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The Redwood Microbiome: Microbial community composition and functional consequences of plant-microbe interactions for the tallest species on Earth

By

Claire E Willing

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Environmental Science, Policy, and Management

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Todd Dawson, Chair
Professor Thomas Bruns
Professor Mary Firestone
Professor David Ackerly

Summer 2019

The Redwood Microbiome: Microbial community composition and functional consequences of plant-microbe interactions for the tallest species on Earth

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Claire E Willing

Abstract

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The plant microbiome has proven to be an essential and often overlooked aspect of plant physiological ecology. Plant interactions with microbes are thought to have enabled the transition of plants from aquatic to terrestrial systems, thus co-evolving for millions of years. Consequently, microbes have important consequences for plant performance, nutrient cycling, and potentially even plant species ranges. However, our knowledge of these processes derives from only a few model systems. My dissertation explores these relationships in coast redwood forests where, despite their notoriety, plant-microbial relationships remain unexplored. Redwoods are the largest and some of the oldest plants on Earth, yet their geographical ranges are very constrained. As such, redwood forests serve as an excellent system to explore plant-microbial interactions and how they might expand or limit species ranges.

In *Chapter 1,* I demonstrate two unexpected and important findings related to the redwood root mycobiome. First, I show for the first time that, in addition to its expected association with arbuscular-mycorrhizal fungi (AMF), redwood roots are also associated with with ecto- and ericoid mycorrhizal fungi. To our knowledge, this is the first evidence that this predominantly AMF-associated tree is also colonized by the other two primary types of mycorrhizal fungi. These findings challenge the common practice of AMF-specific sequencing methods and the paradigm that plant species are assumed to associate with only one type of mycorrhizal fungi. This is important because it may link this tree species to the surrounding trees and shrubs that co-occur in these habitats. Secondly, I describe unique structures, which I call "rhizonodes", that show many parallels in microbial community assembly to well-described nodules in N-fixing plants where rhizonodes act as reservoirs of AMF in the redwood roots. The presence of rhizonodes similarly are a unique finding that seem to occur in other predominantly AMF-associated tree species, but have been widely overlooked. We posit that these structures may be important domiciles for AMF in many of the upwards of 80% of plant species predicted to form relationships with this group. These findings also suggest that arbuscular mycorrhizal fungi (AMF) may play key roles in redwood physiology.

In *Chapter 2*, I focus on redwood association with AMF and the functional consequences of these interactions for plant hosts. I found that mycorrhization of coast redwood proceeds a number of downstream consequences for redwood hosts including in morphology, growth, allocation, hormonal physiology, drought-response, and leaf-level physiology. In addition, I

found that mycorrhizal seedlings demonstrate progressively higher rates of photosynthesis compared to non-mycorrhizal seedlings in response to rising CO_2. Together, these findings indicate that mycorrhizal fungi are likely to become increasingly important more broadly for plant response to climate change.

In *Chapter 3,* I demonstrate the importance of site-specific water-availability on the root-associated bacterial community in redwood. Previous work on crop plants has shown that artificial drought selects for monoderm bacteria, or bacterial taxa with a single, thick cell-wall layer. I present evidence of similar trends for a long-lived tree species in response to local water-availability. While the consequences of interactions with these types of bacteria remain unknown, as increased intensity and severity of drought are predicted as a result of climate change, I highlight the importance of testing the downstream consequences of monoderm versus diderm-dominated bacterial communities on plant physiology, nutrient cycling, and ecosystem function.

Taken together, these studies suggest that the root microbiome is an important axis in understanding the response of redwood forest to climatic and environmental change predicted in the Anthropocene. Further, our study of a basal gymnosperm highlights important gaps in our understanding and categorization of plant-microbe interactions and emphasizes the importance of future investigations on non-model plant organisms.

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Chapter 1:

Novel root structures and unexpected mycorrhizal types discovered in coast redwood, *Sequoia sempervirens*

Abstract

Plant associations with fungi are ubiquitous and represent an important frontier in plant ecology. Leveraging improvements in DNA sequencing, we demonstrate strong biotic filtering in the rhizosphere of the iconic coast redwood (Sequoia sempervirens). In addition to the expected arbuscular mycorrhizal fungi (AMF) community, we find numerous ecto- and ericoid mycorrhizal fungi associated with redwood roots, potentially linking redwoods to surrounding trees and shrubs. Further, we describe for the first time the previously unseen role of root structures (which we call "rhizonodes") as domiciles for AMF, demonstrating the capacity of plants to harbor fungal symbionts within a specialized root organ. These results challenge the common practice of AMF-specific sequencing that considers only a fraction of the root mycobiome. Moreover, our study of a basal gymnosperm brings into question whether redwoods make or break rules about mycorrhizal relationships, host specificity, and the role of plant organs in root mycobiome assembly.

Introduction

The rhizosphere microbiome is home to a diversity of fungi that directly impact nutrient acquisition in plants (Augé *et al.*, 2001; Smith & Smith, 2011; Behie *et al.*, 2012). Consequently, considerable emphasis has been placed on elucidating the factors influencing the biogeography of fungi associated with the rhizosphere, which includes the soils adjacent to roots as well root environments themselves (Talbot *et al.*, 2014; Tedersoo *et al.*, 2014; Davison *et al.*, 2015; van der Linde *et al.*, 2018). While the rhizosphere hosts a diversity of fungi, particular emphasis has been placed on a group of symbiotic, root-associated fungi known as mycorrhiza. Mycorrhizas are categorized into one of five different types (arbuscular, ecto-, ericoid, arbutoid, and orchid mycorrhizal fungi) based on their taxonomic identity, morphology of fungal growth within the root, and identity of their plant host, where a given plant species is assumed to associate with a single mycorrhizal type (Smith & Smith, 2011).

Due to methodological constraints, few studies have investigated the rhizosphere mycobiome of plants associated with the most ubiquitous of mycorrhizal types, arbuscular mycorrhizal fungi (AMF) (Hart *et al.*, 2015; Lekberg *et al.*, 2018), which form associations with upwards of 80% of vascular plant species (Brundrett, 2002). Studies focused on AMF-associated plant species have historically employed small subunit (SSU) rRNA primers that are AMF-specific and are thereby limited to low taxonomic resolution (Bruns & Taylor, 2016) and a fragment of the functional profile of the mycobiome (Van Geel *et al.*, 2014; Hart *et al.*, 2015; Lekberg *et al.*, 2018). Furthermore, most research has focused on either root or soil fungal community composition, but few studies have explored the composition of both of these communities in tandem across geographic or environmental gradients (Öpik *et al.*, 2006; Van Geel *et al.*, 2014; Davison *et al.*, 2015; Gorzelak *et al.*, 2017). Recently developed ITS2 rRNA primers recognize all fungal groups and allow for the examination of paired root and soil samples in AMF-associated plant hosts, thereby enabling us to fill these knowledge gaps (Taylor *et al.*, 2016).

Interestingly, the mycobiome associated with the world's tallest trees, the coast redwood, (*Sequoia sempervirens*, D. Don, Cupressaceae) has never before been studied. While this iconic species spans significant environmental gradients, it remains by far the most dominant species throughout its range, maintaining a mean basal stem area of approximately 97% (Van Pelt *et al.*, 2016). Redwoods are also a basal gymnosperm shown to form associations with AMF (Mejstrik & Kelley, 1979). As such, the redwood system provides a unique opportunity to test rhizosphere mycobiome dynamics across environmental gradients while controlling for the plant host and surrounding plant community.

Turnover in the soil mycobiome, or the variance in fungal community composition, is related to both the dispersal capacity of fungal species and environmental filtering (Peay *et al.*, 2010; Talbot *et al.*, 2014; Tedersoo *et al.*, 2014; Glassman *et al.*, 2017). However, while community turnover in the soil mycobiome has been a major focus of mycology in recent years, far less is known about how shifts in this pool of fungi might impact root mycobiome dynamics (Goldmann *et al.*, 2016; Gao *et al.*, 2018). Root mycobiomes are then horizontally assembled from the surrounding community in the soil in a process known as biotic filtering (Gao *et al.*, 2018). In this study, we aim to understand how turnover in the soil fungal community corresponds with turnover in the root mycobiome. We hypothesize that (H1) roots will select for specific fungal communities and therefore soil fungal community turnover will be more tightly coupled with changes in both geographic distance and soil pH than the corresponding turnover in

root fungal community composition, and (H2) that root fungal communities will exhibit strong biotic filtering of the surrounding soil mycobiome, thus containing a small subset of the taxa found in the larger soil environment throughout the redwood ecosystem.

In addition to the biotic filtering that we predict at the ecosystem scale, plant roots are heterogeneous environments containing microhabitats that may act as additional biotic filters. For example, root nodules legumes and *Alnus* have been shown to play an important role in root microbiome assembly where these nodules house a specialized, predominately N-fixing bacterial community that differs from adjacent root tissue (Zgadzaj *et al.*, 2016). In AMF-associated tree species similar swollen, nodule-like root structures have been observed (Grand, 1969; Beslow *et al.*, 1970; Dickie & Holdaway, 2010). While mycorrhizal fungi are capable of colonizing these structures, little research has investigated the fungal community composition of these structures, herein referred to as "rhizonodes" (Duhoux *et al.*, 2001; Russell *et al.*, 2002). As parallel genetic pathways have been shown in plant signaling for both N-fixing bacteria and AMF, the common occurrence of rhizonode structures on trees known to associate with AMF suggests that they might also act as domiciles for a specialized fungal community (Bonfante & Genre, 2010). Accordingly, we predict (H3) that rhizonodes will host fungal communities that are a subset of the community found in the adjacent root tissue, thus acting as an additional biotic filter at the scale of the individual root.

Methods

Site descriptions: The geographic range of *S. sempervirens* extends from the central Californian coast, USA to just north of the Oregon boarder (36.08°N, 121.60°W to 41.77°N, 124.11°W) in a narrow band characterized by mild temperatures and coastal fog water inputs. For our study, eight sites spanning this range were sampled in a nested design (Fig. 1) based on a network previously established by the Redwoods and Climate Change Initiative (RCCI) (Van Pelt *et al.*, 2016). The redwood forest has been categorized based on its associated plant communities into three regions—the northern, central, and southern zones. Sub-dominant plant taxa throughout this range include *Polystichum munitum*, *Oxalis oregona*, *Vaccinium spp*, *Pseudotsuga menziesii*, and *Notholithocarpus densiflorus*. Thirty-year average climatic data were extracted from 1985-2015 for plot locations from the Basin Characterization Model, which has 270m spatial resolution (Flint & Flint, 2014). Based on these extracted values, mean annual precipitation at these sites ranged from 720 to 2210 mm per year and maximum temperatures ranged from 16.5°C to 21.2°C. Due to the importance of fog-water input in this system (Dawson, 1998; Simonin *et al.*, 2009; Johnstone & Dawson, 2010), we chose to focus on actual evapotranspiration (AET) as a proxy for water-availability for the purposes of this study, as well as the mean minimum winter temperatures for December-January-February (DJF) and mean maximum summer temperature for June-July-August (JJA). Further plant community data for these sites can be referenced in (Van Pelt *et al.*, 2016).

Sampling description: Sixteen soil cores (15cm deep and 8cm diameter) were collected at each site between September and October 2015 (Fig. 1). This time point was selected as this follows the most active periods of photosynthesis and likely represents the greatest demand for both water and nutrients from the soil. In sampling, the duff layer was physically removed and cores were taken from the top 15cm of soil; redwood roots were abundant in all cores as this species is shallow-rooted. Cores were collected into freezer-safe bags, transported from the field, and kept

on ice until returning back to the lab. They were then kept at 4°C until processing, which occurred within 3-5 days of returning to the lab (and no more than 1 week post-collection).

To determine the community composition of the different root zones, three different redwood trees were sampled in August 2018 in Albany, CA. Three root samples were collected from each tree and washed thoroughly in DI water. Rhizonodes were excised from adjacent root tissue for each of the nine root samples and approximately 100mg of rhizonode tissue was used for each extraction. DNA extractions were performed using the MoBio Power Soil kit (Qiagen, Germantown, Maryland, USA) per protocol instructions. Sequencing and downstream bioinformatics steps followed the protocol as detailed below.

Soil chemistry: After sieving soil and collecting soil for sequencing, the remaining soil from each soil core was dried on the bench-top. To quantify total carbon and total nitrogen content, each soil sample was ground using mortar and pestle, weighed into tin capsules and assessed using a CE Instruments NC 2100 Elemental Analyzer (Rodana, Milano, Italy) at the University of California, Berkeley. All remaining soil chemistry data were measured at the University of Massachusetts, Amherst using the protocols for Routine Soil Analysis. Soil chemistry data included pH, total P, K, Ca, Mg, Zn, B, Mn, Cu, Fe, Pb, Al, Na, and S, as well as cation exchange capacity (CEC), exchangeable acids, and scoop density.

DNA extractions: Soil cores were sieved to 2mm and 0.25g of soil was collected and put directly into MoBio Power Soil buffer (Qiagen, Germantown, Maryland, USA). From the same core, 0.70g of fine roots from coast redwood were collected, washed in running distilled water 5 times to remove particles adhered to the root surface, and frozen at -80°C prior to DNA extractions. Soils were immediately processed according to manufacturer's instructions and soil DNA was stored at -80°C. Roots were ground in liquid nitrogen using a mortar and pestle and immediately transferred into the Powersoil buffer. DNA was then extracted according to manufacturer's instructions and concentration of extracted DNA was quantified using a NanoDrop 2000 (Thermo Fisher Scientific, Indianapolis, IN). Remaining soil was bench-dried for subsequent soil chemical analyses.

Molecular identification of species: The PCR primers used targeted the ITS2 rRNA region, which can discriminate against plant DNA and provide more accurate measures of fungal diversity and abundance (Taylor *et al.*, 2016). These primers have also successfully been used to study the succession of AMF communities through a growing season (Gao *et al.*, 2018), making them ideal primers for this study. The primers used in the present study were adapted to Illumina platform for single-step PCR based on best-practices for high-throughput amplicon sequencing, including hetero-spacers to help increase base-pair diversity thereby increasing accuracy (Lundberg *et al.*, 2013). DNA concentrations for each sample were quantified using NanoDrop 2000 (Thermo Fisher Scientific, Indianapolis, IN) and diluted to 10ng/µL for each sample before PCR amplification. Samples were randomized within three 96-well plates that included negative controls (both DNA extraction kit controls and PCR controls). 25µl reactions were set up for PCR using 2.5µl of forward primer, 2.5µl of reverse primer, 2µl of DNA (from 10ng µl/µL dilutions), 7µl of water, 10µl of Kapa HiFi ready-mix (Kapa Biosystems, Boston, MA, USA) and 1µl of BSA. PCR conditions were: 96°C for 2 min, 34 cycles of 94°C for 30 sec, 58°C for 40 sec, 72°C for 2 min, and 72°C for 10 min before 4°C hold. PCR products were run on a gel to verify product and then quantified via Qubit dsDNA HS kit. Samples that did not initially produce PCR products were amplified again and these were used for subsequent sequencing. Samples were then pooled at equimolar concentrations (100ng PCR product per sample) and

Pippin Prep (Sage Science, Beverly, MA, USA) was used to purify for PCR products between 200-800bp (as PCR products included hetero-spacers and Illumina adapters). Bioanalyzer trace was used to detect that products were of the correct length and purity. Both Pippen Prep and Bioanalyzer trace were performed by the Functional Genomics Laboratory at the University of California, Berkeley. Paired-end (2 x 300 bp) sequencing was performed on the Illumina Miseq (Illumina Inc., San Diego, CA, USA) operating v3 Chemistry at the Vincent J. Coates Genomics Sequencing Facility (also at the University of California, Berkeley) upon receipt of clean Bioanalyzer trace. Details of PCR, library prep, and sequencing can be found in the SI Appendix.

Sequencing data were processed using the AMPtk pipeline (Jusino *et al.*, 2019). Briefly, reads were merged, custom primers were trimmed, and PhiX was removed from reads. Reads were then clustered at 97% sequence similarity and filtered to remove chimeric reads, singletons, and index bleed at a rate of 0.005% as per AMPtk standards. Taxonomy was assigned using AMPtk's hybrid approach, which uses Bayesian methods to select consensus lineages between Global Aligment, UTAX, and SINTAX (Jusino *et al.*, 2019). For the rhizonode data, the same parameters were retained with an additional filtering step used via AMPtk's Lulu pipeline. FUNguild was then used to parse OTUs into ecological guilds (Nguyen *et al.*, 2016).

Confirmation of host plant: Though these primers are designed to discriminate against plant DNA, plant DNA was still picked up through our sequencing. We chose to utilize this plant DNA in order to confirm that our root samples were from intended host. Pre-rarefaction, 81.7% of root samples contained plant DNA solely from the intended target host *S. sempervirens*. 12.7% of root samples contained no plant DNA. Given that only 3 of these samples remained after rarefaction, we presumed that these samples had low read-depth, likely explaining why they did not pick up plant DNA. The remaining 5.6% of samples contained both redwood and DNA from one other plant: *Pseudotsuga menziesii* (five samples), *Acer sp.* (one sample), *Juglans sp.* (one sample), and *Oxalis sp.* (one sample). Because these samples also contained redwood DNA, we choose to retain these reads in our analyses and used caution in interpreting the OTUs from these samples. Plant OTUs themselves were removed in Phyloseq (McMurdie & Holmes, 2013) by parsing out any OTUs that did not belong to Kingdom Fungi.

Statistics: All data were analyzed in R version 3.3.2 (R Core Team). The OTU table was loaded into R using Phyloseq (McMurdie & Holmes, 2013) and data were filtered to retain only fungal reads. Data were then rarefied to 1000 reads in Phyloseq. Ordinations were produced in Phyloseq on rarefied data using both Bray-Curtis and Jaccard distances and barplots were produced using Phyloseq on rarefied data. All plots were produced using ggplot2 (Wickham, 2009).

