Biotic and abiotic drivers of plant symbionts determine plant performance, the maintenance of diversity, and response to global change

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DEDICATION

To my family and friends.

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ABSTRACT

Interactions among organisms regulate the structure and function of ecosystems and the response of ecosystems to global change. The outcome of species interactions is shaped by the partners involved in the interaction and the climate contexts of the systems in which they reside. Global change is altering the distributions of organisms as well as the climate contexts of the systems they reside within, shifting the biotic and abiotic factors shaping species interactions. Thus, predicting the response of ecosystem structure and function to global change remains unresolved. For my dissertation, I explored how the interactions among plants and their mutualistic communities alter individual plant, community, and ecosystem function and how these interactions are shaped by changing biotic and abiotic factors. To do this, I combined a series of experiments and observations across scales, from detailed root-microbe experiments in laboratory mesocosms to coupled observations and experiments conducted along elevation gradients. Overall, my work provides mechanistic insights on how microbial community composition shapes the morphology and physiology of plant hosts, how plant community composition shapes the structure and function of microbial communities, and how abiotic and biotic contexts shape fungal endophyte assemblages at global scales.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Background and key questions

Interactions among organisms influence a range of ecological patterns and processes from community assembly (Hausmann & Hawkes 2010; HilleRisLambers *et al.* 2012) to ecosystem function (van der Heijden *et al.* 1998; Wardle *et al.* 2004). The outcome of these interactions can be shaped by the partners involved (Grime 1998; Palmer *et al.* 2000), climate (Callaway 2007) as well as by other ecosystem properties (Fierer & Jackson 2006; Johnson *et al.* 1997). Mutualistic interactions, which are typically defined as a fixed positive interaction between two species, are globally ubiquitous interactions, critical for the maintenance of global biodiversity (Janzen 1985; Bronstein 2015). However, mutualistic interactions are often more complex than the idealized +/+ interaction, typically involving a complex network of interacting partners that exchange services that maximize their own fitnesses (Janzen 1980; Olesen *et al.* 2007; Bascompe & Olesen 2015). Additionally, partners differ in the quality and quantity of the services they provide (Bever *et al.* 2009; Palmer *et al.* 2010; Kiers *et al.* 2011). Thus, the outcome of mutualistic interactions exists on a continuum ranging from wholly beneficial to neutral to pathogenic depending on the partners involved (Bronstein 1994; Palmer *et al.* 2010) and climate contexts of the systems that they reside in (Johnson *et al.* 1997; Hoeksema *et al.* 2010). The composition of mutualists cascades to affect the functioning of the entire ecosystem (van der Heijden *et al.* 1998; Rodriguez-Cabal *et al.* 2013). For example, mutualisms between plants and endophytic bacteria and fungi regulate plant community composition and diversity (Grime *et al.* 1987; Hartnett & Wilson 2002), plant productivity (van der Heijden *et al.* 1998; 2008; Wagg *et al.* 2014), nutrient cycling (Read & Perez-Moreno 2003), carbon storage (Rillig 2004; Verbruggen *et al.* 2013; Hilesalu *et al.* 2014; Clemmensen *et al.* 2015), and soil stability (Rillig *et al.* 2002; Rillig 2004; Duchicela *et al.* 2013).

Plant-associated fungi and bacteria, such as arbuscular mycorrhizal fungi (AMF) and dark-septate endophytes (DSE) and root endophytic bacteria, associate with nearly all land plants (Smith & Read 2009). Fungal and bacterial endophytes provide plants with nutrient resources, protection against pathogens, and allow plants to better cope with biotic and abiotic stress (Smith & Read 2009; Bever *et al.* 2010; van der Heijden *et al.* 2008; Friesen 2013). While endophytic bacteria and fungi provide numerous positive benefits to plant hosts (Smith & Read 2009; Friesen *et al.* 2011), they cost plants up to 20% of total fixed carbon (Allen 1991; Wright *et al.* 1998). Root symbionts are reliant on plant-derived carbon and changes in carbon allocation from plant to symbiont, shape the composition and function of symbiotic communities (Smith & Read 2008; Hammer *et al.* 2011). Additionally, endophytic bacterial and fungal catabolism and production of plant signaling compounds, like auxin and cytokinin, shape plant morphology and physiology (Harris *et al.* 1985; Friesen 2013; Henning *et al.*

2016). Changes to plant morphology, especially plant functional traits, and physiology regulate how plants interact with their biotic and abiotic environments (Lau & Lennon 2012; Wagner *et al.*, 2014). Thus, direct and indirect interactions and feedbacks among plants and their symbionts may shape ecosystem functioning and host interactions with their environment.

