhizal colonization (Fig 2, 4). Most likely these increases in rhizosphere enzyme activity and N mineralization were driven by a combination of greater C transfers to rhizosphere microbes and hydraulic lift of water by ECM tree roots (Raczka et al., In review). Previous findings at the experiment site support this mechanism in two ways. First, ingrowth core assays show that ECM roots enhance rhizodeposition under the water stress treatment. Second, results showing that ECM treatment plots had lower soil moisture declines than AM treatment plots relative to controls provide some evidence for hydraulic lift (Raczka et al., In review). This propensity of ECM trees to hydraulically lift water during times of water stress is supported as well by stable isotopic analyses of soil pore water in oak-savannahs (Querejeta et al., 2007). Importantly, this potential for ECM trees to maintain optimal conditions for decomposition in the rhizosphere during times of water stress may also provide a mechanism for why microbial N transformations have been found to decrease in dry soils in the lab, but are less sensitive to precipitation in the field (Homyak et al. 2017). As such, by fueling rhizosphere enzyme production and maintaining soil

moisture at levels that allow for the diffusion of enzymes and substrates, ECM trees may be able to maintain N nutrition to support net primary productivity when faced with water stress.

While shifts in root processes appeared to be the primary control on microbial responses to the water stress treatment, the year-to-year differences we observed in the response of soil respiration in both ECM and AM plots were likely due to interannual variability in climate and the effect of the treatment on root biomass (Fig 1). In 2017 and 2018, soil respiration was maintained in the AM treatment plots and enhanced in the ECM treatment plots relative to their controls (Fig 1a, c). These responses corresponded to the experimental site receiving 18% more precipitation than the past 30-year average (Table S3). Thus, even with reductions in soil moisture from the water stress treatment, there was enough precipitation inputs to meet evaporative demand. As a result, throughfall exclusion likely pushed soil moisture in the treatment plots to lower levels that provided more optimal aerobic conditions than the control plots (Davidson et al. 2012). However, precipitation in 2019 was 15% below the 30-year average (Table S3). This reduction, together with 90% coverage in the water stress treatment, led to less water available in the soil for evaporative demand. As a result, the treatment soils experienced large soil moisture deficits, likely promoting the reduction in soil respiration in the AM treatment plot and the lack of difference in soil respiration in the ECM treatment plot relative to their respective control plots (Fig 1b, d). Additionally, the declines in heterotrophic respiration in the AM treatment plot were likely even greater given that autotrophic respiration may have had a larger contribution to total soil respiration. This potential for greater autotrophic contribution reflects previous findings that showed greater root biomass in the AM treatment plot relative to its control (Raczka et al., In review). Collectively, the observed soil respiration

responses to the water stress treatment highlight that there are important interactions between soil moisture and root responses to drought that feedback on soil C fluxes in temperate forests.

Both AM and ECM soils showed shifts in enzyme activity in response to the water stress treatment, but the direction, magnitude, and the apparent mechanism driving the shifts differed by mycorrhizal association. In AM soils, lower oxidative enzyme activity but higher NAG enzyme activity is likely a consequence of stress responses by microbes in AM rhizospheres during water stress (Fig 4a, c, e). NAG activity can metabolize C and N from expired fungal cell walls, which may be more accessible and less costly to microbes during water stress than utilizing oxidative enzymes to break down complex substrates (Schimel, 2018). In support of this mechanism, research shows microbial communities adapted to less complex litter, such as AM litter, allocate more resources to mitigating stress with drought than for growth (Malik et al., 2020). Opposingly, in the ECM treatment, rhizosphere microbes continued to invest in phenol oxidase and NAG enzymes, as well as increased their investment in peroxidase enzymes (Fig 4b, d, f). These contrasting results suggest that the water stress treatment may have promoted stressful conditions in the AM rhizosphere that initiated microbial stress responses at the expense of resource acquisition; whereas in ECM rhizospheres, microbes appeared to be buffered against stressful conditions and continued to invest in a broad suite of resource acquisition enzymes.