To test the factors associated with fungal turnover in the respective root and soil communities (H1), we first selected both soil and environmental factors that we predicted might be meaningful to fungal community composition based on previous studies (Talbot *et al.*, 2014; Glassman *et al.*, 2017). These included: actual evapotranspiration (AET), mean minimum temperature for December, January, and February (DJF), mean maximum temperature for June, July, and August (JJA), soil pH, soil phosphorus, soil cation exchange capacity, soil C:N, and latitude and longitude of samples. To calculate distances between samples, the location of each tree was recorded using a handheld GPS device and each sample collected at a given tree was collected with a 3m arc of that tree. Distances were calculated between each of the different samples using the SoDA package in R (Chambers). We then ran a correlation analysis in R using the Hmisc package to test for autocorrelation of these parameters (Harrell *et al.*, 2018) using a cutoff of 0.6; soil cation exchange capacity and DJF were found to be auto-correlated with

longitude and soil phosphorus, so both were omitted from models as both longitude and soil phosphorus were thought to be more important for fungal community composition than the minimum winter temperature (Talbot *et al.*, 2014; Tedersoo *et al.*, 2014). We ran generalized dissimilarity models (GDM) (Manion *et al.*) for both root and soil samples, which accommodate non-linear changes in species turnover (Glassman *et al.*, 2015). Results were also analyzed with a multiple-matrix regression with randomization (MMRR) approach (Wang, 2013), which resulted in similar ecological interpretations. Because each of the models include the distances between samples, the nested design was implicitly accounted for in both GDM and MMRR approaches. We also used Mantel tests (Goslee & Urban, 2017) to compare the correlation between individual parameters and community similarity for both soil pH and geographic distance (the two parameters that were retained as significant using both GDM and MMRR) using vegan (Oksanen *et al.*, 2013). Indicator species were determined for root versus soil communities across the range of soil pH using TITAN (Baker *et al.*). In order to test the potential affects of soil pH on fungal community richness, linear models were produced using lme functions in R. Analyses were run on rarified OTU tables to ensure equal sampling depth.

To test the role of biotic filtering in fungal community composition in between roots and soils (H2) and rhizonodes and roots (H3), we ran nested PERMANOVAs. The first PERMANOVA tested for significant differences between root versus soil samples and differences across plots within those two groups using the "adonis" command in vegan (Oksanen *et al.*, 2013). An additional nested PERMANOVA was used to test for significant differences between the rhizonodes versus the adjacent root tissues. Further, we tested for differences in the richness and evenness of the fungal community using the Chao1 and Shannon indices across sites and between sample types. To test for significant differences in relative abundance of AMF between root and rhizonodes, data were log-transformed to attain a normal distribution and compared using Student's t-test. Lastly, we ran indicator species analyses to compare the indicator species between root and soil samples and between rhizonodes and adjacent root tissues (Dufrêne & Legendre, 1997) using rarefied data. All reported statistics and figures are based on rarefied data and Bray-Curtis dissimilarity, unless otherwise noted.

Results

Compositional variance of the mycobiome is smaller for root compared to soil habitats

We found strong support for our hypothesis (H1) that the soil fungal community turnover is more tightly coupled with changes in both geographic distance and soil pH than the corresponding turnover in root fungal community composition (Figs. 2&3). Geographic distance and soil pH both contributed significantly to turnover in the fungal community based on multiple-matrix regression with randomization (MMRR) analyses (Table 1; R^2=0.2234, $p<0.01$ and R^2=0.0555, $p<0.01$ for soils and roots, respectively) and Generalized Dissimilarity Models (GDM) (Table 1; R^2=0.3254, $p<0.0001$ and R^2=0.3338, $p<0.001$, respectively). Mantel tests supported the hypothesis that soil community composition was more correlated with geographic distance (Mantel R=0.3992, p=0.001) and soil pH (Mantel R=0.1993, p=0.001) than root communities (Mantel R=0.1321, p=0.001 and Mantel R=0.1196, p=0.005, respectively; Fig. 2).

The response of individual taxa in both the root and soil communities to shifts in pH further revealed divergent patterns between root and soil communities. TITAN analyses demonstrated a large degree of negative selection against fungal taxa at low pH (Fig. S1). Root

associated taxa, by comparison, demonstrated more equal replacement of taxa at across the range of measured soil pH. Low pH also showed a trend of slightly higher fungal richness (Fig. S2).

Redwood roots are strong biotic filters

Our analyses revealed strong biotic filtering of the soil community by roots in coast redwood (H2). Ordinations and statistical analyses revealed that soils and roots host distinct communities from one another (nested PERMANOVA R^2=0.0946, P<0.001; Fig. 3) and that origin of collection was a strong predictor of fungal community composition for both soil (nested PERMANOVA R^2=0.2499, P<0.001) and root communities (nested PERMANOVA R^2=0.1999, P<0.001). Analyses demonstrated significantly higher richness in soil fungal communities (127.478 ± 50.027) than was observed for roots (82.367 ± 32.366), t(201.79)= -8.149, P<0.001.

Soil communities were dominated by taxa that were found sparingly in root samples. For instance, while Mortierellales were dominant in soil samples across much of the redwood range, this group was nearly absent from root samples (Fig. S3). We found that Helotiales and Agaricales dominated root samples (groups that were present, though not particularly abundant in the top 100 soil OTUs) in consistent relative proportions across sites. Indicator species analyses revealed that *Philaocephala*, *Alatospora*, *Lachnum*, and *Pezicula, Oidiodendron* (Helotiales) and *Mycena* and *Hydropus* (Agaricales) were all top indicator taxa that were significantly enriched in root compared to soil samples. Though AMF comprised only 0.8% of the total sequencing depth of root samples, we identified 158 AMF taxa in this study, nine of which were significantly enriched in roots compared to soils across sites. No AMF taxa were enriched in soil samples.

As redwoods are an AMF associated host we were surprised to recover a number of other mycorrhizal types associated with redwood roots, even after vigorous washing of the roots 5 times in DI water prior to extractions and sequencing. In total, we identified 357 potential EMF and 25 ericoid-mycorrhizal OTUs throughout this study based on FUNGUILD delineations of "highly probable" and "probable" matches to the respective functional groups (Nguyen *et al.*, 2016). Of these, 206 (~58%) EMF OTUs were found in association with roots. However, based on indicator species analyses, we identified only 2% of EMF OTUs showing any significant enrichment in roots compared to soils and similar trends were seen for ericoid-mycorrhizal fungi; only one ericoid OTU was found to be significantly enriched in roots compared to soils.

Rhizonodes act as domiciles of additional biotic filtering at the scale of the individual host

We found strong evidence that rhizonodes act as an additional biotic filter at the scale of the individual root and that rhizonodes demonstrate enrichment of certain fungal taxa compared to the adjacent root tissue (H3). While the most important factor in determining fungal community composition was the geographic location of collection (PERMANOVA R^2=0.19037, P<0.001), we demonstrate evidence of tissue-specific biotic filtering, indicating that the rhizonode was a subset of the adjacent root community. We found 12 taxa to be significantly enriched in the adjacent root compared to the rhizonode, emphasizing the potential for selection against these taxa in the rhizonode tissue itself (Table 2). We were surprised to find that AMF taxa comprised a significantly higher proportion of the sequencing depth in rhizonodes, averaging at 11.6% of rarefied sequence reads compared to 1.84% in the surrounding root tissue and 0.8% across the larger study (t(14.72)= 3.43, P<0.004; Figs. 4, S4, and S5). In another study using these same primers, the highest read depth comprised by AMF taxa was approximately 2% and was more

commonly 0.5 to 0.8% of the total sequence reads from root samples (Gao *et al.*, 2018). Interestingly, two of taxa that were significantly enriched in the root compared to the rhizonode were from a specific clade of AMF belonging to the Diversisporaceae indicating specific selection against these AMF taxa contrasted against trends of enrichment of another AMF clade (Glomeraceae) within the rhizonodes themselves. We also demonstrate a trend of higher richness in root versus rhizonode communities (140.98 ± 55.67 versus 120.20 ± 36.85, respectively). While the overall community composition between roots and rhizonodes was not found to be significantly different (nested PERMANOVA R^2=0.07245, P=0.2298), these communities separate out in ordination space (Fig. 5). We attribute these non-significant PERMANOVA results to a relatively small sample size in our nested sampling design (n=3) and the otherwise large overlap in taxa between both sample types where roots already demonstrate strong filtering on community composition.

Discussion

Redwoods roots exert strong biotic filtering of fungal communities across strong geographic and environmental gradients. Furthermore, redwoods associate with not only AMF, but also EMF and ericoid mycorrhizal fungi. These findings challenge current paradigms about the promiscuity of mycorrhizal fungi. Additionally, in studying this evergreen, basal gymnosperm, we describe for the first time how specialized structures in plant roots, or rhizonodes, act as reservoirs of AMF, and even specific AMF taxa.

Geographic distance and soil pH have consistently emerged as important factors in the turnover of fungal communities (Peay *et al.*, 2010; Talbot *et al.*, 2014; Glassman *et al.*, 2017; van der Linde *et al.*, 2018) . Here we identify that the relative importance of these factors differ for soil versus root fungal communities. We also observe lower rates of species turnover in root communities across geographic and environmental gradients. One feasible explanation is that root-associated fungal communities are important in redwood physiology and thus under the processes of strong selection. Alternatively, the root environment itself may buffer against changes in abiotic conditions that result in higher rates of species turnover in the soil community (Goldmann *et al.*, 2016). Given the importance of the rhizosphere mycobiome in plant acquisition of nutrients and drought response, the identification of the factors influencing the assembly of the rhizosphere mycobiome remains an important frontier (Augé *et al.*, 2001; Wu & Xia, 2006; Smith & Smith, 2011; Bárzana *et al.*, 2012).

We also demonstrate evidence of biotic filtering across multiple scales where root fungal communities appear a subset of the soil community and where rhizonodes represent a further subset of the fungal community. While we demonstrate lower turnover in the root community across geographic and environmental gradients, it is also important to emphasize that root and rhizonode communities represent a subset of the taxa available in the larger soil mycobiome, and are thus are spatially restricted and still vulnerable to abiotic factors that shift the soil mycobiome, particularly in the face of climatic and environmental change seen in the Anthropocene (Loarie *et al.*, 2009). This pattern where soil communities demonstrate the highest species richness has previously been shown in *Arabidopsis thaliana*, a non-mycorrhizal host plant, demonstrating the potential specificity of plant-fungal interactions across diverse plant hosts and highlighting the need to understand the role not only of mycorrhizal fungi, but the larger fungal community in rhizosphere dynamics and plant physiology (Behie *et al.*, 2012; Smith *et al.*, 2017; Urbina *et al.*, 2018). Further, our demonstration of decline in species richness

from the root and rhizonodes demonstrates tissue-specific biotic filtering and emphasizes how specificity of plant fungal interactions may occur across multiple scales.

It is typically assumed that a given plant species associates with a single mycorrhizal type, however, our findings challenge this paradigm (Smith & Smith, 2011). While there have historically been occasional reports of EMF in non-EMF hosts, these findings have been largely written off as misdiagnoses (Brundrett, 2009). However, in recent years there have been increasing reports of EMF taxa associated with roots of AMF-host plants (Dawkins & Esiobu, 2017; Schneider-Maunoury *et al.*, 2018). In *S. sempervirens*, we find not only abundant EMF OTUs associated with redwood roots, but also a number of ericoid mycorrhizal taxa. Characteristic structures of EMF associations (root tips with Hartig net structures) were not observed on redwood roots indicating that, while EMF are able to access redwood roots, they do not form typical EMF-associations (Smith & Smith, 2011). It is however possible that these taxa act as endophytes within non-hosts as the ectomycorrhizal habit is thought to have evolved from former root endophytes (Selosse *et al.*, 2009; Smith *et al.*, 2017). Moreover, the occurrence of both ecto- and ericoid mycorrhizal fungi in a non-host suggests that these fungi may connect redwoods to the surrounding trees and shrubs in the understory. The concept of a common mycorrhizal network has been well established, however plant taxa associated with different mycorrhizal type were thought to function within their own networks (Simard *et al.*, 1997; van der Heijden *et al.*, 2015; Klein *et al.*, 2016). Our data demonstrate that it is possible that AMF, EMF, and ericoid networks might also actually connect on AMF-hosts. Particularly for coast redwood, our findings imply that these old growth forests are even more interconnected than we had previously thought. While AMF-specific primers have application in certain contexts (Öpik *et al.*, 2006; Van Geel *et al.*, 2014; Davison *et al.*, 2015), evidence is emerging that the root environment hosts a broad of suite fungal taxa important to plant and ecosystem function, including other types of mycorrhizal fungi, with important ecological and evolutionary implications (Selosse *et al.*, 2009; Smith *et al.*, 2017; Selosse *et al.*, 2018; Schneider-Maunoury *et al.*, 2018).

During the course of our investigations, we were excited to discover that rhizonodes act as domiciles for symbiotic AMF taxa in *S. sempervirens,* akin to a mycorrhizal version of an N-fixing root nodule. While our findings are the first to note the enrichment in AMF taxa in the rhizonode, we posit that this is a result of the prevalence of AMF-specific primers that would likely not detect differences in these two communities, particularly with respect to the relative abundance of AMF between these two root structures (Van Geel *et al.*, 2014; Hart *et al.*, 2015; Bruns & Taylor, 2016; Lekberg *et al.*, 2018). The common presence of these structures on other AMF-associated tree species provides an exciting opportunity to further explore the function of these structures not only in redwood, but in other plant taxa (Grand, 1969; Beslow *et al.*, 1970; Duhoux *et al.*, 2001; Russell *et al.*, 2002; Dickie & Holdaway, 2010). One potential functional benefit to host plants is in concentrating mycorrhizal activity into a specialized hub, particularly for a tree notorious for its extensive rooting zone, allowing for more efficient plant-fungal signaling in the development of symbiosis (Noss, 1999; Akiyama *et al.*, 2005; Bonfante & Genre, 2010). AMF structures in plant hosts are also ephemeral, living only days to weeks in plant roots, and the formation of the rhizonodes may represent a more efficient means of reinfection for host plants compared to construction of entirely new roots (Staddon & Fitter, 2001; Dickie & Holdaway, 2010). These findings have important implications not only for plant

strategies of nutrient acquisition, but also for the evolution of plant-mycorrhizal relationships across different plant lineages.

These data also add an additional angle to the ongoing debate of plant specificity in mycorrhizal interactions and the precision of such selection (Klironomos, 2000; Kiers *et al.*, 2011; Dawkins & Esiobu, 2017). We demonstrate not only EMF and ericoid colonization of redwood roots, but also highlight specific selection against even certain AMF taxa within the rhizonode. These results lend support to the notion that mycorrhizal fungi are able to colonize broader host ranges than our current paradigm suggests, yet plants may demonstrate fine-scale specificity within specialized host organs (Smith & Read, 2010). Additionally, these rhizonode structures may play an important role in plant colonization by AMF and functional benefit of this symbiosis. Together, our findings emphasize the importance of studying the mycobiome holistically, particularly in AMF-hosts, hereby opening up exciting new paths of research in host specificity and biotic filtering in plant-fungal interactions.

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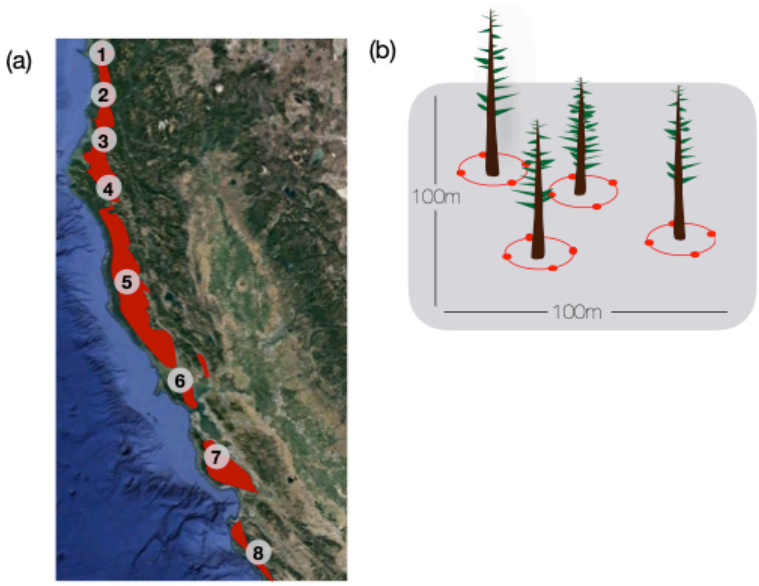

Figure 1. Sampling locations and design throughout the redwood range. (a) The approximate redwood range is denoted in red and sampling locations are labeled by plot identity. The plots span an approximately 700 km range. (b) Plots were sampled in a nested design, where red dots denote sampling locations 3m from DBH of tree in the north, south, and east directions. Map data: Google, DigitalGlobe, 2017.