Global change, such as climatic warming, nutrient deposition, and invasion, alters plant community composition and productivity globally (Suding *et al.* 2005; Fay *et al.* 2015). Changes aboveground cascade belowground through changes in carbon allocation to symbionts (Hawkes *et al.* 2008, Hammer *et al.* 2011) and ecosystem inputs (Bardgett *et al.* 1999) to alter the structure (Treseder 2004; Egerton-Warburrton *et al.* 2007; Camenzind *et al.* 2014; Henning *et al.* in review) and function (Nadelhoffer 2000; Staddon *et al.* 2003; Stevens *et al.* 2015) of fungal and bacterial endophytes. Further, changes in belowground community structure and function can feedback to affect plant community structure and ecosystem function (Henning *et al.* in review). Thus, interactions among plants and endophytic bacteria and/or fungi likely mediate the response of ecosystems to global change in a myriad of ways.

The responses of both plant and endophyte communities to global change are contingent upon site-specific ecosystem properties like the initial community composition, historical legacies, environmental characteristics (Treseder & Allen 2002; Johnson *et al.* 2003; Steven *et al.* 2015), as well as interactions among multiple global change pressures (Yang *et al.* 2013; Stevens *et al.* 2015; Zhang *et al.* 2015). For instance, nitrogen deposition generally reduces the reliance of plants on their symbiotic communities. Under increased nitrogen availability, plants reduce carbon allocation to symbionts and increase biomass allocation toward leaves and stems, altering soil inputs (Treseder & Allen 2000; Egerton-Warburton and Allen 2000; Hoeksema *et al.* 2010). However, the response of plant community composition to N addition varies greatly across systems and likely depends on dominant and subordinate plant species as well as soil properties and climate (Foster & Gross 1998, Xia & Wan 2008; Johnson *et al.* 2015; Fay *et al.* 2015; Farrer & Suding 2016). However, few of these studies have simultaneously investigated the role root-associated fungi play in determining these idiosyncratic outcomes (Urcelay *et al.* 2009; Farrer & Suding 2016). Taken together, interactions between aboveground and belowground communities will mediate the response of ecosystems to global change; however, our ability to generalize across systems and predict future ecosystem function requires a deep understanding of how climate and soil properties shape these interactions.

To fill this knowledge gap, my dissertation explores how manipulating symbiotic communities alters performance of the host, how changes in plant community composition shape symbiont communities, and how environment simultaneously shapes the structure and function of plant and symbiont communities. To do this, my dissertation combines a series of observations and experiments across scales, from detailed root-microbe experiments in laboratory

mesocosms to coupled observations and experiments conducted along elevation gradients. Specifically, my dissertation consisted of four chapters:

Chapter I: *How do plant morphological and physiological traits respond to colonization by common root endophytes?* Here, I used a mesocosm study to measure the physiological and morphological response of *Populus trichocarpa that were* inoculated with single strains of common, endophytic bacteria.

Chapter II: *How does plant phenotype respond to changes in endophyte community composition among closely related bacterial strains?* As an extension of my findings in Chapter 1, I use a mesocosm experiment to measure plant resource allocation and nutrient acquisition patterns of *Populus deltoides* that were inoculated with mixed communities of endophytic bacterial communities.

Chapter III: *Do changes in plant community composition and soil nutrient status interact to alter root communities in ways that feedback to alter plant performance?* Here, I coupled observational and greenhouse plant-soil feedback experiments to explore how changes in nutrient availability and plant community composition feedback to shape plant performance in the field and in the greenhouse.