The divergent responses of N mineralization to the water stress treatment between the AM and ECM plots demonstrate the integrated impact of water stress and root processes on the availability of soil N (Fig 2). In the ECM treatment plots, our proposed mechanism of hydraulic lift coupled with more C inputs during water stress likely enhanced N mineralization in the treatment rhizospheres relative to control rhizospheres (Fig 2b). This increase in N mineralization in the rhizosphere as a consequence of water stress suggests that future drought

events will not enhance N limitation in ECM forests. Oppositely, in the AM treatment, water stress did not lead to roots priming microbial activity which led to reductions in N mineralization across all three soil fractions relative to the control plots (Fig 2a, c, e). Even though water stress led to enhanced uptake capacity of AM trees through increases in the scavenging surface area of roots and mycorrhizae; these reductions in N mineralization suggest that future drought events could hinder N uptake by AM trees and reduce net primary production (Sardans and Peñuelas 2010).

When we place our results in the context of other throughfall experiments located in AM or ECM systems that examined belowground processes, it appears that the impact of the water stress treatment on microbial activity mirrors the water status of the ecosystem. At a comparatively wet site in Tennessee (MAP = 1352 mm), the researchers found no difference in AM and ECM fine root biomass or mineral soil N fluxes to the water stress treatment (Hanson et al. 2001, Johnson et al. 2002). By contrast, two throughfall experiments located in comparatively drier climates in Spain (MAP = 503, 658 mm) found reductions in enzyme activity and N availability in ECM tree stands with water stress (Sardans and Peñuelas 2010, Bastida et al. 2017). Our results and the climate conditions of our experimental site both appear to lie somewhere in the middle and suggest, particularly for the soils of ECM trees, that there may be a threshold in ambient climate where water stress promotes or hinders microbial activity. While this link is speculative, it highlights the need to assay belowground responses to throughfall manipulations in forested ecosystems that span more climatic conditions.

Much of the research on water stress in temperate forests has focused on either plant responses or microbial responses independently of each other to understand how drought may impact belowground nutrient cycling and net primary production (de Vries et al. 2018, Glassman

et al. 2018, Fuchs et al. 2020, Krüger et al. 2021). Here, building upon previous research, we investigated the integrated response of plants and microbes to the water stress treatment. Past research at this site has shown that the water stress treatment led to differences in root biomass and mycorrhizal colonization in ECM and AM treatment plots compared to the control (Raczka et al., In review). In this study, we found divergent responses in microbial activity between the ECM and AM treatment plots (Fig 1, 2, 3). Importantly, we show that microbial activity was greater in the ECM treatment than in the control during water stress (Fig 2b, Fig 4b, d, f). This response suggests ECM roots maintained soil moisture and transferred greater C to the rhizosphere to promote N nutrition to support net primary productivity. In contrast, microbial activity declined in the AM rhizospheres in response to water stress, likely due to lower resource investment to rhizosphere microbes by AM roots (Fig 2a, 4a, c, e). Importantly, this downregulation of microbial activity in the rhizosphere cascaded across all soil fractions leading to less N mineralization to support net primary productivity in the AM treatment plots (Fig 2c, e). Given that forests in the Eastern US are predicted to experience more periods of water stress (Dai 2013, Xu et al. 2019), our results provide an important conceptual advance by showing that plant-microbial interactions appear to drive the magnitude and direction of the response of soil C and nutrient cycling to water stress.

4.6 Tables and Figures

Figure 4-1. Mean sampling date soil respiration for AM (a) and ECM plots (c) for each year.

Grey areas indicate when the treatment started to when the treatment ended for the growing season. 90% coverage for July 2018 indicated in the light red area. Growing season averages were taken for each year from DOY 150-250 for AM (b) and ECM plots (d). Asterisk indicates $p < 0.05$. Error bars represent SE.

Figure 4-2. Nitrogen mineralization was altered by water stress for AM and ECM trees over three years. Box plots depicting minimum and maximum values of nitrogen mineralization for each growing season of each year ($n=6$) in (a) AM and (b) ECM rhizosphere, (c) AM and (d) ECM 0-15 cm mineral soils, and (e) AM and (f) ECM 15-30 cm mineral soils. Asterisks represent $p<0.05$.