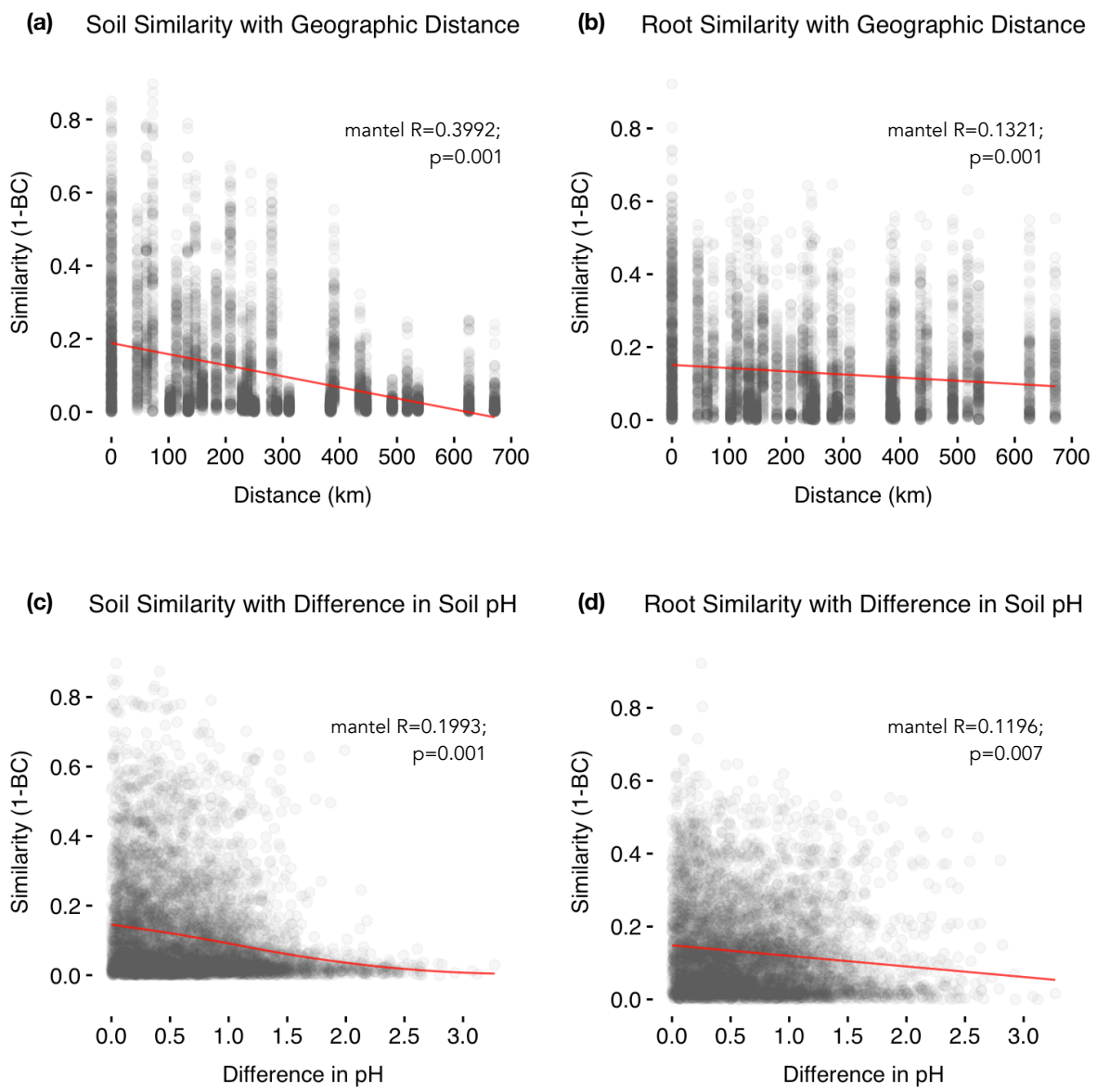

Figure 2. Compositional variance of fungal communities across factors significantly associated with fungal community turnover. Community similarity (1-Bray-Curtis) versus geographic distance for (a) soil fungal communities and (b) root fungal communities; dots are transparent, so darker colors indicate higher density of samples. Similarity (1-Bray-Curtis dissimilarity) versus soil pH for (c) soil fungal communities and (d) root fungal communities; dots are transparent, so darker colors indicate higher density of samples. Mantel coefficients are reported for each plot individually and red line indicates regression trend. Plots are based on rarefied data for roots (n=111) and soils (n=118).

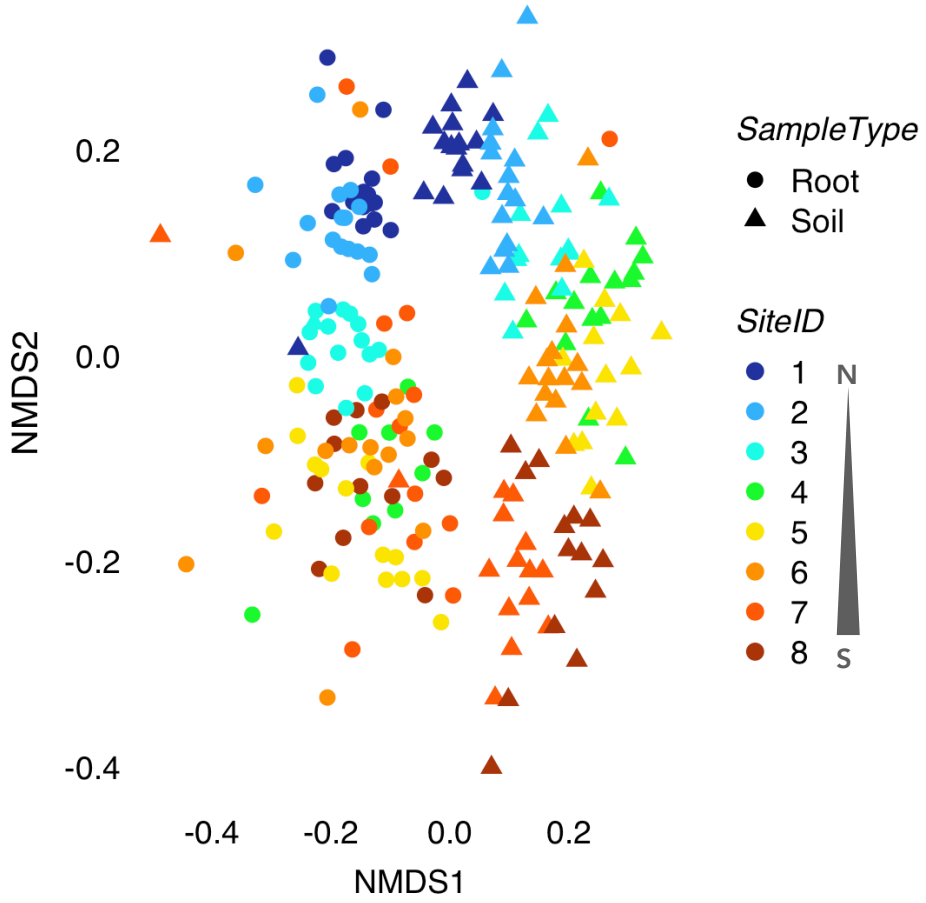

Figure 3. Fungal community similarity across sites and sample type. Ordination based on NMDS (non-metric multidimensional scaling) using Bray-Curtis dissimilarity for total fungal community of roots and soils (Stress=0.2622). Samples were rarefied to 1000 reads per sample and are colored by site of origin. Shapes denote sample type (either root or soil). After rarefaction, 111 root samples were retained 118 soil samples were retained.

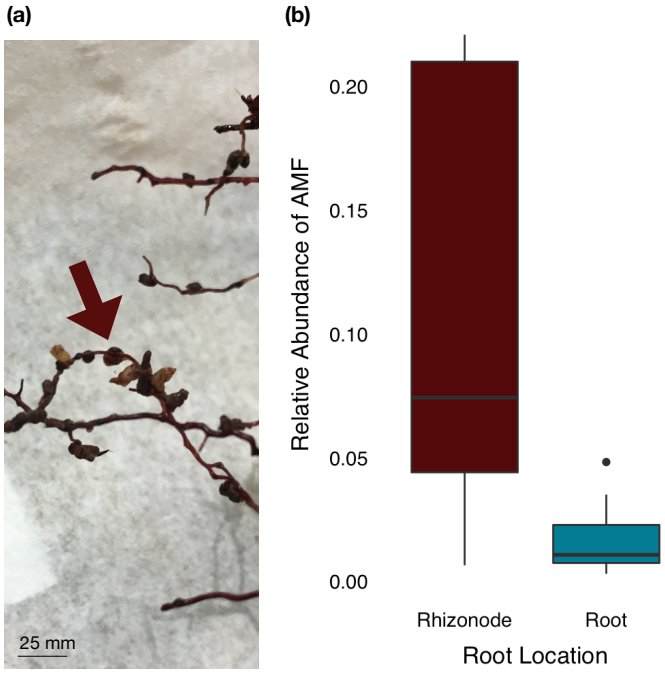

Figure 4. Rhizonode structures and enrichment in AMF taxa within the rhizonode. (a) Photo with arrow pointing towards a cluster of typical rhizonodes and adjacent root tissue. (b) Relative abundance of AMF increases from a mean of 1.8\% in adjacent root tissue to 11.6% in the rhizonode. Data here are shown as raw relative abundance based on the top 100 operational taxonomic units (OTUs) from the rarefied data (n=9). Horizontal lines represent the median, the box represents the lower 25th and upper 75th quartiles. Circles represent values that fall outside of the 1.5 x interquartile range (whiskers). To test for significant differences, data were first log transformed to attain normality prior to running Students t-test t(14.726) = 3.416, p = 0.004).

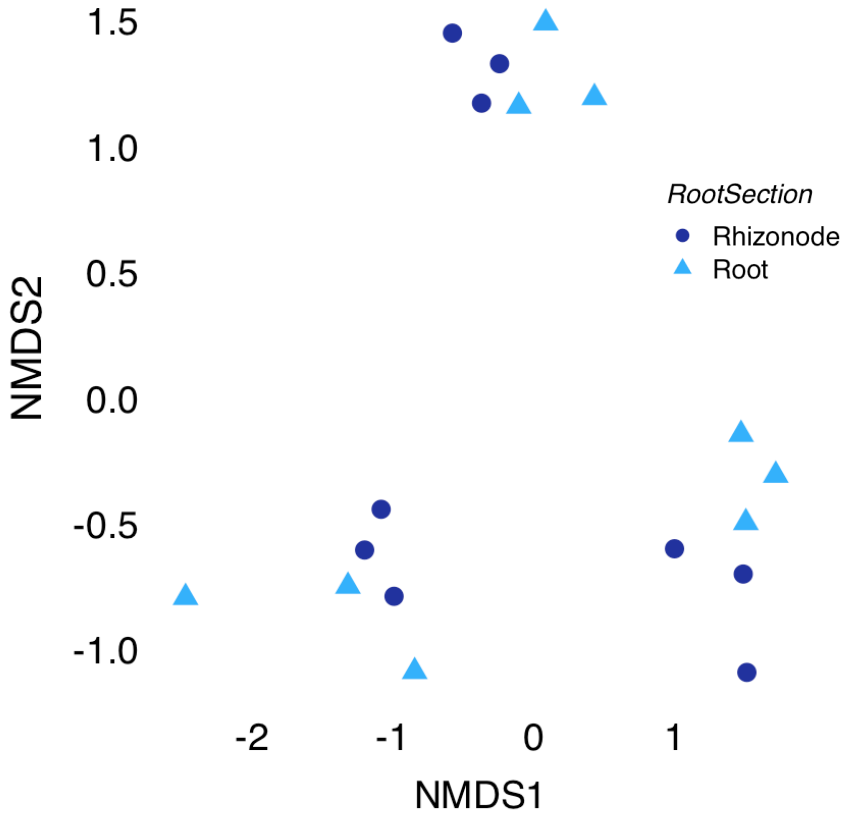

Figure 5. Total fungal composition of rhizonode and root communities. Ordination based on NMDS (non-metric multidimensional scaling) using Bray-Curtis dissimilarity for between rhizonodes and the adjacent root tissue (Stress=0.0841). Root and paired rhizonode samples cluster by location of tree sampled (n=3) and rhizonodes are offset from root samples (n=3)

Table 1. Environmental factors correlated with turnover in the fungal community. Results from Multiple Matrix Regression (MMRR) and from generalized dissimilarity model (GDM) for soils (left) and roots (right) predicting fungal community composition of roots and soils based on geographic and climatic factors. For GDM, values reported are best on best model which only retained predictors that were determined to be significant in backwards elimination model selection. Bolded parameters were significant in models. AET refers to actual evapotranspiration.

MMRR Soil Data		
	*Model R^2=0.1763; p=0.001**	
Factor	Coeff	p val
Distance	**0.0004**	**0.001**
Soil pH	**0.0396**	**0.001**
Soil CN	0	0.679
Soil P	0	0.564
AET	-0.0001	0.061

GDM Soil Data		
	Deviance explained=33.38%; p=0.0000	
Factor	Coeff	p val
Distance	**43.49966**	**0.000**
Soil pH	**32.6889**	**0.000**

MMRR Root Data		
	Model R^2=0.0283; p=0.02	
Factor	Coeff	p val
Distance	**0.0001**	**0.010**
Soil pH	**0.0225**	**0.035**
Soil CN	-0.0007	0.693
Soil P	0.0001	0.752
AET	-0.0001	0.393

GDM Root Data		
	Deviance explained=32.54%; p=0.00000	
Factor	Coeff	p val
Distance	**71.27319**	**0.000**
Soil pH	**10.7696**	**0.000**

Table 2. Indicator species analysis for root versus rhizonodes reporting indicator value (IV) and p-values. No taxa were determined to be significantly enriched in the rhizonode compared to the root.

Group	IV	p val	Phylum	Class	Order	Family
Root	0.5696	0.001	Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae
Root	0.4621	0.001	Basidiomycota	Agaricomycetes	Auriculariales	NA
Root	0.4527	0.001	Basidiomycota	Agaricomycetes	Sebacinales	NA
Root	0.5000	0.001	Basidiomycota	NA	NA	NA
Root	0.5807	0.001	Rozellomycota	NA	NA	NA
Root	0.5461	0.001	Glomeromycota	Glomeromycetes	Diversisporales	Diversisporaceae
Root	0.5273	0.001	Basidiomycota	Agaricomycetes	Agaricales	Stephanosporaceae
Root	0.5000	0.001	Ascomycota	Pezizomycetes	Pezizales	NA
Root	0.7396	0.001	Glomeromycota	Glomeromycetes	Diversisporales	Diversisporaceae
Root	0.6111	0.001	Ascomycota	Eurotiomycetes	Chaetothyriales	NA
Root	0.4791	0.001	Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae
Root	0.5653	0.001	Ascomycota	Pezizomycetes	Pezizales	NA
Rhizonode	NA	NA	NA	NA	NA	NA

Appendix

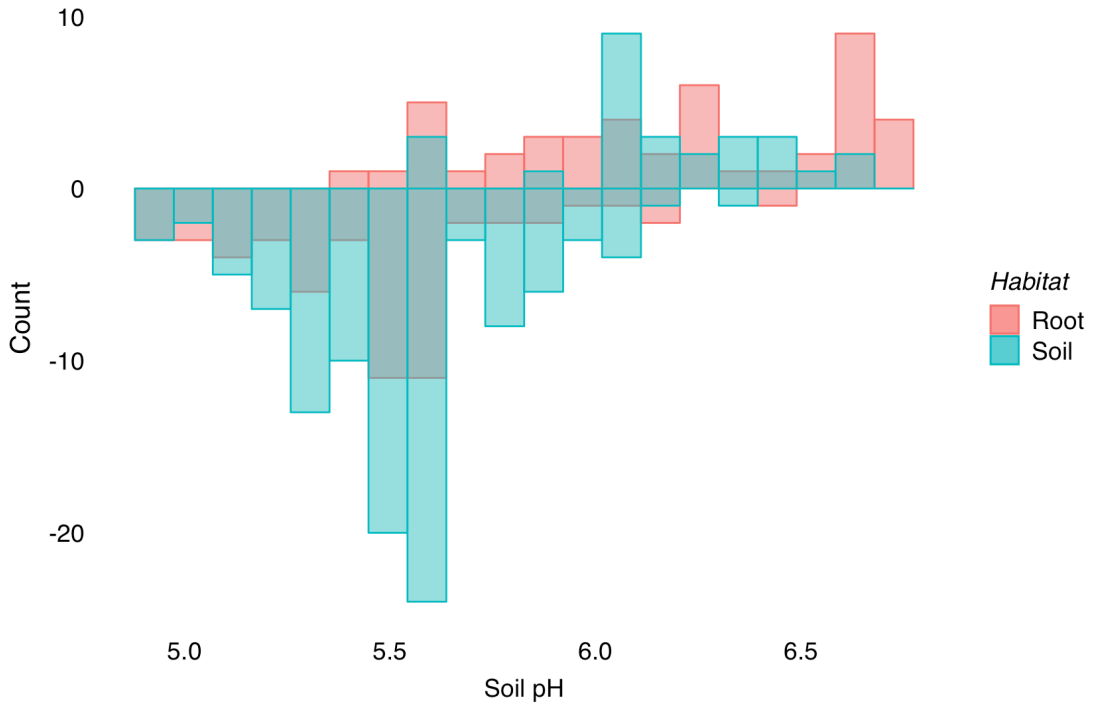

Figure S1. Taxa significantly selected for or against across measured soil pH. Results from threshold indicator taxa results (TITAN) indicating that soils showed more negative selection than positive selection for taxa, particially at low pH as represented in the histogram where bars that fall below the zero line represent counts of taxa at that bin of pH and taxa in above the zero line represent counts of taxa that are positively selected for at that pH.

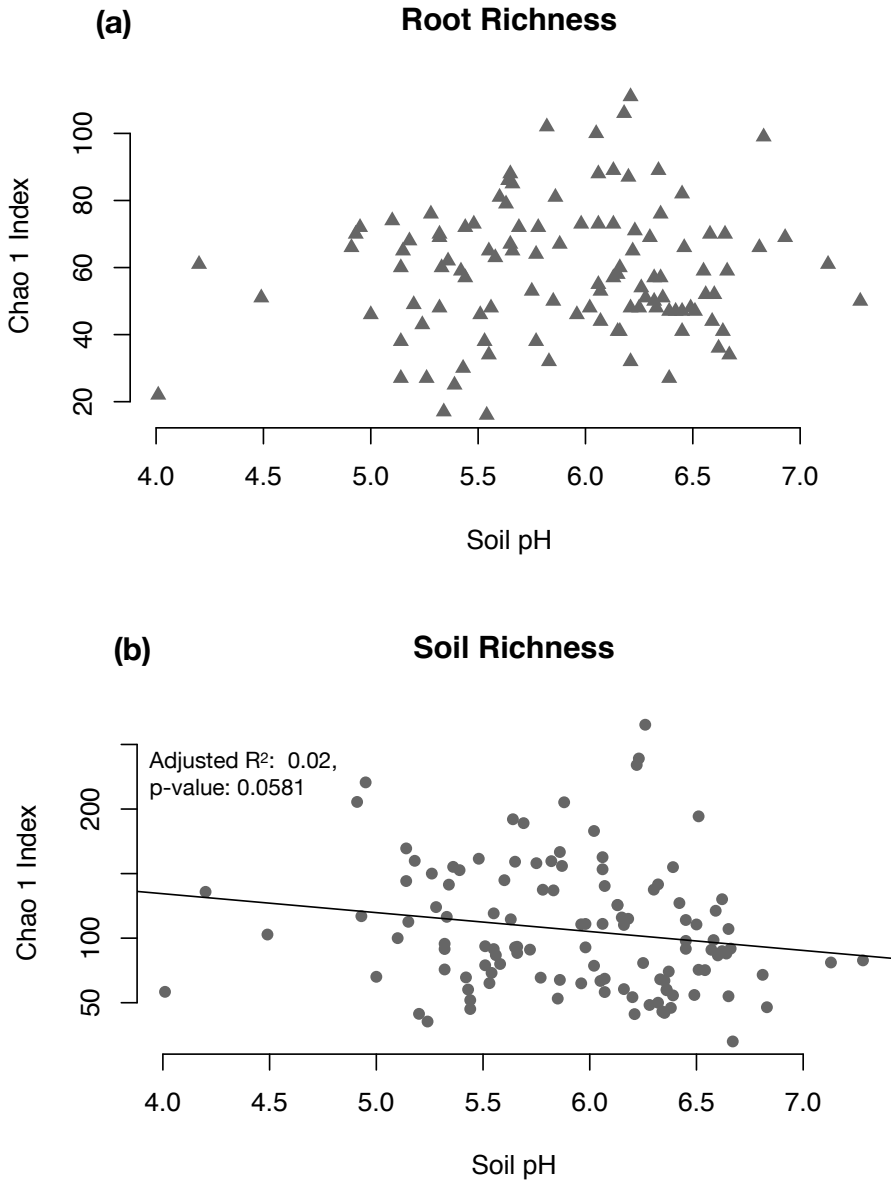

Figure S2. Richness of fungal community across soil pH. Fungal richness by soil pH for (a) roots and (b) rhizosphere soil samples. There was no relationship found for richness in root community composition by soil pH.