Chapter IV: *How is the biodiversity of plant-associated fungal communities distributed across climatic gradients, and what are the drivers of fungal biodiversity patterns?* In this chapter, I explored how climatic and edaphic factors simultaneously shape distributions of plants and endophytic fungal communities along elevational gradients. To do this, I conducted a global synthesis of fungal colonization patterns, combining results I collected along elevational gradient sites in North America, Europe, and Asia with previously published data. The global synthesis generated several testable hypotheses of the factors that shape plant-fungal mutualists across elevational gradients. Next, I conducted multiple observational and manipulative experiments to test these hypotheses at a representative gradient site in Colorado, USA.

The four chapters of my dissertation combined with the collaborative projects that I conducted allow me to explore the linkages among: aboveground-belowground systems, ecosystem and community ecology, and small-scale experiments and large-scale observations.

Chapter summaries

In Chapters I and II, I manipulated the composition of root endophytic bacterial communities in plant hosts, *Populus deltoides* and *P. trichocarpa*, to explore how host morphology and physiology respond to bacteria that were inoculated as single-strains and in mixed communities. To understand plant morphological and physiological responses to endophyte infection, I chose three different bacterial strains that differed in predicted metabolic capabilities, plant hormone synthesis and metabolism, and secondary metabolite synthesis. Overall, I found that single bacterial strains did not significantly increase plant carbon fixation rates or total plant biomass, but their presence altered where and how carbon was allocated within the plant. I found that bacterial root endophyte

infection increased root growth rate up to 184% and leaf growth rate up to 137% relative to non-inoculated control plants, evidence that the inoculated bacteria modified overall plant morphology. Building on Chapter I, in Chapter II, I measured how plant phenotype responded to compositional changes in mixed communities using the same strains as Chapter I. Contrary to the expectations that dominant individuals drive the functioning of communities, I found that a strain that contributed 98-99% of the bacterial community relative abundance, contributed very little to allocation and resource patterns in *Populus*. I found that plant biomass allocation toward leaves, stems, or roots, was determined by the combination of subordinate bacterial strains in the community that composed 1-2% of the total community relative abundance.

In Chapter III, I explored how dominant plant species and nitrogen availability independently and interactively, shape the local distributions of fungal communities and how changes in fungal communities feedback to affect plant performance. First, I measured fungal colonization in a common meadow plant, *Helianthella quinquenervis,* within an existing N addition × dominant plant species (*Festuca thurberi*) removal experiment near Gothic, Colorado (Read *et al.* 2017). To understand how N addition and dominant plant removal altered *Helianthella* performance in the field, I measured *Helianthella* height in the field. Overall, I found that distance from *Festuca* but not nitrogen addition or *Festuca* removal predicted *Helianthella* fungal colonization. Specifically, DSE colonization decreased by 1% for each 1 cm increase in distance from *Festuca*, although AMF colonization was consistently high (~78%) and independent of distance from *Festuca*. Thus, neighbors and legacy of past neighbors are strong determinants of fungal community composition. Second, I conducted a plant-soil feedback experiment to explore how plant-neighbors shape fungal communities and determine how changes in fungal communities shape plant performance. I used field-collected soils from field-measured colonization patterns as inoculum for the plant-soil feedback experiment. In the greenhouse experiment, I found a positive relationship between seedling survival and distance from *Festuca*. Surprisingly, I found that if Helianthella survived in soils that originated near Festuca, there were no performance tradeoffs in the field or in greenhouse-grown *Helianthella*. Our results suggest that fungal root-endophytes may play a critical role in the resistance and resilience of ecosystems facing ongoing global change.

In Chapter IV, I conducted a synthesis on the distribution of fungal root-endophytes and their plant hosts along global elevational gradients. I combined plant community, fungal colonization, climatic and edaphic data from four elevational gradients in the Colorado Rocky Mountains, northern Sweden at Abisko, the Swiss Alps, and the Tibetan Plateau, with eight previously published datasets. To understand the predictors of fungal distribution at broad spatial scales, I tested for relationships between plant hosts and functional groups, soil properties, and environmental factors with AMF and DSE distribution. Overall, I found no consistent pattern of fungal distribution across elevation sites, however, I found significant correlations between fungal endophytes, environmental

factors, and plant functional groups at global scales. The global synthesis generated testable hypotheses to explore the linkages between environment, plants, and fungi. For instance, my global synthesis predicted that the relative abundance of dark septate endophytes should be higher in cold, wet systems, whereas arbuscular mycorrhizal fungal colonization should be higher in hot and dry systems. However, when I measured fungal colonization across the growing season and in a soil moisture manipulation experiment, fungal colonization patterns did not match predictions of synthesis. My results suggest that the regional and local factors structuring fungal communities may differ or interactions among edaphic, climatic, and the different plant hosts residing within the community may drive overall colonization patterns. Both hypotheses provide fruitful avenues for future research.