Figure 4-3. Water stress altered enzyme profiles in AM and ECM soils. Scatterplot is NMDS1 and NMDS2 of the enzyme profiles. There was both a main effect of mycorrhizal type on enzyme investment ($p < 0.01$) and a significant interaction between mycorrhizal type and treatment ($p < 0.01$).

Figure 4-4. Water stress altered rhizosphere enzyme activity for AM and ECM plots. Values are July averages (n=6) of (a & b) N-Acetylglucosaminidase, (c & d) Peroxidase, and (e & f) Phenol oxidase. Error bars are SE. Asterisks represent $p < 0.05$.

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Chapter 5. Conclusions: Advancing our understanding of the role of plant-microbial interactions in mediating soil C and nutrient cycling in response to water stress

Summary of results

My dissertation examined how differences between AM and ECM nutrient acquisition strategies lead to divergent microbial diversity and function that can impact soil C storage and ecosystem responses to global change. To do this, I sampled soils from ECM and AM plots in a ~ 120-year-old forest in West Virginia under ambient conditions and also built a throughfall exclusion experiment at the same site to answer three broad questions: 1) Does microbial diversity drive function and the resulting products of decomposition in temperate forest soils?; 2) To what extent do temperate forest trees shift their investment of C above vs. belowground under water stress?; and 3) How do plant-microbial interactions impact decomposition in temperate forests under water stress? Across all three questions, I focused on how these processes varied across plots dominated by AM vs. ECM trees.

Overall, I found evidence that differences in nutrient acquisition strategies led to AM soils harboring greater diversity of fungi and bacteria than ECM soils. This difference in diversity appears to have led to divergent functional capabilities, whereby in the AM soil, greater diversity led to more decomposition pathways due to dynamic shifts between the identity of the active decomposers depending upon the chemistry of the substrate. Further, this dynamic decomposition led to a greater diversity of decomposition products that could form stable soil organic matter. In the less diverse ECM soils, the decomposition pathway was more static leading to few decomposition products that could potentially limit stable soil organic matter formation. Additionally, I found that during water stress ECM trees maintained belowground C investment of root biomass and mycorrhizal colonization at the same level, while AM trees upregulated belowground C investment to ensure access to water. Finally, these divergent belowground responses between AM and ECM trees impacted the microbial response to the

water stress treatment. In ECM soils, it appeared that the ability of roots to maintain an optimal rhizosphere environment in ECM soils in response to the treatment led to greater enzyme activity and N mineralization in the rhizosphere, along with greater overall soil respiration, during water stress. In AM soils, the treatment induced decreases in enzyme activity, N mineralization and soil respiration, likely reflecting a reduction in root C transfers to the rhizosphere. Together, these three major results provide evidence that plant nutrient acquisition strategies shape soil microbial diversity and function. Additionally, the response of soil C and nutrient cycling to drought may be driven by differences in the strength of plant nutrient acquisition strategies between AM and ECM trees. Below, I will further provide details on how each dissertation chapter answers one of the broad research questions above.

Chapter 2 – Are microbial community identity and function linked with the products of decomposition in temperate forest soils?

In *Chapter 2*, I examined how known differences between AM and ECM trees in their nutrient acquisition strategies lead to differences in microbial diversity, function, and decomposition products. To do this I coupled quantitative stable isotope probing and metabolomics to track the fate of ^{13}C enriched substrates that varied in chemical composition as they were assimilated and transformed by microbes from soils collected at Tom's Run Natural Area in West Virginia, USA. I observed that differences in nutrient acquisition strategies led to greater diversity of fungi and bacteria in AM soils than ECM soils. I also found that shifts in identity of active decomposers, and the number of metabolic products present in AM soils were driven by substrate type, while in ECM soils these metrics did not respond to substrate chemistry. Importantly, I found that the shifts between the decomposition of substrate types

were controlled by active taxa in the AM soil that were not present in the ECM soil, indicating decomposition relies upon the identity of actively decomposing microbes. The more diverse AM community generated a greater amount of diverse decomposition products than the ECM community. AM soils with a greater amount of diverse decomposition products have the potential to generate more stable soil organic matter. Collectively, these findings advance our understanding of mechanistic links between decomposition and C storage by showing that more diverse microbial communities have the potential to generate more stable soil organic matter.