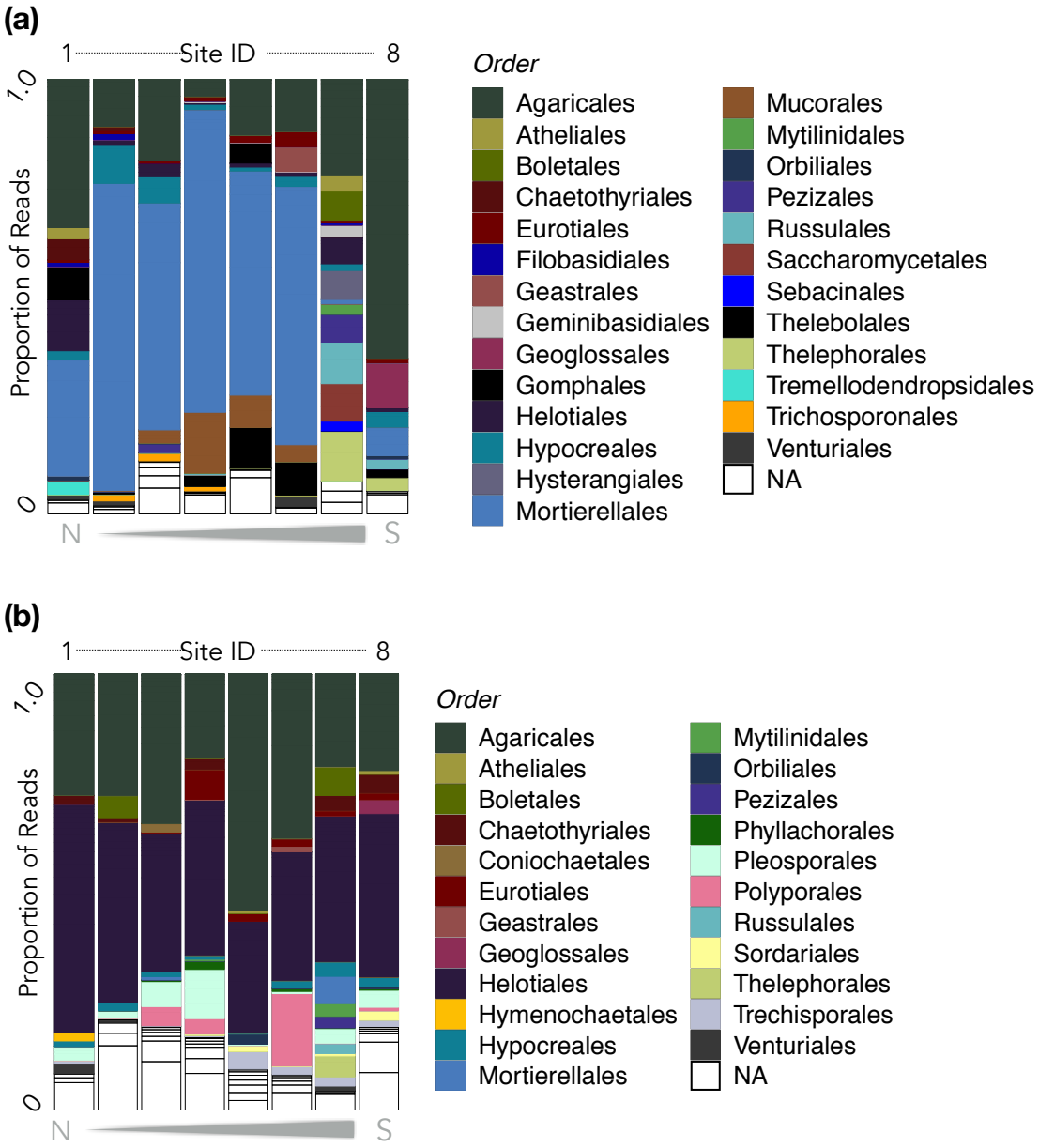

Figure S3. Taxonomic composition of root and soil fungal communities across sites. (a) Bar graph of the 100 most abundant fungal operational taxonomic units (OTUs) in soils by relative abundance at each site based on fungal order. (b) Bar graph of the 100 most abundant fungal OTUs in roots by relative abundance at each site based on fungal order.

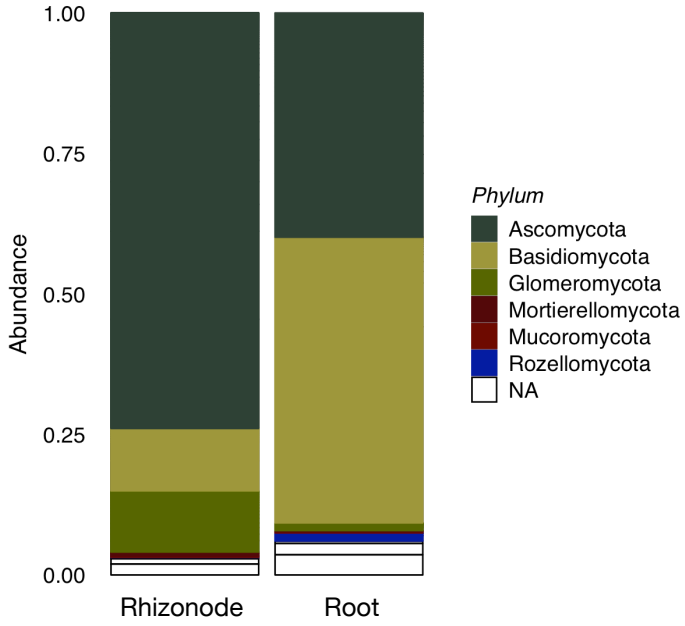

Figure S4. Taxonomic composition of rhizonode versus root samples. Plots are shown as relative abundance of the Top 100 OTUs from roots and rhizonodes at the phylum level. AMF all fall within the single, monophyletic group referred to here are Glomeromycota (now, Phylum Zygomycota, Subphylum Glomeromycotina).

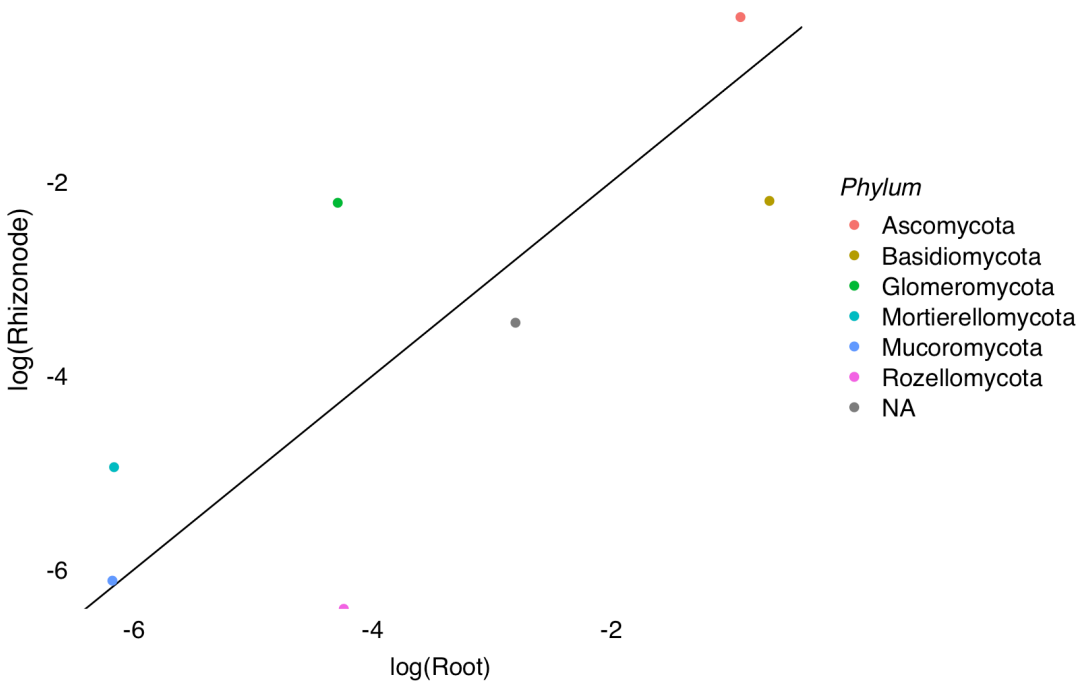

Figure S5. Enrichment of AMF (Phylum Glomeromycota) in root versus rhizonode samples. Log transformed plot comparing root and rhizonode phyla where the 1:1 line represents equal relative abundance of a phylum between sample types. Taxa above the 1:1 line show enrichment in rhizonode samples and sample below the 1:1 line show enrichment in root samples. The gray arrow points to where the AMF fall off the 1:1 line.

Chapter 2:

Mycorrhizal fungi shift plant hormone production and influence plant architecture, physiology, and drought response

Abstract

Biotic interactions are known to have important consequences for plants, however, they are frequently omitted from studies of plant eco-physiology. Root-associated mycorrhizal fungi are ubiquitous and form associations with the vast majority of plant species. Further, mycorhizal fungi are thought to have co-evolved with the first land plants, where mycorrhizal fungi can be thought of as an extension of the plants own rooting system, acting to increase root surface area. While the potential benefits of a nutrient-for-carbon trade between the plant and fungus is clear, the processes involved in the association of plants with this group of fungi is actually quite complex, involving a system of signaling processes and coordination between the plant and the fungi in order to escape their respective immune responses. Interestingly, few studies have investigated the downstream consequences of these interactions for plant physiology, particularly in light of future climatic change. Here we demonstrate that association of a basal gymnosperm (*Sequoia sempervirens*) with root-associated mycorrhizal fungi affects aboveground seedling architecture, photosynthetic demand, responsiveness to increasing concentrations of atmospheric CO_2, as well as stomatal response to drought. Our results highlight that plant association with mycorrhizal fungi, in addition to impacting plant capacity for nutrient acquisition, also implications for other plant processes with many downstream consequences. Further, we highlight the need for more integrative approaches in investigating plant response to the environment.

Introduction

While biotic interactions, particularly with bacteria and fungi, have been shown to alter strategies of nutrient acquisition, defense, and allocation of carbon to root growth, little work has investigated the downstream ecophysiological implications of biotic interactions in plants (Eissenstat, 1992; Lerdau *et al.*, 1994; Smith & Smith, 2011; Friesen *et al.*, 2011; Huang *et al.*, 2018). One of the most ubiquitous biotic interactions is that between plants root-associated arbuscular mycorrhizal fungi (AMF), which form relationships with approximately 70% of vascular plant taxa (Brundrett, 2002). Plant associations with this particular group of mycorrhizal fungi are thought to have enabled the transition of plants to land, coevolving with plants and potentially playing fundamental yet often overlooked roles in their ecophysiological strategies (Brundrett, 2002; Delaux *et al.*, 2015). Given the ubiquity of these interactions and the rate of predicted climatic and environmental change (Loarie *et al.*, 2009), understanding the role of mycorrhizal fungi in basic plant function is paramount to predicting how our ecosystems will respond.

While the majority of tree species interact with AMF, the consequences of these interactions for tree physiology remain poorly resolved. One particularly iconic species, the California coast redwood (*Sequoia sempervirens*, D. Don, Cupressaceae) has been shown to form unique structures, or rhizonodes, that select for AMF, akin to root nodules that house N-fixing bacteria in legumes and *Alnus* (Willing*,* Chapter 1). In conjunction with other root attributes (e.g. the absence of root hairs, evergreen strategy), redwood should demonstrate strong dependence on mycorrhizal fungi (Smith & Smith, 2011; Eissenstat *et al.*, 2015). These attributes place coast redwood in an important context both ecologically and evolutionarily to test how biotic interactions with AMF might impact allocation strategies and ecophysiology. Additionally, as the tallest trees on Earth, these forests represent an important C sink where redwood forests fix and sequester three to five time more CO_2 per unit area than the Amazon rainforest (Van Pelt *et al.*, 2016).

The process of mycorrhization begins with the exudation of plant hormones (Bonfante & Genre, 2010). These plant exudates have been identified as strigolactones, a class of plant hormones first identified for their role in triggering the germination of a group of parasitic plants in the genus *Striga* (Akiyama *et al.*, 2005). The production of strigolactones by plants has been linked to nutrient deficiencies, whereby plants increase strigolactone production in response to both N and P limitation (López-Ráez *et al.*, 2011). Strigolactone production then triggers the germination of mycorrhizal spores and the subsequent mycorrhization of roots, which can improve plant-access to soil nutrients (Smith & Smith, 2011). In contrast with triggering hyphal branching of AMF, strigolactones appear to have the opposite role in plant hosts, suppressing the branching of plant shoots in *Pisum sativum* and *Arabidopsis thaliana* (Gomez-Roldan *et al.*, 2008). While production of strigolactones has been demonstrated in a small subset of predominately model plant organisms to date, a recent study demonstrated strigolactone production in three different gymnosperm species, including Japanese cedar (*Cryptomeria japonica*, D. Don), which like coast redwood, is a member of the Cupressaceae (Yoneyama *et al.*, 2018a,b). The production of these hormones associated with the ancestral mycorrhizal state calls for further investigation into both the potential production in other plant species and the ecophysiological consequences of strigolactone production as it relates to both biotic interactions and plant architecture.

Interestingly, an apparent tradeoff exists between strigolactone and absisic acid (ABA) production as both plant-produced hormones appear to share the same precursor molecule, a β-carotenoid (López-Ráez *et al.*, 2010; Mostofa *et al.*, 2018). ABA is an important plant hormone that signals stomatal regulation in response to declining water availability. While much literature in plant-water relations has focused on xylem vulnerability to embolism, further work is beginning to indicate that plants typically signal for stomatal closure far before reaching these vascular thresholds in order to avoid cavitation (StPaul *et al.*, 2017). Despite their clear capacity to alter strigolactone production and potentially impact ABA production, few studies have explored the role of mycorrhizal fungi in mediating these responses (López-Ráez *et al.*, 2010; Mostofa *et al.*, 2018).

In addition to the potential hormonal and drought-response axes of plant-mycorrhizal interactions, the longtime paradigm regarding these interactions is that mycorrhizal fungi typically improve N and P acquisition by plants (Smith & Smith, 2011). In the context of an atmosphere of increasing CO_2 concentrations, higher susceptibility to drought, and declining fog frequency and consequently water resources for coast redwood (Dawson, 1998; Simonin *et al.*, 2009; Loarie *et al.*, 2009; Johnstone & Dawson, 2010), greater access to nutrients may also play an important role in plant physiological ecology. For instance, increased nutrient uptake might allow for greater investment in photosynthetic enzymes such as RuBisCo, which is often limited by plant access to nutrients (Niinemets *et al.*, 1999; Niinemets & Kull, 2005; Tissue *et al.*, 2010; Zhu *et al.*, 2014). Investment in photosynthetic enzymes is predicted to increase photosynthetic and intrinsic water use efficiency (iWUE), enabling more efficient CO_2 assimilation (Tissue *et al.*, 2010). As such, associations with mycorrhizal fungi may have disproportionate impacts of photosynthesis and iWUE, making these relationships increasingly important in future climate scenarios.

In this paper, we leverage greenhouse experiments to examine the relationship between coast redwood and AMF-symbionts. We predict (H1) that mycorrhizal redwoods will increase shoot branching compared to nonmycorrhizal redwoods, likely as a result of decline in strigolactone production related with alleviation of nutrient deficiency in mycorrhizal seedlings. Further, as ABA is produced via the same pathway as strigolactones, we infer a tradeoff in the production of these two hormones in coast redwood. Accordingly, we predict (H2) that in response to water-deficit, mycorrhizal seedlings will have higher concentrations of ABA leading to greater stomatal regulation. Additionally, we predict (H3) that mycorrhizal seedlings, given the affect of mycorrhization on nutrient acquisition, will have higher photosynthetic capacity than non-mycorrhizal seedlings. Lastly, we predict (H4) that the magnitude of the effect of mycorrhization on photosynthetic capacity will be accentuated with increasing atmospheric CO_2 concentrations.

Methods

Seedling germination

Redwood seeds were donated by CalFire, which sources seeds by provenance. Seeds used in this study were collected from Santa Cruz County, California. To stratify, seeds were first rinsed for 48 hours in a running water bath (DI water) and were then kept in a sealed Ziploc bag at -20°C prior to stratification process. Seeds were allowed to stratify at 4°C in Ziploc bags for 3 weeks prior to germination. After stratification and one week prior to planting, seeds were germinated on 1% water-agar. Seeds were then placed into a growth chamber with a 16-hour light period.

Mycorrhizal treatments

Soil inoculum took one of two forms: control or mycorrhizal. AMF inoculum was purchased from the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM, West Virginia University) and was a 50:50 mix by volume of a *Rhizophagus intraradices* (Accession number: UT126A) and *Glomus "white"* (Accession number: IL205). These cultures were selected due to the relatively high proportion of Glomeraceae found in association with redwood (Willing, Chapter 1). In order to mimic as much of the general soil community as possible while removing only the fungal component, the total inoculum was homogenized and roots were chopped into 2 mm pieces using a sterile razor blade. The total mass was taken and divided into two parts: one portion was retained for the mycorrhizal treatment and placed at 4°C; the other portion for the non-mycorrhizal treatment was passed through a series of sterilization and filtration processes. For the filtration process, one half of the inoculum was filtered through 20 µm mesh in a large filter overnight and then #1 Whatman for about 8 hours (total volume was allowed to filter); the 20 µm mesh removed most species of fungal spores and likely most hyphae. The filtrate was kept at 4°C overnight. The control inoculum was autoclaved for 30 minutes, allowed to sit overnight and then re-autoclaved the next day. The control inoculum was then allowed to dry at 60°C overnight to help reduce any phytotoxic compounds that might have been released through autoclaving process.