CHAPTER I
ROOT BACTERIAL ENDOPHYTES ALTER PLANT PHENOTYPE, BUT NOT PHYSIOLOGY

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The use of “we” in this chapter refers to my co-authors and me. As the lead author of this article I was responsible for this paper. J.A.H., D.J.W., D.A.P., and A.T.C. designed the experiment, J.A.H. conducted the experiment with assistance from S.S.J. and C.M.T. J.A.H. analyzed data and J.A.H. wrote the manuscript. All other co-authors assisted with writing and revision.

Abstract

Plant traits, such as root and leaf area, influence how plants interact with their environment and the diverse, microbiota living within plants can influence plant morphology and physiology. Here, we explored how three bacterial strains isolated from the *Populus* root microbiome, influenced plant phenotype. We chose three bacterial strains that differed in predicted metabolic capabilities, plant hormone production and metabolism, and secondary metabolite synthesis. We inoculated each bacterial strain on a single genotype of *Populus trichocarpa* and measured the response of plant growth related traits (root:shoot, biomass production, root and leaf growth rates) and physiological traits (chlorophyll content, net photosynthesis, net photosynthesis at saturating light - A_{sat}, and saturating CO_2 - A_{max}). Overall, we found that bacterial root endophyte infection increased root growth rate up to 184% and leaf growth rate up to 137% relative to non-inoculated control plants, evidence that plants respond to bacteria by modifying morphology. However, endophyte inoculation had no influence on total plant biomass and photosynthetic traits (net photosynthesis, chlorophyll content). In sum, bacterial inoculation did not significantly increase plant carbon fixation and biomass, but their presence altered where and how carbon was being allocated in the plant host.

Introduction

A recent review exploring microbiome-mediated plant traits found that plant-associated microbes can modify fourteen out of thirty commonly measured functional traits (Cornelissen *et al.* 2003; Friesen *et al.* 2011). For example, inoculation with common root-colonizing bacterial strains influenced root and leaf architectural traits, such as specific leaf area and specific root length, as well as plant physiological traits such as carbon fixation and chlorophyll content (Harris *et al.* 1985; Ma *et al.* 2003; Friesen 2013). Further, inoculation by different members of the plant microbiome may differentially alter plant phenotype (Zamioudis *et al.* 2013; Timm *et al.* 2016). The presence of unique bacterial

strains in legume genotypes explained more variation in shoot biomass, root biomass, and plant height than plant genotype did (Tan & Tan 1986). Inoculation of common endophytes can also inhibit primary root elongation and promote lateral root formation and root hair production (Zamioudis *et al.* 2013; Weston *et al.* 2012). Recent breakthroughs in the multitude of the –omics fields have allowed for unprecedented mechanistic investigations of microbe-induced changes in host function (Verhagen *et al.* 2004; Walker *et al.* 2011; Weston *et al.* 2012; Vandenkoornhuyse *et al.* 2015; Timm *et al.* 2015; 2016) and have been the subject of multiple recent reviews (Friesen *et al.* 2011; Friesen 2013; Vandenkoornhuyse *et al.* 2015; Hacquard & Schadt 2015; Lebeis 2015; and many others). This work demonstrated that plant growth promoting bacteria elicit numerous changes in host gene expression through multiple and simultaneous hormonal and immune response pathways (Verhagen *et al.* 2004; Walker *et al.* 2011; Weston *et al.* 2012; Drogue *et al.* 2014; Timm *et al.* 2016). However, these studies fall short in explaining how changes in gene expression influence the overall plant phenotype or plant function. Thus, understanding the response of plant traits and overall plant phenotype to microbial strains remains a research gap.