Chapter 3 – To what extent do temperate forest trees shift their investment of carbon above vs. belowground under drought stress?

Increasing evidence shows that mycorrhizal association may control drought responses. Aboveground measurements have shown that ECM trees can maintain greater rates of photosynthesis than AM trees and drought reduces AM tree growth and C storage to a greater extent than ECM trees (Au et al., 2020; Brzostek et al., 2014; Roman et al., 2015). These results have led to the understanding that ECM trees may be more drought-tolerant, while AM trees may be more drought-sensitive aboveground. However, the belowground responses between AM and ECM trees to water stress have been less straightforward (Kannenberg et al., 2018; Liese et al., 2019). As such, in *Chapter 3*, my main objective was to understand the extent to which AM and ECM trees alter their C investment in belowground resource acquisition during times of water stress (Phillips et al., 2013). I developed two hypotheses of how water stress may impact tree species differently belowground. First, the ‘Parallel hypothesis’ predicted that drought-tolerant ECM trees would ramp up their belowground C investment in response to water stress, similar to their aboveground responses. Oppositely, AM trees, known to be more drought-sensitive,

would maintain or reduce belowground C investment since less C is available due to water stress restricting photosynthesis. The second hypothesis, the ‘Inverse hypothesis’ predicted that with water stress, AM trees would upregulate belowground C investment to ensure access to water, while ECM trees would maintain or downregulate their belowground C investment.

To test these hypotheses, I built a multi-year water-exclusion experiment in Tom’s Run and measured several indices of belowground C investment between AM and ECM trees including fine root biomass, mycorrhizal colonization, and root-derived C in soils. The results supported the ‘Inverse hypothesis’, whereby ECM trees maintained their belowground C investment in root biomass and mycorrhizal colonization during the water stress treatment at the same level in both the treatment and control plots. Research has shown that ECM trees continue to grow aboveground with water stress (Au et al., 2020), and our findings suggest that ECM tree maintenance belowground exhibits an ability to leverage a higher baseline level of belowground C investment to continue taking up water. By contrast, AM trees upregulated belowground C investment in the AM treatment compared to the control plot. AM trees are known to reduce growth aboveground with water stress and as a result may have increased root biomass and mycorrhizal colonization belowground to ensure access to water. Further, the cumulative effects of the water stress treatment combined with interannual variability likely drove the shifts between AM and ECM belowground C investment over the course of the three-year experiment. These results collectively show that differences between AM and ECM trees in their strategies to maintain belowground resource acquisition during water stress is inversely linked to their aboveground strategies. As temperate forest composition shifts from ECM to AM dominated species, there is the potential that these shifts, coupled with predicted increases in water stress during the growing season, will alter the balance of C allocation to above vs. belowground pools.

Chapter 4 – How do plant-microbial interactions impact decomposition in temperate forests under drought stress?

In *Chapter 2*, I found that known differences in nutrient acquisition strategies between AM and ECM trees under ambient conditions can foster microbial communities that are different in their diversity and their function in decomposition. Further, in *Chapter 3*, I found that AM and ECM trees diverge in their belowground C investment with water stress, in which there was greater belowground C investment by AM trees in root biomass and mycorrhizal colonization. While most research has focused on plant and microbial responses to water stress that have been independent of each other (Brunner et al., 2015; Deng et al., 2021; Liese et al., 2019; Manzoni et al., 2012), limited research has investigated the integrated response of plant-microbial interactions to water stress. As such, in *Chapter 4*, my objective was to understand the degree to which the divergent belowground responses between AM and ECM trees may impact microbial activity and how these responses feedback on C and nutrient cycling. I hypothesized that the greater belowground C investment by AM trees would lead to a stimulation of microbial activity and continue decomposition during water stress. Further, I hypothesized that since ECM trees maintained belowground C investment in the treatment relative to the control, microbial activity would mirror this response. I leveraged the water-exclusion treatment built in AM and ECM plots at Tom's Run to test this hypothesis. I coupled my previous results on belowground C investment with additional measurements of decomposition processes, including soil respiration, N mineralization, and extracellular enzyme activity over three growing seasons in the treatment and control plots.