Cone-tainers™ were filled with nylon fill to help prevent the loss of large quantities of soil when watering. Next, 10 ml of sterile sand (autoclaved for 40 minutes) were added on top of the pillow-fill to promote drainage, followed by 125 ml of homogenized organic potting soil in each cone-tainer™. This soil was confirmed not to be sterilized prior to purchase, and typical soil microbes (and thus processes of nutrient transformation) were assumed to be present. Though AMF were also likely present in the soil mixture, pilot studies indicated that they were likely in low abundance or had low viability (Willing, unpublished data). This observation was also corroborated where studies of field soils found that in spite of an abundance of spores, only about 6% were viable (McGee *et al.*, 1997). 10 g of bulk inoculum (either sterilized or live for respective treatments) was mixed into potting soil for each treatment. Seedlings were then planted into cone-tainers™ using surface-sterilized forceps. Non-mycorrhizal (NM) treatments were watered with 550 mL of the control inoculum to inoculate the NM treatment with the bacteria associated with the mycorrhizal treatment, but exclude the mycorrhizal spores themselves. Mycorrhizal treatments (M) were watered with the equivalent volume of sterilized DI water. Seedlings were watered twice per week using 10 mL of DI water. Seedlings were fertilized using 10 mL of a dilute 20-20-20 fertilizer solution at concentration of 25 ppm once per week.

Dry-down experiment and assessment of plant response

Approximately 9 months after germination, seedlings were randomized into either well-watered or dry-down treatments. Well-watered seedlings continued with a normal watering regime of 10 mL of water 2 days per week and the typical fertilizing regime was replaced with the standard 10 mL of water to avoid additional fertilization effect. Seedlings were assessed at 0 days (well-watered treatment), 3 days, 5 days, and 7 days without watering. Measurements of stomatal conductance (gsw) were made at maximum photosynthetic capacity conditions (A_{max} of 1200 PAR) and standard LiCor6800 chamber conditions. Leaf area encapsulated by the chamber was

marked using permanent marker and this leaf area was subsequently digitally scanned for determination of leaf area. Leaf area was measured using ImageJ with a scale bar for calibration and leaf area values were adjusted in LiCor to allow for more accurate calculations of gas exchange based on the true leaf area encapsulated by the chamber.

After taking these measurements, water potential was determined on the main stem. Foliage was wrapped in a moistened paper towel and placed directly into the pressure chamber (Scholander *et al.*, 1965). The water potential was recorded in PSI and samples were checked carefully to make sure that water retreated back into stems after measurements to ensure that the water-potential measurements were not artificially inflated. A portion of leaf tissue was then quickly removed from the pressure chamber and placed immediately into 10 mL of methanol-BHT solution on ice for ABA assessment (see below). For the seedlings measured at days 5 and 7 post-watering, the full seedling was then harvested and soils were compared for soil volumetric water-content by comparing fresh to dry mass. Because water-potential measurements were destructive for these seedlings, seedlings assessed at days 5 and 7 after imposing dry-down were fully harvested on those respective days. Seedlings from the day 3 post-watering were kept alive (water-potential was not taken on these seedlings) and harvested the following week due to logistical constraints in sample processing.

Assessment of growth

The harvesting process consisted of a full assessment of plant biomass and allocation. First, total stem height, stem diameter, and total branch length were determined using calipers and branch number was determined. Next, shoots were excised from roots and a small section of roots was removed and stored in EtOH and mass was recorded. The remaining root area was rinsed in DI water, patted dry, and stored at 4°C for determination of specific root length using the grid-intersect method (Giovannetti & Mosse, 1980). Stems were then defoliated, and then total leaf area was scanned for determination of total leaf area using ImageJ2 v2.0.0 (Rueden *et al.*, 2017). The fresh mass of the leaves was taken to assess water content and then leaves were dried at 60°C for >3 days prior to taking dry mass for determination of leaf water content.

Assessment of leaf nutrient content

Dried leaf material from the dry-down experiment was used to assess carbon and nitrogen content and stable isotope ratios. Leaf samples were ground into a fine powder and approximately 7 mg was weighed into tin capsules for assessment using elemental analyzer/continuous flow isotope ratio mass spectrometry at the University of California, Berkeley Center for Stable Isotope Biogeochemistry. This facility uses an Isoprime100 continuous flow mass spectrometer using dual-element analysis mode interfaced with a CHNOS elemental analyzer. Long-term precision for C and N isotope determinations is ±0.10‰ and ±0.20‰, respectively. Isotope values are expressed as a ratio of the heavy to the light stable isotope relative to the standard.

Dried and homogenized samples were also used to determine leaf P content at University of California, Davis Analytical Lab using a closed vessel microwave digestion in nitric acid/hydrogen peroxide and Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES). Long-term method detection limits for P at this lab is 0.01%.

Analysis of ABA

Samples were collected for ABA by cutting approximately 0.15-0.30 g of leaf material with ethanol-sterilized scissors, recording the mass to the nearest 0.001 g, and rapidly placing into 10 mL of methanol-BHT solution on ice. Leaf material was then cut into ~2 mm fragments and placed directly into -20°C prior to overnight shipment on dry ice to Purdue University for analysis based on an established protocol (McAdam & Brodribb, 2014; McAdam, 2015). In brief, organic-phase extraction was used to extract ABA and an isotopically labeled standard was included in each extraction to determine extraction efficiency. Samples were run using ultra-performance liquid chromatography for determination of ion abundance and then sample concentrations were determined based on extraction efficiency and fresh mass of leaf tissue.

A-Ci Curves

A-Ci curves were generated after assessing stomatal conductance at maximum photosynthesis on well-watered seedlings using the automated program in the LiCor 6800. PAR was set to 1200 $\mu M/m^2s$ as these seedlings grow best in lower-light conditions. The seedlings were run through 6 different CO_2 concentration scenarios (5, 150, 250, 400, 700, 1000 ppm) in the automated setup and allowed to come to equilibrium for up to 8 minutes between concentrations. Seedling leaves were marked prior to placement in the chamber as per above and measurements were calibrated based on actual measured leaf area.

Statistical analyses

All statistics were performed in R v3.5.3 (R Core Team) using the ggplot2, dplyr, and lme4 packages (Wickham, 2009; Bates, 2010; Wickham *et al.*). Raw data were checked for normality using Shapiro-Wilk tests and data were transformed when necessary using log or square-root transformations, and then tested again for normality. If transformed data still did not meet assumptions of normality, non-parametric tests were used.

Seedling architecture was compared between mycorrhizal and non-mycorrhizal seedlings using two-tailed Student's t-tests and Wilcoxon signed rank test where necessary (for data that did not meet parametric assumptions). For the dry-down experiment, control (well-watered) plants in both the mycorrhizal and non-mycorrhizal measurements were kept well-watered and assessed throughout the duration of the experiment. To control for potential differences in stomatal conductance intrinsic to each mycorrhizal treatment, the averages of the well-watered control seedlings for their respective mycorrhizal treatments were taken and a response-ratio was then calculated for each of the seedlings in the dry-down treatment relative to the control. In testing for a potential effect of mycorrhization on seedling drought response, measurements were destructive due to the size of the seedlings. As a result, each measurement represents a single individual at a single time point for a given treatment (individual seedlings were not tracked through time). Thus, we considered each time point of the dry-down to be statistically independent and analyzed seedling responses between mycorrhizal and non-mycorrhizal seedlings at each time point using two-tailed Student's t-tests. To test if mycorrhization increased N or P acquisition and $\delta^{13}C$, we compared the two treatments using Student's t-tests. Because the same seedling was used throughout the measurements of the $A\text{-}C_i$ curve, we used a linear-mixed effects approach using the lmer function with main effects of mycorrhizal treatment and CO_2 concentration, an interaction term for mycorrhizal treatment by CO_2 concentration, and random effects of seedling individual (Bates, 2010). Residuals were visually checked to confirm normality.

Results

Mycorrhizal colonization increases shoot branching

While redwood seedlings colonized by AMF showed no significant change in height related to fungal colonization ($T_{1,44}$=0.493, p=0.6244; Fig. 1a), significant increases in shoot branching were observed in the mycorrhizal treatment ($T_{1,26}$=5.0499, p<0.00001; Fig. 1b). Mycorrhizal seedlings also had significantly lower specific root length (W=22, p=0.001), a potential marker of decreased investment in fine roots associated with nutrient acquisition.

Mycorrhization increases ABA concentrations in dry-down resulting in greater stomatal control

Mycorrhizal seedlings exhibited increased ABA concentrations in response to dry-down. Concentrations continued to increase throughout the dry-down experiment becoming significantly higher in mycorrhizal seedlings by the seventh day of dry-down ($T_{1,5}$=2.796, p=0.036; Fig. 2a). While we saw an initial spike in ABA concentrations in non-mycorrhizal seedlings at the onset of water-deficiency, ABA concentrations quickly plummeted to pre-water deficit values for the remainder of the dry-down. This is in contrast to the mycorrhizal treatment where ABA concentrations continued to ramp up throughout the dry-down experiment.

Stomatal response to dry-down mirrored trends in ABA production where mycorrhizal seedlings more tightly regulated stomata in response to water-deficit (Fig 2b). Initially, non-mycorrhizal seedlings demonstrated a decline in stomatal conductance in coordination with the spike in ABA concentrations. However, no further stomatal closure was demonstrated in this treatment following that initial response. This contrasts to the mycorrhizal seedlings which continued to decrease stomatal conductance throughout the dry-down, ultimately attaining significantly lower stomatal conductance by the seventh day of dry-down ($T_{1,5}$= -2.742, p=0.036).

Mycorrhizal seedlings have higher photosynthetic capacity than non-mycorrhizal seedlings

Leaf N content increased by approximately 20% ($T_{1,44}$= 3.7803, p<0.0005) in the mycorrhizal treatment and leaf P increased by approximately 17% ($T_{1,18}$= 1.9828, p=0.0629). This increase in leaf nutrient content did not correspond to significant differences between treatments, but there was a trend of increasing instantaneous photosynthesis in mycorrhizal (A=3.5 μmol/m^2s) compared to nonmycorrhizal seedlings (A=2.9 μmol/m^2s) at ambient concentration of CO_2 ($T_{1,15}$= 1.741, p=0.101). Integrated over time, $\delta^{13}C$ at ambient CO_2 demonstrated modest, but significant differences in photosynthetic efficiency between treatments where mycorrhizal seedlings showed a 1‰ offset from nonmycorrhizal seedlings, incorporating significantly more of the heavy carbon isotope ($T_{1,43}$= 3.706, p<0.001). A linear mixed effects model determined that there was a significant interaction between mycorrhizal treatment and atmospheric CO_2 concentrations where there is an effect of mycorrhization and CO_2 at high (700 and 1000 ppm CO_2), but not low (0—400 ppm CO_2) concentrations of CO_2 on photosynthesis where significantly higher rates of fixation were observed in mycorrhizal compared to nonmycorrhizal seedlings at 700 and 1000 ppm CO_2, but not a ambient and low levels of atmospheric CO_2 (Fig 3; Table 1).

Discussion

While biotic interactions are often ignored in plant physiological ecology, we demonstrate that these relationships can have substantial implications for plant response to the environment, particularly in the face of ongoing and predicted climatic and other environmental change (Loarie *et al.*, 2009). We show how the mycorrhization alters plant hormonal profiles with cascading affects for plant architecture, physiology, and drought response. We also present for the first time, a shoot branching phenotype in association with mycorrhization. While the long-standing paradigm has been that mutualistic relationships with mycorrhizal fungi improve host nutrient uptake, we demonstrate evidence of important shifts in plant architecture, physiology, and hormonal profiles. The characterization of these processes in an ancient gymnosperm has important evolutionary implications for physiology and hormonal processes related to mycorrhization for potentially a much broader range of plants associated with AMF than previously shown.

Mycorrhizal relationships are traditionally regarded for their benefits to plant nutrition (Smith & Read, 2010). In addition to impacting nutrition in host plants, we also present evidence of the potential links between mycorrhization, plant hormone production, and shifts in plant growth and architecture both above- and belowground. It has been established in other plant lineages that 1) strigolactones trigger mycorrhizal germination (Akiyama *et al.*, 2005) and 2) that strigolactones inhibit shoot branching (Gomez-Roldan *et al.*, 2008). Through the changes observed in shoot branching of mycorrhizal versus nonmycorrhizal seedlings, we demonstrate potential phenotypic evidence of these processes occurring simultaneously in coast redwood and highlight the need for further investigation of redwood production of strigolactones and in tree species more broadly. Conifers grow from a single, dominant shoot leader, exhibiting strong apical control (Cline, 1997). As the tallest species on the planet, coast redwood displays potentially the most extreme example of apical control (Noss, 1999). Thus our demonstration that association with mycorhizal fungi reduces apical dominance is particularly intriguing for this host species. In addition, this shift in architecture this might have adaptive implications, particularly for this species where seedlings are typically shaded in a dense understory and investment in shoot branching is likely to enhance total canopy light capture.

Further, we demonstrate how mycorrhization allows for increased ABA production leading to tighter stomatal control of mycorrhizal seedlings in response to water-deficit. Stomatal closure in response to drought is emerging as one of the most important thresholds predicting capacity of species to survive and endure drought (StPaul *et al.*, 2017). While it is clear that ABA plays a critical role in signaling for stomatal closure in response to water deficit, this phytohormone has also been shown to have linkages to plant immunity response and pathogen defense (Asselbergh *et al.*, 2008). Given the prediction of higher incidence and severity of drought in future climate scenarios, this data contributes to a greater body of literature which suggests the importance of inclusion of biotic interactions in plant response to both abiotic and biotic stresses that are predicted to increase in severity with climatic change (Logan *et al.*, 2010; Anderegg *et al.*, 2015; Karst *et al.*, 2015). The multi-faceted nature of ABA and the capacity for ABA concentrations to be altered by mycorrhization calls specifically for further investigation into the connections between ABA, mycorrhization, and biotic interactions more broadly.

Mycorrhizal seedlings also demonstrated greater N and P acquisition and higher photosynthetic capacity than their nonmycorrhizal associates. Further, we demonstrate that the effect size of mycorrhization on redwood photosynthetic capacity is accentuated with increasing

atmospheric CO_2 concentrations. Though mycorrhizal seedlings showed no significant difference in instantaneous photosynthetic rates at ambient levels of CO_2, we demonstrate that mycorrhizal seedlings are able to take better advantage of elevated CO_2, likely due to increased nutrient acquisition and greater investment in photosynthetic enzymes (ie RuBP, NADPH, ATP) (Ågren *et al.*, 2012). Further, $\delta^{13}C$ measurements revealed greater intrinsic water use efficiency in mycorrhizal seedlings at ambient CO_2 highlight potential for greater photosynthetic demand at the cellular level when seedlings were grown in association with mycorrhizal fungi. These findings suggest that plant associations with mycorrhizal fungi may offer not only greater potential for fixation in well-watered conditions, but also potentially allow for more efficient C-fixation (including less water loss) in conditions of water-deficit. This is noteworthy particularly for coast redwood, where fog water and the water subsidies that it represents for this species have been in decline as a result of increasing sea-surface temperatures (Dawson, 1998; Simonin *et al.*, 2009; Johnstone & Dawson, 2010).

Together, our results highlight the importance of the inclusion of biotic interactions in plant physiology and response to climatic and environmental change. Our demonstration of the importance of AMF in plant response to both water-deficit and increasing CO_2 in an ancient conifer species highlights the potential for similar types of responses across a much broader range of host plants than has previously been considered. Future work should further investigate hormonal underpinnings of the changes in physiology observed as a result of mycorrhization, particularly across different plant taxa so that we might be able to build these types of interactions into strategies of restoration, conservation, and models of plant distribution with climate change.

Relevance of work to conservation

Our research shows that mycorrhizal fungi are likely to be increasingly important to redwood growth and physiology with continued climate change. Our findings that AMF increase not only nutrient uptake, but photosynthetic efficiency indicate that restoration efforts should consider inoculating seedlings with AMF prior to planting of seedlings in field as this may help seedlings to establish. Additionally, our findings that mycorrhizal redwoods are better able to regulate water-status in response to dry-down indicate that inoculation of redwood seedlings may also help seedlings to establish in the face of hotter and drier climates predicted along the California coastline. Further, activities that could result in the loss of naturally occurring mycorrhizal inoculum (e.g., loss of contiguous redwood forests) could have important consequences for redwood regeneration. Because our work was limited to seedlings, our inferences related to mycorrhizal function in mature trees (beyond providing inoculum for the next generation of redwoods) are also more limited. However, given the importance or mycorrhizal fungi for seedlings form and function, mycorrhizal fungi could also play an important role in mature tree response to climatic and environmental change, particularly given the shallow and extensive root systems of coast redwood.

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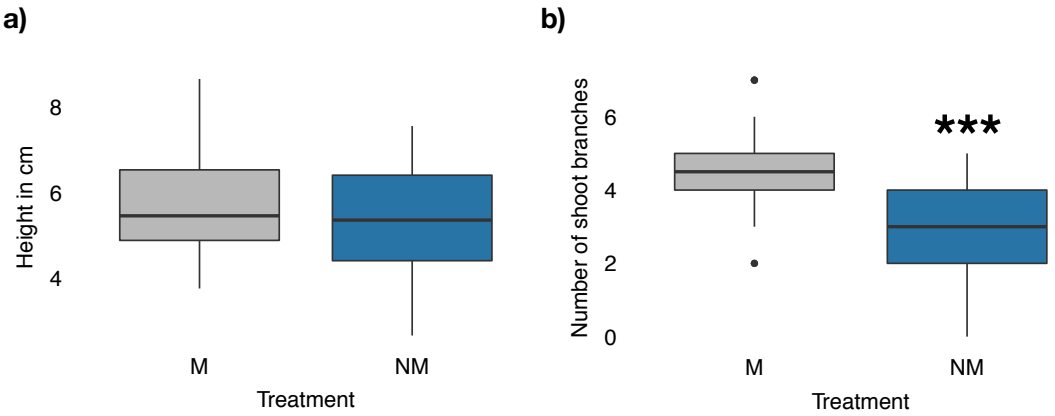

Figure 1. Height (a) and shoot branching (b) by treatment where (M) refers to mycorrhizal vs (NM) non-mycorrhizal redwood seedlings. No significant difference was found in the height of the seedlings at time of measurement, however mycorrhizal seedlings produced significantly more shoot branches by Students t-test t(46.448) =2.838, $p < 0.007$, n=25. Circles represent values that fall outside of the 1.5 x interquartile range (whiskers).