Here, we inoculated three endophytic bacterial strains (*Pseudomonas fluorscens* GM41, *Pseudomonas fluorscens* GM30, and *Burkholderia* sp. BT03), originally isolated from wild *Populus*, on a single genotype of *Populus trichocarpa* and measured plant phenotypic response to bacterial inoculation. We measured a suite of traits commonly measured in the functional trait ecology literature to explore how phenotype is influenced by bacterial strains within the pre-existing functional trait framework. Plant functional trait ecology has largely ignored microbiome contribution to plant phenotype. Bacterial strains belonging to the *Pseudomonas fluorscens* group are common plant growth promoting bacteria that are abundant in the *Populus* microbiome (see Gottel *et al.* 2011). *Pseudomonas fluorscens* accounted for approximately 34% of the sequences found in the *Populus* endosphere, but only 2-3% of the sequences in the rhizosphere and soil samples originating from the same roots (Gottel *et al.* 2011). *Pseudomonas* strains can alter plant host function by modifying plant growth (Kloepper *et al.* 1980; Lugtenberg & Kamilova 2009; Timm *et al.* 2015), nutrient allocation (Bisht *et al.* 2009), hormone signaling (Stearns *et al.* 2012), up-regulating/down-regulating of gene expression pathways (Timm *et al.* 2016), and immune function (Verhagen *et al.* 2004; Weston *et al.* 2012). Additionally, the *Pseudomonas fluorscens* clade has a large amount of functional diversity (Jun *et al.* 2016), thus selecting two *Pseudomonas* strains allows us to explore how plant traits and overall phenotype respond to closely related bacterial strain genomes. To contrast with these two strains, we selected a distantly related, but enriched in *Populus* endosphere (Gottel *et al.* 2011), bacterial strain from the genus *Burkholderia*.

We predicted that aboveground and belowground traits of *Populus trichocarpa* would respond to *Burkholderia* and *Pseudomonas* strains and

inoculation of different bacterial strains would result in different plant phenotypes. Further, we predicted that the two *Pseudomonas* strains would produce a plant phenotype that was more similar to one another than to that produced in the presence of *Burkholderia* because of phylogenetic relatedness, i.e. more shared functionality. To test our predictions, we first conducted a genomic comparison using COG (clusters of orthologous groups) database to predict the functional differences among strains. Next, we inoculated each bacterial strain on *Populus trichocarpa* and measured a suite of physiological and architectural plant traits including the root:shoot, biomass production, root and leaf growth rates, chlorophyll content, net photosynthesis, and net photosynthesis at saturating light - A_{sat}, and saturating CO2 - A_{max}. We chose to measure overall trait response to bacterial endophytes without measuring the pathways involved because we were interested in understanding down-stream consequences of bacterial inoculation on overall plant phenotype

Materials and Methods

Populus trichocarpa genotype “93-968” (Labbe *et al.* 2014) was propagated in tissue culture following standard procedures (see Kang *et al.* 2009). Briefly, in vitro cultures were established from actively growing shoot tips collected from greenhouse-grown *Populus* plants. We sterilized shoot tips by soaking fresh cut tips in a 1% Tween 20 solution for 5 min, 70% Ethanol solution for 1 min, a 0.525% sodium hypochlorite solution for 15 min and then rinsed them three times in sterile H2O for 5 min. Shoot tips were trimmed to 2 cm in length and transferred to a magenta box (Sigma-Aldrich, St. Louis, MO) containing 80 mL of tissue media (1× Murashige & Skoog (MS) basal medium (Murashige & Skoog 1962) supplemented with MS vitamins (Caisson Labs, North Logan, UT, USA), 0.05% 2-(N-morpholino) ethanesulfonic acid (MES hydrate) (Sigma-Aldrich, St. Louis, MO, USA), 3% sucrose, 0.1% PPM™ (plant protective mixture) (Plant Cell Technology, Washington, DC, USA), 0.5% activated charcoal (Sigma-Aldrich, St. Louis, MO, USA), and 0.15% Gelzan (Plantmedia, bioWORLD, Dublin, OH, USA). Plants were sub-cultured until it was determined, using microscopy and colony formation units with R2A medium, that the plants were axenic.