In contrast to my hypothesis, I found that the microbial responses to water stress did not mirror the shifts in root biomass or mycorrhizal colonization between AM and ECM trees. The

water stress treatment led to declines in soil respiration, N mineralization and oxidative enzyme activity in the AM treatment plot compared to the control. These results suggests that AM trees did not prime soil microbial activity, and coupled with a reduction in soil moisture, microbes likely became C limited and their ability to synthesize enzymes for decomposition was reduced (Ficken & Warren, 2019; Schimel et al., 2007). Similarly, I did not find support for my hypothesis that microbial activity would mirror ECM tree responses to water stress. I found that microbial activity was higher in the ECM treatment rhizospheres than the control, likely driven by more optimal soil moisture conditions and potentially more root C inputs in ECM rhizospheres. As such, these results show that differences in the response of plant-microbial interactions to water stress between AM and ECM trees lead to divergent trajectories in soil C and nutrient cycling.

Future directions and research

In this dissertation, I used plant nutrient acquisition strategies as a lens to investigate the extent to which differences between tree species impact microbial traits and function, as well as how forest ecosystems respond to stress induced by global change. While the majority of my PhD research has focused on temperate trees, my goal is to utilize the tools I have acquired from this work to build my future research program focusing on plant nutrient acquisition strategies in tropical forests. Specifically, I will focus on the strategies that plants use to acquire nutrients, such as mycorrhizal fungi or N-fixing rhizobia, that drive variability in C storage as well as control the magnitude and direction of global change responses. Though mycorrhizal-driven strategies have been shown to drive differences in C and nutrient cycling between AM and ECM stands in temperate forests, there has been limited research investigating whether these patterns

hold true in tropical forests (Frey, 2019; Phillips et al., 2013a). Given that tropical forests contain one-third of global soil C stores (Pan et al., 2011), there remains a grand challenge to integrate these traits, processes, and strategies that are endemic to tropical forest ecosystems into a rule of life that cuts across biome boundaries.

To begin meeting this grand challenge, I plan to develop a research program that will allow me to test a conceptual model (Fig. 1) that I developed which theoretically predicts the extent to which differences in the dominant nutrient acquisition strategies of tropical forest trees feedback at the organismal scale on keystone microbial traits that control soil C, N and P cycling. Along an axis of a decreasing ratio in organic to inorganic nutrient availability, I predict that the acquisition of nutrients will shift from ECM strategies fueled by plant C subsidies to symbiotic and free-living microbes to mine nutrients in the rhizosphere to NF-AM strategies that enhance the scavenging of inorganic nutrients across the entire bulk soil volume. Intermediate to these two endmembers are two lower carbon cost strategies: the release of N-rich exudates to fuel nutrient mining by NF trees (Nasto et al., 2014) and the ability of AM trees to use their symbionts to enhance nutrient scavenging (Phillips et al., 2013). The resulting feedbacks of these strategies on microbial traits is controlled by where plant C is transferred, either to rhizosphere miners or symbiotic scavengers. Focusing on the endmembers, the larger transfer of C to rhizosphere miners by ECM trees promotes greater fungal to bacterial ratios, higher rhizosphere microbial CUE (i.e., proportion of total C consumed that is used to grow new biomass) and turnover, and greater nutrient acquisition enzyme production. By contrast, in soils where trees rely on NF-AM scavenging, plant C investments are localized to symbiotic microbes leading to greater dominance of bacteria and little difference between microbial traits in the rhizosphere and bulk soil. Understanding the degree to which plant nutrient acquisition

strategies feedback on keystone microbial traits is critical because these traits are a dominant control on the balance between stable soil C formation and decomposition (Domeignoz-Horta et al., 2020; Geyer et al., 2016).

5.1 Figures

Figure 5-1. Dominant plant nutrient acquisition strategies and resulting microbial response.