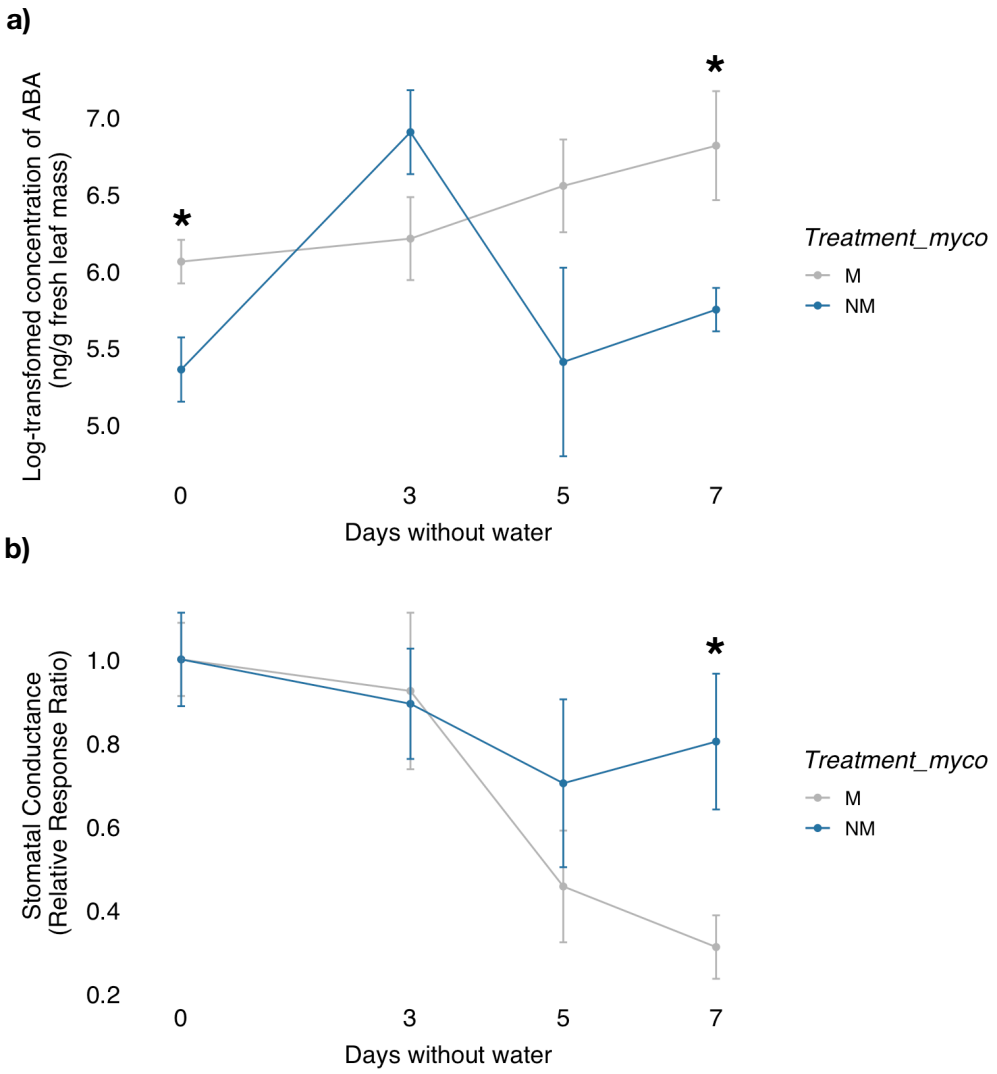

Figure 2. Drought response of mycorrhizal (M) versus non-mycorrhizal (NM) seedlings. (a) Log-transformed abscisic acid (ABA) concentration in seedling foliage measured as ng of ABA per fresh weight of leaf tissue. (b) Mycorrhizal seedlings showed greater stomatal responsiveness to dry-down experiment. Stomatal conductance (mol/m^{2}s) was measured using Licor-6800. For both ABA and stomatal conductance measurements sampling was destructive, so means and standard error bars data were calculated based on unique individuals at each timepoint. ABA data were log transformed to attain a normal distribution and data shown here are the transformed values. Stomatal conductance is presented as a ratio of drought treatment compared to the mean of the well-watered control seedlings. Bars represent SE, $p<0.05$ = (*), n=5.

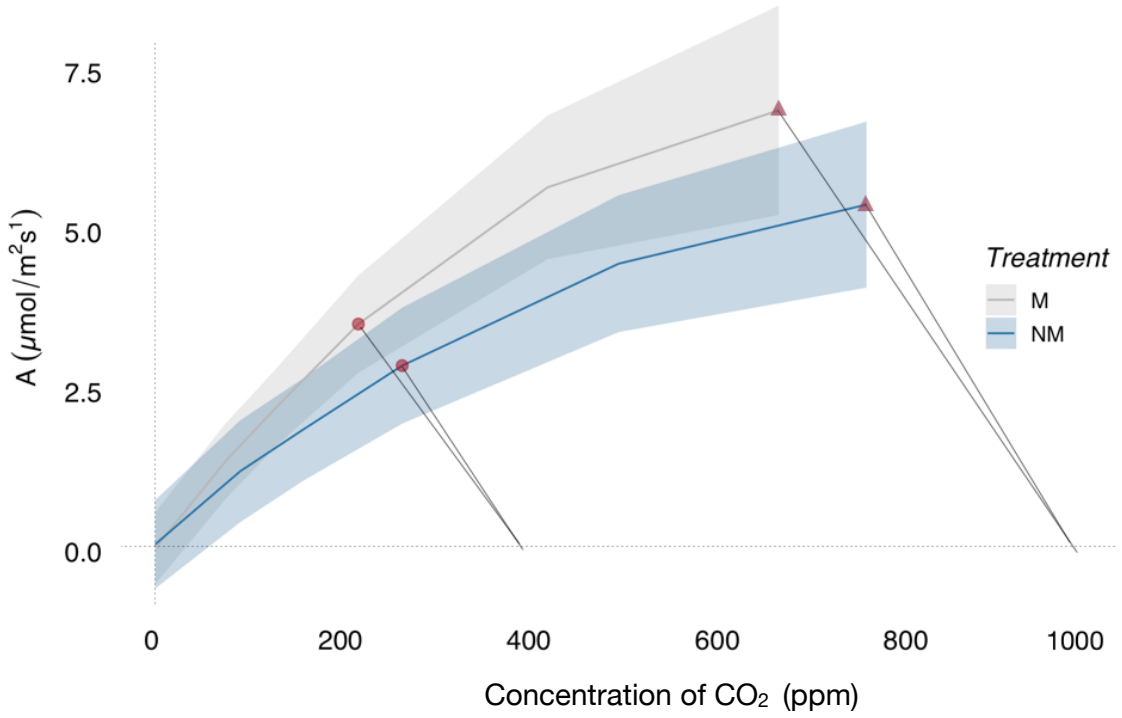

Figure 3. Photosynthetic CO_2 response curve for mycorrhizal (M) versus non-mycorrhizal (NM) redwoods. Photosynthesis (A) is plotted against internal concentration of CO_2 (ppm) measured at increasing chamber CO_2 concentrations (from 0 to 1000 ppm). There was no significant effect of mycorrhizal treatment on photosynthesis, however there was a significant interaction between mycorrhizal treatment and CO_2 on photosynthesis at chamber CO_2 concentration of both 700 and 1000ppm demonstrating that mycorrhizal and non-mycorrhizal seedlings showed significantly different responses in photosynthesis with increasing concentrations of CO_2 (see Table 1 for results from mixed-effects model). Ribbons represent SE. Lines indicate chamber CO_2 concentrations of 400 (circles) and 1000ppm (triangles) compared to measured internal CO_2 concentration of leaves for respective treatments and associated rates of photosynthesis indicating greater draw-down of internal CO_2 concentrations and associated higher rates of photosynthesis in mycorrhizal versus non-mycorrhizal seedlings.

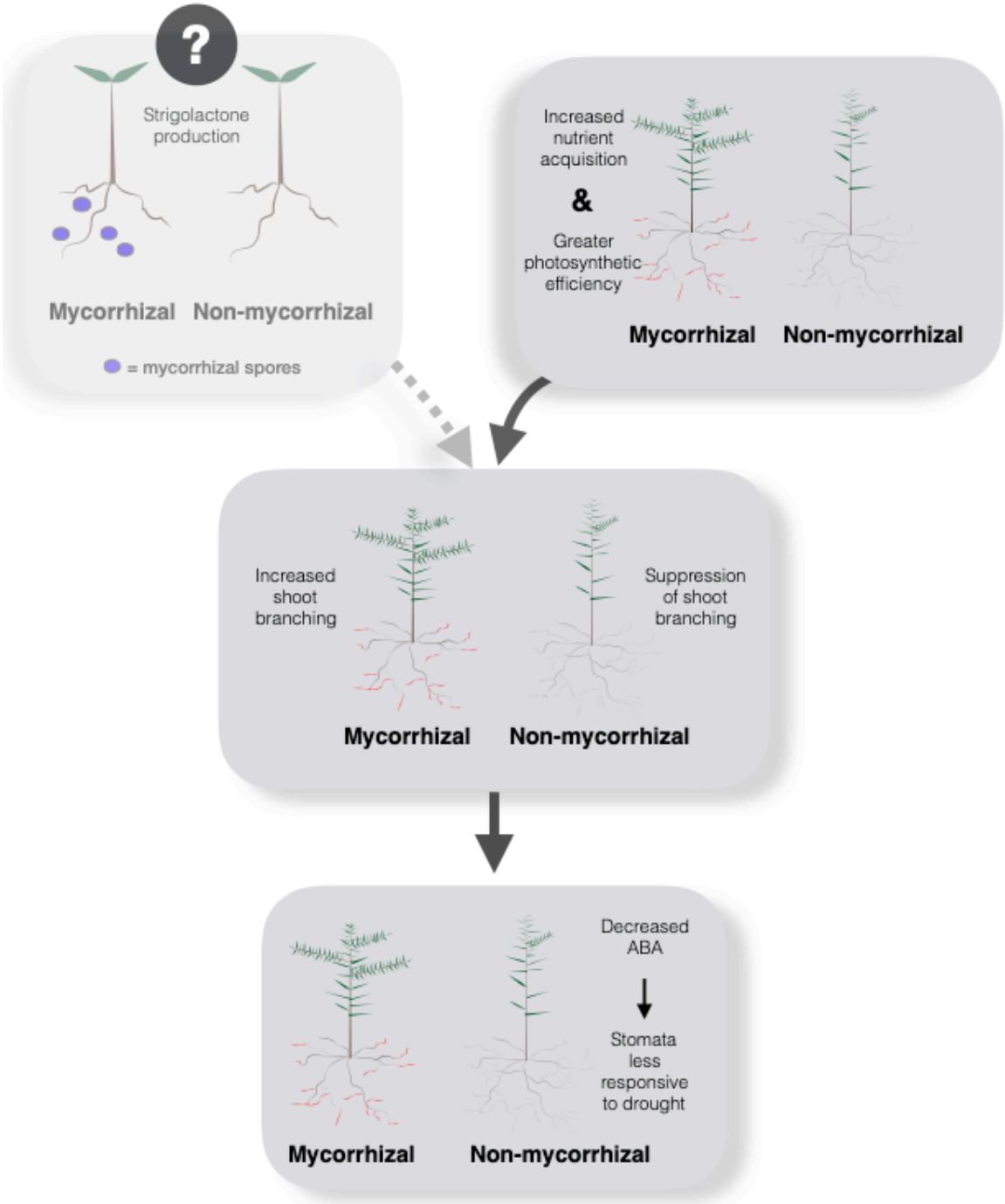

Figure 4. Conceptual figure of how mycorrhization interacts with plant hormone production and nutrient acquisition with cascading effects on plant physiology. We predict that strigolactone production related to mycorrhization underpins our findings of suppression of shoot branching and decreased ABA concentrations in response to drought in nonmycorrhizal seedlings.

Table 1. Results from mixed-effects model for A-C_i curves and respective mycorrhizal treatments. Model indicates that there is no significant main effects of mycorrhiza on photosynthesis, but there is a significant interaction between mycorrhizal status and the two highest atmospheric CO_2 concentrations (700 and 1000ppm). There are significant main effects of CO_2 concentrations on photosynthesis at all values.

Predictors	**Estimates**	**CI**	**P val**
(Intercept)	0.06	-0.60 – 0.73	0.850
Mycorrhiza	0.05	-0.83 – 0.94	0.905
CO_2 (150ppm)	1.35	0.80 – 1.90	**<0.001**
CO_2 (250ppm)	2.21	1.66 – 2.76	**<0.001**
CO_2 (400ppm)	3.50	2.95 – 4.05	**<0.001**
CO_2 (700ppm)	5.64	5.09 – 6.18	**<0.001**
CO_2 (1000ppm)	6.84	6.29 – 7.39	**<0.001**
Mycorrhiza: CO_2 150ppm	-0.21	-0.94 – 0.53	0.579
Mycorrhiza: CO_2 250ppm	-0.41	-1.15 – 0.32	0.276
Mycorrhiza: CO_2 400ppm	-0.70	-1.44 – 0.03	0.064
Mycorrhiza: CO_2 700ppm	-1.25	-1.98 – -0.51	**0.001**
Mycorrhiza: CO_2 1000ppm	-1.53	-2.27 – -0.79	**<0.001**
Random Effects			
σ^2			0.31
$\tau_{00\ SampleID}$			0.60
ICC			0.66
$N_{SampleID}$			18
Observations			108
Marginal R^2 / Conditional R^2			0.832 / 0.942

Chapter 3:

Site-specific water-availability predicts proportion of monoderm bacteria in coast redwood forests

Abstract

Climate change is predicted to increase the frequency and severity of drought. While the role of water-availability in shaping plant community composition is well understood, little is known about the influence of water-availability on bacterial community composition. Recent studies have shown drought in experimental systems results in both the delay in the formation of the bacterial community and the enrichment in monoderm bacteria, or a classification of bacteria based on the presence of a thick, single cell wall layer. Leveraging a naturally existing gradient of water-availability in the coast redwood forests of California, we investigated the role water-availability on bacterial community composition and the potential for similar trends of enrichment in monoderm bacterial taxa with increasing water-deficit in a natural system. Here we report that site-specific water-availability is important to bacterial community composition in both the rhizosphere soil and root environments and that water-availability predicts abundance of monoderm taxa associated with coast redwood. These results highlight that the increasing abundance of monoderm taxa in the rhizosphere in response to drought occurs in both natural and experimental systems and across a broad range of plant-hosts types. While the consequences of interactions with these types of bacteria remain unknown for coast redwood, as increased intensity and severity of drought are predicted in California, we highlight the importance of testing downstream consequences of these shifts in bacterial community composition on plant physiology, nutrient cycling, and ecosystem function.

Introduction

Climate change is predicted to increase the frequency, severity, and duration of droughts (Diffenbaugh *et al.*, 2015). The associated decline in water-availability highlights an important area of research focused on understanding how ecosystems will respond to novel and unprecedented rates of climatic change (Breshears *et al.*, 2005). While much research has focused on above-ground physiological plant responses to water-deficit and how plant communities might respond, adapt, or migrate in response to novel climatic regimes (McDowell *et al.*, 2008; Skelton *et al.*, 2015; Oliveira *et al.*, 2018; Feng *et al.*, 2019), the impact of water-deficit on the rhizosphere and potential downstream consequences are less understood (Naylor *et al.*, 2017; Naylor & Coleman-Derr, 2018; Xu *et al.*, 2018).

The mutualistic and commensal bacterial associations of plants, often referred to as the root microbiome, has been lauded for its role in plant disease resistance (Ryu *et al.*, 2003b; Friesen *et al.*, 2011), increasing plant access to nutrients (Gyaneshwar *et al.*, 2002; DeAngelis *et al.*), and even for impacting plant growth and fitness (Ryu *et al.*, 2003a; Friesen *et al.*, 2011). In particular, the bacterial microbiome associated with roots and the surrounding soil, or the rhizosphere, has been likened in importance and function to the human gut microbiome, shown to have important consequences for macro- and micronutrient absorption and many other downstream processes, including in pathogen defense, photosynthetic capacity, and drought tolerance (Ramírez-Puebla *et al.*, 2013). Further, evidence suggests that the root microbiome may play an important role in plant response to drought (Naylor & Coleman-Derr, 2018; Xu *et al.*, 2018). As such, it is essential to understand the factors that shape rhizosphere bacterial dynamics both in ambient and future climatic conditions so that we might understand some of the negative and positive feedbacks between drought, microbial community composition, and plant physiological ecology.

Given the importance of the microbiome to plant physiology, defense, and acquisition of nutrients and water, it is essential to better understand the role of shifting water-availability on rhizosphere microbiome assembly. While the relationship between water-availability and plant community composition is well-established (Whittaker, 1970), our understanding of the role of water-availability on bacterial community composition is less well-understood, even for those bacterial taxa closely associated with plant hosts. Experimental drought has been demonstrated to delay the development of the root microbiome community and enrich representation of monoderm bacteria in grasses (Barnard *et al.*, 2013; Naylor *et al.*, 2017; Xu *et al.*, 2018). In contrast to diderm bacteria, monoderm bacteria lack an outer membrane and have thicker cell walls, posited to improve their ability to withstand drought. Further, in addition to the anatomical traits that may assist their survival in water-limiting conditions, monoderm taxa have been demonstrated to have positive growth benefits to plant hosts during drought (Mayak *et al.*, 2004; Kohler *et al.*, 2008; Figueiredo *et al.*, 2008; Xu *et al.*, 2018). Yet in spite of the potential benefits of the root microbiome under water-limiting conditions, studies investigating the response of rhizosphere microbial communities to water-limitation have predominately been focused on grasses and agricultural systems (Barnard *et al.*, 2013; Naylor *et al.*, 2017; Xu *et al.*, 2018). Given the increase in drought frequency and severity with climate change (Loarie *et al.*, 2009; Diffenbaugh *et al.*, 2015), there is an urgent need to understand the role of water-deficit in natural and forested systems on rhizosphere microbial communities and the potential functional consequences for plants and ecosystems (Breshears *et al.*, 2005).