Plant cultures were rooted in a growth room at 25 °C under a 16 h photoperiod. After root establishment, plants that were similar in size and developmental stage were selected for experimentation. Plants were weighed and scanned to account for initial plant size differences among treatments. To ensure sterility during scanning, plants were placed between two (21.59 x 27.94 cm) sheets of cellulose acetate that were sprayed with 100% ethanol. Scans were performed with a portable scanner (VuPoint Solutions Inc., City of Industry, CA, USA) at 600 × 600 dpi. Scanned images were analyzed in WinRhizo (Regent Instruments, Quebec City, Canada) to determine initial root surface area, root

length, stem length, and leaf surface area. After scanning, plants were transferred into experimental microcosms.

Experimental design

We constructed closed microcosms by interlocking two sterile Magenta boxes (Sigma-Aldrich, St. Louis, MO, USA) with a coupler (Sigma-Aldrich, St. Louis, MO, USA). We added 150 ml calcined clay (Pro's choice Sports Field Products, Chicago, IL, USA) and 70 ml of 1× Hoagland's nutrient solution (Sigma-Aldrich, St. Louis, MO, USA) to each microcosm. We drilled two 7 mm holes on adjacent sides of the upper magenta box and covered the holes with adhesive microfiltration discs (Tissue Quick Plant Laboratories, Hampshire, United Kingdom) to allow air to flow into and out of the microcosms and to prevent outside microbial contamination. Prior to microbial addition, we double sterilized each closed microcosm by autoclaving on a 60 m dry cycle on consecutive days. *Pseudomonas fluorscens* strains (GM30 and GM41) and *Burkholderia* sp. (BT03), hereafter termed *Pseudomonas* GM30, *Pseudomonas* GM41, and *Burkholderia* BT03 were isolated from *Populus deltoides* endospheres from east Tennessee and western North Carolina, USA (originally described in Brown *et al.* 2012). For full isolate descriptions, see Brown *et al.* 2012; Weston *et al.* 2012; Utturkar *et al.* 2014; and Timm *et al.* 2015; 2016. We selected these three strains because previous work (*Pseudomonas* GM30 – Weston *et al.* 2012, Labbe *et al.* 2014; *Pseudomonas* GM41 – Labbe *et al.* 2014, Timm *et al.* 2016; *Burkholderia* Bt03 – Timm *et al.* 2016) had given us indication that strains were able to influence traits in *Arabidopsis thaliana* (Weston *et al.* 2012), were able to manipulate plant gene expression and hormonal signaling in *P. deltoides* (Timm *et al.* 2015; 2016), and were able to influence host interactions with mycorrhizal symbionts (Labbe *et al.* 2014). Although strains were isolated from *P. deltoides*, strains from *Pseudomonas* and *Burkholderia* readily colonize natural *P. trichocarpa* tissues (Moore *et al.* 2006; Xin *et al.* 2009; Knoth *et al.* 2014; Kahn *et al.* 2014; Doty *et al.* 2016). We grew bacterial strains in isolation and at a constant temperature, 25 °C, in 5 ml of R2A medium. After growing overnight they were pelleted and re-suspended in sterile water to an OD600 of 0.01 (~1.0×E7 cells ml^{-1}).

We inoculated each microcosm by adding 10 ml of the bacterial strain (107 cells ml^{-1}) to the calcined clay substrate and stirring for 30 s to distribute the bacteria. After inoculation, we planted the *Populus* clones within each microcosm. Each *Populus* was grown in an individual microcosm in combination with one of the bacterial strains. Thus, the experiment had four treatment combinations – *Pseudomonas* GM30 inoculation, *Pseudomonas* GM41 inoculation, *Burkholderia* BT03 inoculation, and a bacteria-free control. In total, there were 32 microcosms with four treatments (n = 8). The experiment was divided into three different establishment dates in 2014 (1 March, 3 replicated blocks; 25 March, 2 replicated blocks; and 2 April, 3 replicated blocks) because microbiome-free plant tissues were difficult to propagate. Plant-bacteria

combinations were grown in the microcosms for five weeks with a 16 hr photoperiod, at 21°C and 80% relative humidity.