5.2 Literature Cited

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Appendix. Supplementary Tables and Figures

Table S2-1. Soil properties

Variable	Soil	
	AM	ECM
C:N	11 [±] 0.141	14 [±] 0.248
pH	6.5 [±] 0.150	6.5 [±] 0.133
SOM Chemistry		
Amino-Sugar	0.03 [±] 0.005 ^a	0.011 [±] 0.004 ^b
Carbohydrates	0.109 [±] 0.018	0.126 [±] 0.301
Condensed HC	0.187 [±] 0.131	0.091 [±] 0.031
Lignin	0.242 [±] 0.091 ^a	0.375 [±] 0.092 ^b
Lipids	0.304 [±] 0.057 ^a	0.115 [±] 0.114 ^b
Other	0.016 [±] 0.032	0.095 [±] 0.005
Protein	0.037 [±] 0.042 ^a	0.102 [±] 0.009 ^b
Tannin	0.051 [±] 0.021	0.086 [±] 0.021
Unsaturated HC	0.018 [±] 0.003 ^a	0.004 [±] 0.001 ^b

Table S2-2. Summary of PERMANOVA analysis on the effects of substrate type on fungi and bacteria community composition and ^{13}C assimilation (EAF). Bold font indicates significance at $P < 0.05$.

		Soil _(1,16)		Substrate _(1,16)		Soil X Substrate _(1,16)	
		F-value	P-value	F-value	P-value	F-value	P-value
Relative Abundance	Bacteria	0.85178	0.001	0.01805	0.165	0.01622	0.183
	Fungi	0.72108	0.001	0.02916	0.151	0.01591	0.336
Excess Atom Fraction	Bacteria	0.35565	0.001	0.09617	0.035	0.04913	0.250
	Fungi	0.42800	0.001	0.09765	0.010	0.10660	0.008

Table S2-3. Summary of PERMANOVA analysis on the effects of substrate type on SOM chemistry and lipids. Bold font indicates significance at $P < 0.05$.

Variable	Soil		Substrate		Soil x Substrate	
	Pseudo-F _(,16)	P-value	Pseudo-F _(,16)	P-value	Pseudo-F _(,16)	P-value
SOM Chemistry	2.651	0.039	1.8890	0.213	1.349	0.296
Lipids	5.781	0.003	1.951	0.207	2.867	0.031

Table S3-1. Basal area for all species for each plot. Highlight shows the AM-associated trees for the AM plots, and ECM-associated trees for the ECM plots.

<u>AM treatment</u>	Total Basal Area (m²/ha)	Basal Area (%)
Acer rubrum	6.56	21
Acer saccharum	24.33	79
<u>AM control</u>		
Acer saccharum	30.24	59
Fraxinus americana	3.81	7
Quercus rubra	17.10	33
<u>ECM treatment</u>		
Acer saccharum	3.94	4
Carya ovata	0.99	1
Quercus rubra	14.45	16
Quercus velutina	68.08	75
<u>ECM control</u>		
Acer rubrum	1.16	2
Acer saccharum	4.06	7
Betula alleghaniensis	0.31	1
Betula lenta	0.39	1
Quercus rubra	8.13	14
Quercus velutina	44.53	76

Table S4-1. Mean soil moisture for each plot and each year during throughfall exclusion treatment. Mean and SE from DOY 150-250.

Plot	Year	Mean	SE
AM Control	2017	0.293	0.005
AM Control	2018	0.266	0.008
AM Control	2019	0.245	0.007
AM Treatment	2017	0.238	0.005
AM Treatment	2018	0.218	0.009
AM Treatment	2019	0.141	0.007
ECM Control	2017	0.269	0.007
ECM Control	2018	0.236	0.010
ECM Control	2019	0.218	0.010
ECM Treatment	2017	0.226	0.005
ECM Treatment	2018	0.216	0.007
ECM Treatment	2019	0.170	0.008

Table S4-2. Significance of the effects of mycorrhizal association (Myc), Treatment, Year, and Myc x Treatment in microbial activity. Analysis for enzyme activity is only for July of each year. Values in bold italics and bold indicate significant differences at $p < 0.05$ and 0.10 , respectively.