The iconic California coast redwood (*Sequoia sempervirens*) traverses a strong gradient of water-availability where the north-most part of the redwood range receives twice the annual rainfall as southern extent of the range, but where fog plays variable importance in local water-availability (Dawson, 1998; Noss, 1999; Johnstone & Dawson, 2010). Additionally, in spite of this dramatic shift in water-availability, coast redwood is dominant tree species throughout its range, contributing a mean basal stem area of 97% throughout a network of established plots across the range (Van Pelt *et al.*, 2016). Together, the climatic and biotic characteristics of the redwood forest make it an ideal natural laboratory to investigate the role of site-specific water-availability on the rhizosphere microbiome.

Here we predict (1) that lower site-specific water-availability is an important predictor of bacterial community composition for both roots and rhizosphere soils. Additionally, using Actinobacteria as our case study of monoderm bacterial taxa, we predict that (2) that redwood roots from sites with lower site-specific water-availability will demonstrate greater abundance of Actinobacteria, and (3) that because monderm bacteria are thought to be able to withstand drought better than their diderm counterparts as a result of their anatomy, that rhizosphere soils will also demonstrate greater abundances of Actinobacteria taxa.

Methods

Site descriptions: The geographic range of *S. sempervirens* spans from Big Sur, CA through just north of the Oregon boarder (36.08°N, 121.60°W to 41.77°N, 124.11°W) in a narrow band characterized by mild temperatures and coastal fog water inputs. For our study, eight sites spanning this range were sampled in a nested design based a plot network previously established by the Redwoods and Climate Change Initiative (RCCI) as per Willing, Chapter 1. Thirty-year average climatic data were extracted using plot coordinate locations from the Basin Characterization Model, which has spatial resolution at the 270 m scale (Flint & Flint, 2014). Mean annual precipitation at these sites ranged from 720 to 2210 mm per year and maximum temperatures ranged from 16.5°C to 21.21°C (for further descriptions of climatic data for the plots, see Willing, Chapter 1).

Sampling description: Sixteen soil cores (15 cm deep and 8 cm diameter) were collected at each site between September and October 2015 at the end of the dry season in these forests. For further details, please refer to Willing, Chapter 1.

Sample preparation and DNA extractions: Soil cores were sieved to 2 mm and 0.25 g of soil were collected and put directly into MoBio Power Soil kit buffer. From the same core, 0.70 g of fine roots from coast redwood were collected, washed in distilled water 5 times, and frozen at -80°C until extractions could be carried out. Soils were immediately processed according to manufacturer's instructions and soil DNA was stored at -80°C. Roots were ground in liquid N using mortar and pestle and immediately transferred into MoBio Powersoil buffer. DNA was then extracted according to manufacturer's instructions. Remaining soil was kept and bench-dried for subsequent soil chemical analyses.

Soil chemistry: Though many studies of similar design often pool samples for soil chemistry to analyze them at the plot level, we chose to analyze soil from each individual soil core independently, which provided more fine-scale resolution of environmental tolerances and

avoided pseudoreplication. Total carbon and nitrogen content of soils were assessed by grinding using mortar and pestle, weighing them into tin capsules, and running them on the CE Instruments NC 2100 Elemental Analyzer (Rondana, Milano, Italy) at the University of California, Berkeley. All other soil chemical analyses were completed at the University of Massachusetts, Amherst using protocols for routine soil analysis as per Willing, Chapter 1. Soil chemistry obtained includes soil pH, total P, K, Ca, Mg, Zn, B, Mn, Cu, Fe, Pb, Al, Na, and S. In addition to these measurements, an additional field campaign to the north and south-most redwood sites was completed in August 2018 to assess N isotope ratios. Using the same study trees and soil core-sampling methods as were used for DNA analyses, soils and redwood roots were collected trees. Additionally, roots from neighboring *Oxalis oregona* plants were also collected using the corer. Soil and root material was dried at 60°C for >3 days prior to grinding and weighing into tin capsules for analysis using elemental analyzer/continuous flow isotope ratio mass spectrometry at the University of California, Berkeley Center for Stable Isotope Biogeochemistry. This facility uses an Isoprime100 continuous flow mass spectrometer using dual-element analysis mode interfaced with a CHNOS elemental analyzer. Long-term precision for C and N isotope determinations is ± 0.10‰ and ± 0.20‰, respectively. Isotope values are expressed as a ratio of the heavy to the light stable isotope relative to the standard.

Molecular identification of species: DNA concentrations for each sample were quantified using Nanodrop and diluted to 10ng/µL for each sample before PCR amplification. Samples were randomized within 3 96-well plates. The 16S rRNA region was sequenced using dual-indexed primers for the V3-V4 region (341 F 341F, 5'-CCTACGGGNBGCASCAG-3' and 785 R, 5'-GAC TACNVGGGTATCTAATCC-3') and reaction conditions followed established protocols (Coleman-Derr *et al.*, 2015; Naylor *et al.*, 2017). Sequencing data were processed using a previously developed pipeline (Coleman-Derr *et al.*, 2015).

Statistics

All statistical analyses were performed using R version 3.5.3 (R Core Team). Phyloseq was used to important metadata and OTU table and for preliminary filtering and sorting of data where samples were rarified to 5000 reads (McMurdie & Holmes, 2013). After rarefication, 116 and 125 out of 128 original samples were retained for roots and rhizosphere soils respectively. Data were visually assessed using PCoA analysis in Phyloseq.

To test our first hypothesis (H1) that site-specific water-availability was an important factor shaping bacterial community composition across sites, we used generalized-dissimilarity modeling (Manion *et al.*) and paired this analysis with more traditional matrix modeling and confirmed similar biological interpretations from both models (Wang, 2013). Climatic and environmental variables determined to be significantly correlated with community composition from our models were then further tested using Mantel tests to compare the relationship between these individual parameters and community dissimilarity (Goslee & Urban, 2017). We included the same climatic and environmental variables in our models as with a previous study investigating the fungal community composition for the same samples (Willing, Chapter 1) including actual evapotranspiration (AET), which was used as a proxy for site-specific water-availability (30-year average prior to year of sample collection). While a number of metrics for site-specific water-availability, including climatic water-deficit (CWD), were explored, AET was determined to have the greatest explanatory power for this data. While AET and CWD

theoretically represent a reciprocal relationship to one another, they are calculated differently, where CWD is calculated based on the difference between precipitation and evaporative potential, where AET better accounts for occult water inputs, such as fog water (Flint & Flint, 2014). Given the importance of fog in the redwood system where fog subsidies can represent up to 33% of the water budget for redwoods, it was determined that AET was the best metric to determine site-specific water-availability (Dawson, 1998; Simonin *et al.*, 2009; Johnstone & Dawson, 2010).

To test our hypotheses (H2 and H3) that site-specific water-availability was correlated with enrichment in monoderm bacteria in roots and soils respectively, we specifically extracted the abundance of the Phylum Actinobacteria (monoderm taxa) with respect to all other taxa across sites. We chose to investigate these patterns using Actinobacteria as a case study as this phylum has been shown to contain to be predominated by monoderm taxa and was abundant across our samples relative to other monoderm-including taxa (Xu *et al.*, 2018). Using the lme4 package, we analyzed the relative abundance of Actinobacteria using a linear mixed-effects model including main effects of AET and random effects of tree individual nested within site (Bates, 2010). Additionally, indicator species analysis was used to test which taxa were indicators of low, medium, and high AET (Dufrêne & Legendre, 1997). AET data was binned into 3 different bins (500 mm and less, 500 mm to 600 mm, and 600 mm and up for low, medium, and high AET, respectively). The relative proportion of monoderm to diderm indicator taxa were then compared across these bins of site-specific water-availability.

Results

Site-specific water-availability is important to redwood bacterial community composition

Water-availability (AET) and soil pH were both determined to be important factors in community composition of bacteria associated with both redwood roots and rhizosphere soils (Fig.1; Tables 1 & 2). While we also show clear separation between redwood roots and rhizosphere soils in terms of their bacterial composition, root and rhizosphere soil community composition showed similar overall trends shifting composition across sites (Fig. 2). Interestingly, across this ~800 km gradient, geographic distance between samples was a significant predictor of community composition in rhizosphere soils, but was not determined to correlate significantly with shifts in root community composition at this scale.

Phylogenetically, redwood roots were dominated by Proteobacteria and Actinobacteria (Fig. 3a). Rhizosphere soils were colonized by a more diverse array of dominate phyla which included Acidiobacteria, Bacteriodetes, Proteobacteria, and Chloroflexi (Fig. 3b). Interestingly, we also found a number putative N-fixing taxa associated with redwood roots throughout the redwood range. One of the top indicator species associated with redwoods roots compared to rhizosphere soils was a *Rhizobium* species (IV= 0.923, p=0.001; Table S1), and we additionally found a number of *Bradyrhizobium* and Frankiaceae). Natural abundance δ15N for redwood rhizosphere soils had a mean of 0.97± 0.73‰ and redwood roots demonstrated a mean of -1.66± 0.91‰ compared to roots of the reference plant, which demonstrated a mean of -2.12± 1.12‰ (100% of N procured via biological N-fixation would have a value of 0‰).

Higher site-specific water-availability corresponds to low relative abundance of monoderm taxa associated with redwood roots

Lower water-availability corresponded to higher proportion of Actinobacteria found in association with redwood roots ($p=0.0035$; Fig. 4a and Table 3). Further, we used indicator species analysis to determine which taxa were indicators of low, medium, and high AET. We found that these trends held, where sites with lower site-specific water-availability had a much higher proportion of indicator species that were identified as Actinobacteria (approximately 30%) compared to sites with medium (approximately 9%) and high (approximately 9%) site-specific water-availability (Fig. 5).

Rhizosphere soils demonstrate no trend in monoderm relative abundance with respect to site-specific water-availability

In contrast to our findings in redwood roots, rhizosphere soils do not show a significant trend in the relative abundance of Actinobacteria with respect to site-specific water-availability (Fig. 4b, Table 5). Relative abundances of Actinobacteria were also on average lower for rhizosphere soils compared to roots.

Discussion

Our data highlight that site-specific water-availability can have important consequences for the bacterial community associated with the roots of an ancient and iconic tree species. In particular, we demonstrate enrichment of monoderm taxa associated with declining water-availability in a forest system. While previous work has shown that experimental drought in agricultural systems selects for monoderm taxa, we present the first evidence of similar results in a natural system, in this case in association with an ancient conifer species (Barnard *et al.*, 2013; Naylor *et al.*, 2017; Naylor & Coleman-Derr, 2018; Xu *et al.*, 2018). Further, our data suggest that the impacts of site-specific water-availability on bacterial community composition persist across annual scales of water-availability. While the consequences of a more monoderm-dominated rhizosphere remain to be explored for coast redwood, the increasing intensity and severity of drought predicted as a result of climate change necessitates further research into the potential downstream consequences of these shifts in bacterial community composition on plant physiology, nutrient cycling, and ecosystem function.

Site-specific water-availability is important redwood bacterial community composition

Our data demonstrate the importance of both soil pH and site-specific water-availability in the shifts in redwood root and rhizosphere soil bacterial community composition. Soil pH has previously been established as an important factor for bacterial community composition (Fierer & Jackson, 2006). The implications of drought on microbial community composition associated with roots have recently been demonstrated across short timescales and in managed, agricultural systems; however, few studies have examined the role water-availability in natural systems on bacterial community composition (though see (Barnard *et al.*, 2013). Natural ecosystems are becoming increasingly subject to both drought and higher temperatures as a result of climate change and therefore consideration of how these ecosystems might respond to future climatic regimes is essential. We demonstrate that future climates may impact the process of microbial community assembly and will be important to take into consideration while discussing both management and mitigation strategies in forest systems.

Distance-decay in similarity was found to be important in soil, but not root community composition in this study. This is an intriguing result as the geographic distance between samples

would typically be expected to be one of the primary factors correlated with shifts in the bacterial community associated with roots (Horner-Devine *et al.*, 2004; Martiny *et al.*, 2011). We posit that this may be a result of the relatively large range in both soil pH and site-specific water-availability with respect to a relatively small geographic distance. While geographic distance was found to be the best predictor of community similarity of fungi associated with root and rhizosphere soils across this same experimental design (Willing*,* Chapter 1), similar patterns have demonstrated previously where fungi appear to be more responsive to geographic distance than bacteria (Coleman-Derr *et al.*, 2015). One potential explanation is that bacteria are much smaller in size than fungi and may disperse across greater geographic distances more easily, thus demonstrating patterns of distance-decay across different geographic scales. Additionally, bacteria live and grow in association with roots in a different manner than fungi and have different physiological requirements (Philippot *et al.*, 2013). Alternatively, the phylogenetic resolution of bacterial versus fungal primers differ, where bacterial primers are designed to sequence the 16s rRNA region whereas the fungal primers used in the former study sequence the ITS-region (Willing*,* Chapter 1). Because the ITS region is more variable than the 16s region, there is greater phylogentic resolution for ITS (Bruns & Taylor, 2016). As a result, bacterial communities in redwood roots may actually differ across geographic distance, but perhaps our phylogenetic resolution was not fine enough to detect fine-scale changes in species-level compositional shifts that were more reliably detected using fungal-specific methods.

Additionally, we were surprised to find a number of putatively N-fixing bacteria in association with redwood roots. In addition to identifying *Rhizobium* as one of the top species indicating root versus a rhizosphere soil samples, we found a number of *Bradyrhizobium spp.,* one species of *Frankia,* and a number of other Frankiaceae associated with redwood roots. While previous studies have demonstrated that non-symbiotic *Bradyrhizobium spp.* dominate forest soils (these taxa lack the *Nif* gene required for fixation) (VanInsberghe et al., 2015), preliminary data suggest that the redwood roots indeed harbor the *Nif* gene (Willing *et al, in prep)*. In addition, natural abundance nitrogen stable isotope values of redwood roots and soils sit at a range quite plausible for potential N-fixation. Further, redwoods have previously been demonstrated to form rhizonodes, or specialized root structures that house arbuscular-mycorrhizal fungi (Willing, Chapter 1). Given the parallels in molecular signaling between plants and symbiotic arbuscular mycorrhizal fungi and nodule-dwelling N-fixing bacteria (Bonfante & Genre, 2010), we highlight the possibility of N-fixation in redwood forests as the subject of future investigations. The potential for N-fixation in non-leguminous plants and even in association with conifer needles has recently emerged as important gap in our understanding of N-cycling (Moyes *et al.*, 2016) and here we present an additional system in which to test the potential for symbiotic N-fixation in previously unseen plant hosts.

Higher site-specific water-availability corresponds to low relative abundance of monoderm taxa associated with redwood roots

Our results indicate that Actinobacteria are enriched in sites with lower site-specific water-availability. While previous studies have demonstrated enrichment of monoderm taxa associated with grasses over short-term water deficit (Barnard *et al.*, 2013; Naylor *et al.*, 2017; Xu *et al.*, 2018), our data highlight a similar phenomenon in a long-lived tree species in response to annual water-availability. While the mechanisms at play remain to be elucidated, previous studies have highlighted three important axes by which monoderm taxa might experience positive selection

pressure under drought. First, monoderm taxa have thicker cell walls than their diderm counterparts, which may enable them to withstand arid conditions more effectively. Second, in *Sorghum bicolor*, shifts in plant metabolism under drought have been shown to positively select for monoderm bacterial taxa (Xu *et al.*, 2018). Lastly, monoderm taxa have also been demonstrated to have growth benefits to *S. bicolor* under drought, which may result in positive feedbacks for the abundance of monoderm taxa (Xu *et al.*, 2018). As our data indicate that these differences in bacterial community composition persist even across annual, site-specific water-availability, similar mechanisms might be at play for a diversity of plant lineages. Further research should investigate the role of drought in root microbiomes across ecosystems and plant lineages to better understand the generalizability of these results to other plants and ecosystems.

These results also bring to light questions about what drought might mean for forests in the future. With shifts towards a more monoderm-dominated root community, it is possible that there may be important implications for plant physiology, nutrient cycling, or even plant competition. In the context of climatic change, it is important to consider not just the physiological adaptation of plants to drought, but also the role of biotic interactions that might influence plant growth, competition, and tolerance of novel climatic regimes.

Rhizosphere soils demonstrate no significant trend in monoderm relative abundance with respect to site-specific water-availability

Interestingly, while we expected to see a positive feedback of increased relative abundance of monoderm taxa in roots on rhizosphere soil community composition, we did not find this trend to be significant. In general, monoderm taxa were found in lower relative abundance in rhizosphere soils compared to root samples, potentially indicating that these taxa have more specialized habitats within the root themselves. As opposed to fungal taxa that colonize both the root and rhizosphere soil simultaneously (Philippot *et al.*, 2013), bacterial taxa are perhaps more specialized and thus different trends may be expected between these two habitats. Alternatively, AET may be a better predictor of plant (and thus root) water-availability, but a poorer predictor of soil water-availability. Soil water-holding capacity is dependent on a number of characteristics of the soil itself, including clay content and organic matter that may be poorly predicted at the site-level (Jong *et al.*, 1983). While the trend specifically of monoderm relative abundance associated with aridity was not significant, we did demonstrate that the total bacterial community composition of rhizosphere soils was strongly correlated with water-availability. This suggests that there are indeed rhizosphere-dwelling taxa that exhibit a range of tolerance to water-availability, but that perhaps we lacked resolution to detect this within the specific group of monoderm taxa.

The findings reported here also have potential implications for management and conservation. Though further research is required to better understand the physiological consequences of redwood association with monoderm taxa in conditions of increasing water-deficit, it is clear that there may be some benefit of amending certain host plants with monoderm bacterial taxa in drought. However, our results indicate that monoderm taxa may be outcompeted in the rhizosphere even in drought conditions. Careful assessment of different forms of inoculation of plant hosts is therefore necessary in the consideration of the implications of monoderm taxa on plant growth under drought. Further, the specific timing of potential inoculation should also be considered, where the most effective inoculation would likely involve

targeting times of both active root growth and drought stress to best assist potential inoculum in reaching host roots.

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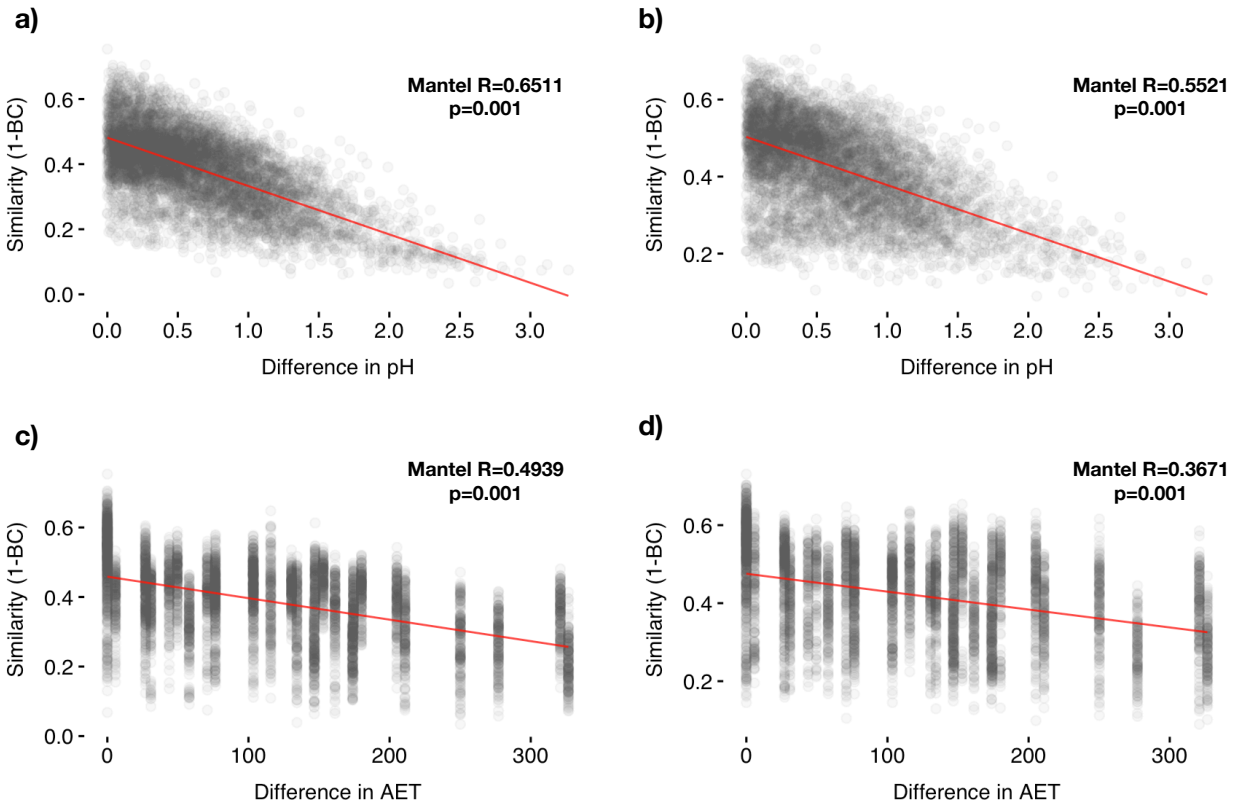

Figure 1. Compositional variance of bacterial communities across factors significantly associated with bacterial community composition. Community similarity (1-Bray-Curtis) versus actual evapotranspiration for (a) soil bacterial communities and (b) root bacterial communities; dots are transparent, so darker colors indicate higher density of samples. Similarity (1-Bray-Curtis dissimilarity) versus soil pH for (c) soil bacterial communities and (d) root bacterial communities; dots are transparent, so darker colors indicate higher density of samples. Mantel coefficients are reported for each plot individually and red line indicates regression trend. Plots are based on rarefied data for roots (n=116) and soils (n=125).

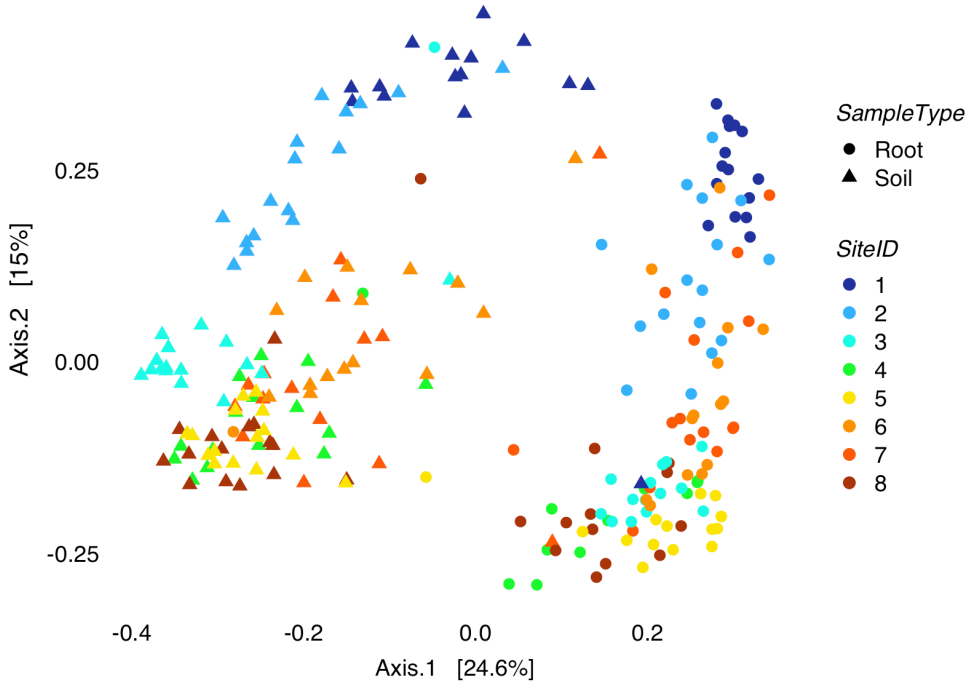

Figure 2. Bacterial community composition across sites and sample type. Ordination based on principle coordinates analysis using Bray-Curtis dissimilarity for total bacterial community of roots and soils. Samples were rarefied to 5000 reads per sample and are colored by site of origin (north to south). Shapes denote sample type (either root or soil). After rarefaction, 116 root samples were retained 125 soil samples were retained out of the original 128 respective samples originally collected and sequenced.

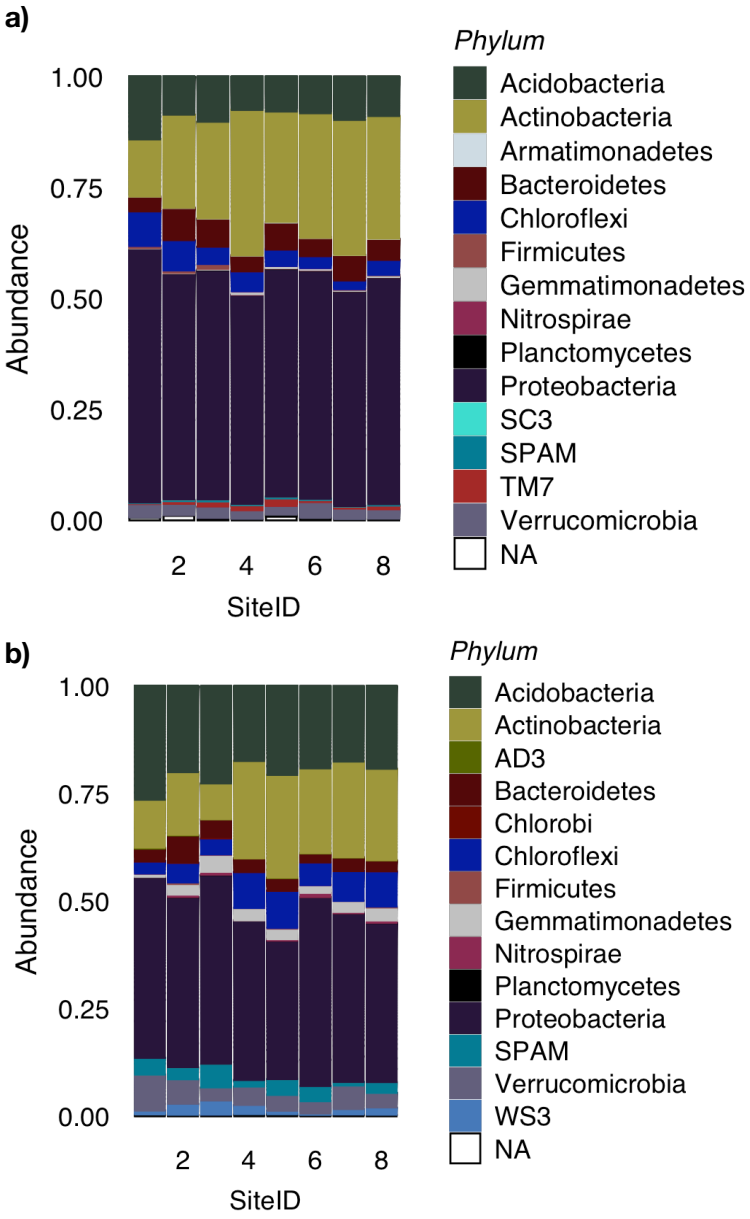

Figure 3. Bacterial taxonomic composition across sites at the phylum level for roots (a) and soils (b). Samples were rarefied to 5000 reads and are shown here as the relative abundance of the top 500 OTUs by site. After rarefaction, 116 root samples were retained 125 soil samples were retained (out of 128 originally sampled).

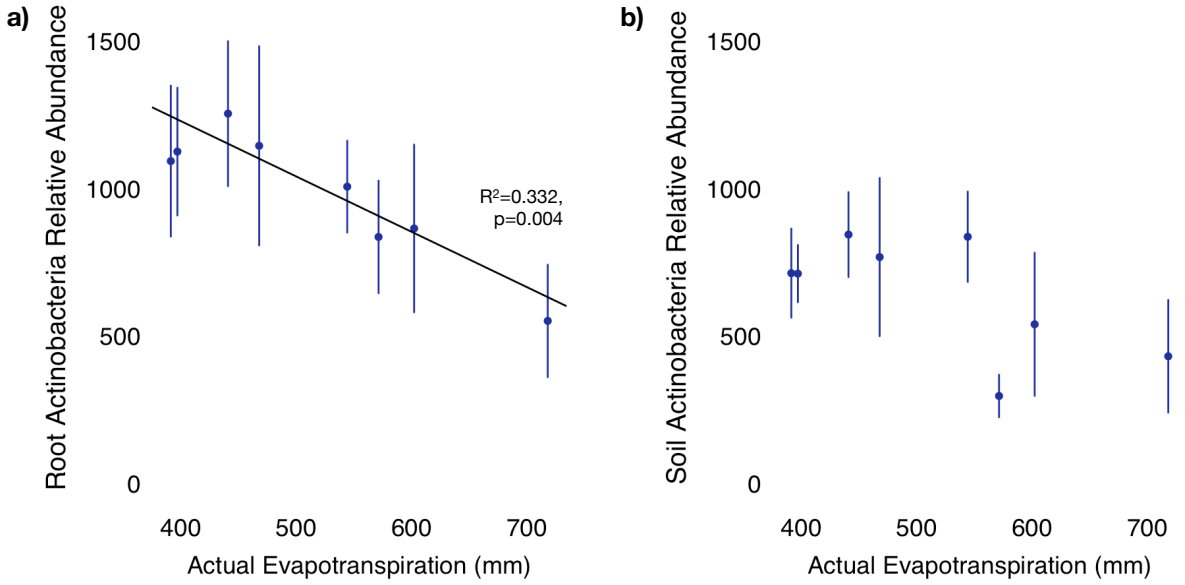

Figure 4. Monoderm bacteria (Actinobacteria) relative abundance by AET. (a) Relative abundance of Actinobacteria in roots and (b) relative abundance of Actinobacteria in soils. Samples were rarefied to 5000 reads. After rarefaction, 116 root samples were retained 125 soil samples were retained (out of 128 originally sampled). The top 500 OTUs were selected by site and the relative abundance of each bacterial phylum was then determined. Dots represent mean relative abundance by plot and bars represent the standard error.

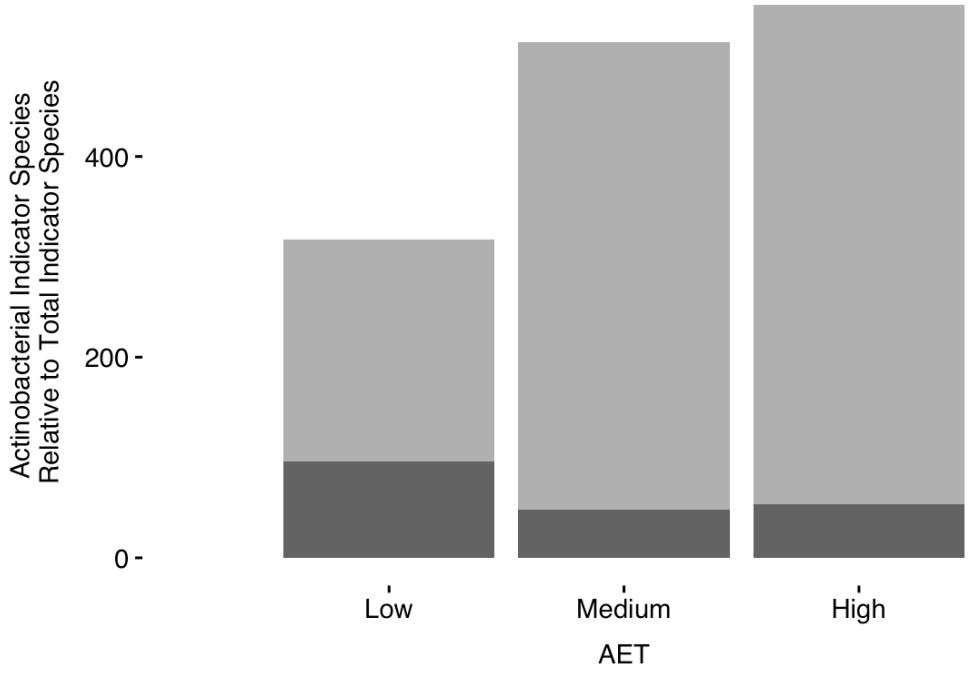

Figure 5. Proportion of indicator species in roots identified as monoderm bacterial taxa (Actinobacteria—dark gray) across low, medium, and high Actual Evapotranspiration (AET) compared to other indicator species taxa (light gray) across by AET. AET for sites was binned as low (500mm or less), medium (500mm-600mm), and high (600mm and up).

Table 1. Results from Multiple Matrix Regression for Roots and Soils (MMRR) predicting bacterial community composition of roots (a) and soils (b) based on geographic and climatic factors.

a)

Factor	Coeff	p val
Distance	0.0000003	0.992
Soil pH	**0.1176276**	**0.001**
Soil CN	0.0006360	0.619
Soil P	-0.0004201	0.140
AET	**0.0003169**	**0.001**
	Model R^2=0.3678; p=0.001	

b)

Factor	Coeff	p val
Distance	**0.0000610**	**0.002**
Soil pH	**0.1234230**	**0.001**
Soil CN	-0.0007766	0.438
Soil P	0.0002785	0.227
AET	**0.0003688**	**0.001**
	Model R^2=0.5489; p=0.001	

Table 1. Results from GDM reported after backwards selection retaining only significant parameters predicting bacterial community composition of roots (a) and soils (b) based on geographic and climatic factors.

a)

Factor	Coeff	p val
Soil pH	**63.18541**	**0.00**
AET	**10.77209**	**0.00**
Deviance explained=45.83%; p=0.00000		

b)

Factor	Coeff	p val
Distance	**4.101646**	**0.00**
Soil pH	**42.624027**	**0.00**
AET	**8.673576**	**0.00**
Deviance explained=57.24%; p=0.00000		

Table 3. Summary from linear mixed-effects models for roots (a) and soils (b) comparing the relative abundance of monoderm taxa (Actionbacteria) by site with main effects of long-term average Actual Evapotranspiration and random effects of specific tree sampled nested within site. Results are base on relative abundance calculated from the top 500 OTUs.

a)

Predictors	Estimates	CI	P val
(Intercept)	1979.20	1570.22 – 2388.18	**<0.001**
AET	-1.88	-2.65 – -1.11	**0.004**
Random Effects			
σ^2			69322.57
$\tau_{00\ Tree:Site}$			3702.63
$\tau_{00\ Site}$			8160.87
ICC			0.15
N_{Tree}			4
N_{Site}			8
Observations			114
Marginal R^2 / Conditional R^2			0.332 / 0.430

b)

Predictors	Estimates	CI	P val
(Intercept)	1240.49	677.37 – 1803.60	**0.005**
AET	-1.16	-2.23 – -0.09	0.077
Random Effects			
σ^2			28863.64
$\tau_{00\ Tree:Site}$			3711.58
$\tau_{00\ Site}$			23803.62
ICC			0.49
N_{Tree}			4
N_{Site}			8
Observations			122
Marginal R^2 / Conditional R^2			0.211 / 0.596

Table S1. Indicator species analysis of root compared to soil samples. The top 10 are reported for root soil samples respectively.

Sample	Indica. Value	P val	Phylum	Class	Order	Family	Genus
Root	0.952	0.001	NA	NA	NA	NA	NA
Root	0.923	0.001	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium
Root	0.919	0.001	Actinobacteria	ActinobacteriaPH	Actinomycetales	NA	NA
Root	0.917	0.001	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	NA
Root	0.907	0.001	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	NA
Root	0.891	0.001	Bacteroidetes	Sphingobacteria	Sphingobacteriales	NA	NA
Root	0.882	0.001	Actinobacteria	ActinobacteriaPH	Actinomycetales	Streptomycetaceae	Streptomyces
Root	0.879	0.001	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Agrobacterium
Root	0.878	0.001	Bacteroidetes	Sphingobacteria	Sphingobacteriales	NA	NA
Root	0.868	0.001	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	NA
Soil	0.95	0.001	Gemmatimonadetes	GemmatimonadetesPH	Gemmatimonadales	NA	NA
Soil	0.946	0.001	Proteobacteria	Betaproteobacteria	Rhodocyclales	NA	NA
Soil	0.933	0.001	Gemmatimonadetes	GemmatimonadetesPH	Gemmatimonadales	NA	NA
Soil	0.93	0.001	Proteobacteria	Betaproteobacteria	Rhodocyclales	NA	NA
Soil	0.925	0.001	SPAM	SPAMPH	SPAMPH	SPAMPH	SPAMPH
Soil	0.904	0.001	Acidobacteria	NA	NA	NA	NA
Soil	0.904	0.001	WS3	WS3PH	WS3PH	WS3PH	WS3PH
Soil	0.904	0.001	Proteobacteria	Betaproteobacteria	Rhodocyclales	NA	NA
Soil	0.898	0.001	Acidobacteria	RB25	RB25CL	RB25CL	RB25CL
Soil	0.896	0.001	Proteobacteria	Betaproteobacteria	Rhodocyclales	NA	NA