Assay	Soil fraction	Myc	Treatment	Year	Myc x Treatment
BG	Rhizosphere	0.540	0.280	0.294	0.393
	0-15cm	0.371	0.942	0.378	0.685
	15-30cm	0.305	0.154	0.123	0.663
NAG	Rhizosphere	<i>0.0006</i>	<i>0.0062</i>	<i>0.0503</i>	<i>0.003</i>
	0-15cm	0.8111	0.8178	0.4935	0.8506
	15-30cm	0.254	0.737	0.523	0.114
Peroxidase	Rhizosphere	0.328	0.729	0.628	<i>0.0974</i>
	0-15cm	<i>0.0228</i>	0.4301	<i>0.0676</i>	0.1292
	15-30cm	0.412	0.5165	0.1655	0.1176
Phenol oxidase	Rhizosphere	<i>0.0246</i>	<i>0.07766</i>	0.191	<i>0.01127</i>
	0-15cm	<i>0.0316</i>	0.2431	0.5219	0.1399
	15-30cm	0.1298	0.6794	0.3587	<i>0.0695</i>
Acid Phosphatase	Rhizosphere	<i>0.0178</i>	0.8984	0.4388	0.4074
	0-15cm	<i>0.06896</i>	0.26263	0.8822	0.7997
	15-30cm	<i>0.0231</i>	0.9542	0.1482	0.1844

Table S4-3. Ambient and experimentally altered growing season precipitation (P, mm) and soil moisture deficits. Historic average normals for precipitation from 1991-2020 are shown. P-PET, precipitation minus potential evapotranspiration (mm) data was previously published.

Year	P	Treatment P	PET	Ambient P-PET	Treatment P-PET
2017	895.60	447.80	433.50	462.10	14.30
2018	974.60	435.28	483.25	491.35	-47.97
2019	674.12	67.41	482.71	191.41	-415.30
Avg historic normals from 1991-2020*	796.04				

*Data from NOAA National Centers for Environmental Information

Figure S2-1. Pie chart showing the relative abundance of bacterial and fungal communities in AM and ECM soils (n=5).

Figure S2-2. Pie chart showing the relative intensity of SOM chemistry groups in control (a) AM and (b) ECM soils (n=5) and in the added (c) AM poplar and (d) ECM oak substrate.

Figure S2-3. Box plots depicting minimum and maximum values of weighted average EAF for microbial type in (a) ECM and (b) AM soils in response to substrate type. Cross bars indicate median (n=5). Asterisks denote differences between bacteria and fungi at $p < 0.05$.

Figure S2-4. Box plots depicting the amount of ^{13}C AM and ECM substrate remaining in each soil after the 21-day incubation. The ^{13}C remaining was calculated by taking the amount of cumulative ^{13}C respiration over the initial amount of ^{13}C substrate added per gram dry weight of soil. Cross bars indicate median (n=5).

Figure S2-5. (a) Active decomposers at only one and at both sites within bacterial families in the AM soil and (b) Bacterial families at the ECM soil. Change in color denotes EAF, symbol the litter substrate and the site of the point denotes relative abundance. Error bars indicate standard error (n=5).

a.

b.

Figure S2-6. (a) Active decomposers at only one and at both sites within fungal families in the AM soil and (b) Fungal families at the ECM soil. Change in color denotes EAF, symbol the litter substrate and the site of the point denotes relative abundance. Error bars indicate standard error (n=5).

Figure S2-7. Bar plots depicting mean values of relative intensity of different lipid classes in AM and ECM soils in response to substrate type. Error bars indicate standard error ($n=5$). Asterisks denote differences in intensity between substrate type in each soil at $p < 0.05$. GL: Diacylglycerols Unk, GL0201: Diacylglycerols, GL0301: Triacylglycerols, GP0101: Diacylglycerophosphocholines, GP0105: Monoacylglycerophosphocholines, GP0201: Diacylglycerophosphoethanolamines, GP0202: 1-alkyl,2-acylglycerophosphoethanolamines, GP0203: 1-(1Z-alkenyl),2-acylglycerophosphoethanolamines, GP0401: Diacylglycerophosphoglycerols, GP1201: Diacylglycerophosphoglycerophosphodiradylglycerols, PR0201: Ubiquinones

Figure S3-1: Photos of the throughfall exclusion experimental design at Tom's Run Natural Area in Morgantown, WV.

Figure S3-2. The aboveground relative growth rates over three years of treatment for (a) AM plots and (b) ECM plots. Values are shown as a percent.