After 35 days of growth, plants were removed from microcosms, submerged in sterilized deionized H2O to remove clay from the root system, weighed, and scanned. Scans were analyzed with WinRhizo to determine final root surface area, total root length, stem length, and leaf surface area. For each plant, the final measurement of root surface area, total root length, stem length, and leaf surface area was subtracted from the initial measurement and divided by the experiment duration to determine tissue growth rates (cm d^{-1} or cm^2 d^{-1}). Additionally, each plant was dried for 48 hours at 70°C and weighed to measure leaf, shoot (leaf + stem) and root and total dry mass. Specific leaf area and the specific root length of each individual were calculated by dividing leaf area by leaf dry mass or by dividing root length by root dry mass, respectively.

To measure host physiological response to different bacterial strains, leaf gas-exchange was measured and used to estimate leaf photosynthesis on our first replicate block (March 1, n = 3). For each plant, gas exchange of the largest leaf of the plant was measured (Li-Cor model 6400, Li-Cor Biosciences, Lincoln, Nebraska, USA) immediately prior to our experimental harvest. The maximum rate of photosynthesis in saturating light under ambient CO2 (A_{sat}), the maximum rate of photosynthesis in saturating light and saturating CO2 (A_{max}), , and the quantum yield of CO2 fixation (Φ) were all measured. Finally, average leaf chlorophyll content was measured on three fully opened leaves (Konica Minolta Chlorophyll Meter SPAD-S02, Ramsey, NJ, USA).

Comparative genomics of microbes

Genomes of *Pseudomonas* GM30 and GM41 and *Burkholderia* BT03 were sequenced at Oak Ridge National Laboratory and genes were identified using Prodigal (Brown *et al.* 2012, Utturkar *et al.* 2014) and are available at NCBI (GM41: AKJN00000000.2; GM30: AKJP02000000.2; BT03: NZ_AKKD000000000.2). Genome annotation, genomes statistics, and annotation comparisons were performed using IMG tools (img.jgi.doe.gov). Genome statistics and COG functional predictions were extracted from Integrated Microbial Genomes (img.jgi.doe.gov) and then they were compared manually for differential inclusion of predicted functions.

Bacterial Colonization

To test for endophytic colonization of *Pseudomonas* GM41, *Pseudomonas* GM30, and *Burkholderia* BT03, we planted cuttings of *P. trichocarpa* into a magenta box using similar methods and treatments described above (n=3). After 2 weeks of growth, all the plant roots, stems, and 1-2 mature leaves were surface sterilized by dipping them in a ~10% bleach solution, followed by 70% ethanol, and then rinsing in water three times. We recorded wet weight of plant tissues and then separately macerated each plant tissue compartment in a sterile mortar and pestle in 1 ml sterile 1× PBS. We transferred macerated plant tissues to a

24-well plate where we serial diluted each sample by 10% with 1×PBS at 1×, 0.1×, 0.01× of original sample concentration. Each sample was streaked onto R2A media plates and allowed to grow for 48 hours at 20ºC. After 48 hours, colony formation was counted. We calculated CFU mg^{-1} of plant tissue by multiplying colony number per plate by $10^{(\text{dilution factor} + 1)}$ and then dividing that number by the dry tissue mass (mg^{1}).

Statistical analyses

We tested all data for normality using the *normalTest* function in the "fBasics" package (version 3011.87, R metrics core team 2014) for R version 3.0.2 (R development core team 2013) and RStudio version 0.98.495 (RStudio 2013). If data were not normally distributed, we performed log transformations or square-root transformations to satisfy the normality assumptions of ANOVA.

To explore plant trait response (root dry mass, leaf dry mass, shoot dry mass, total dry mass, root: shoot, root growth rates, leaf growth rates, change in leaf number, specific root length, specific leaf area) to bacterial strains, we used linear mixed-effect models using the "lme4" package in R (Bates *et al.* 2014). Bacterial strain was a fixed effect in the model and experimental block (three establishment dates) was a random factor. For plant dry mass measures, we incorporated initial measurements of root surface area in the root dry mass model and initial leaf surface area in the aboveground dry mass model as covariates. To test for significance of bacterial strain (fixed effects) and covariate (initial growth measure) we performed a likelihood ratio test to compare models with and without fixed effects and covariates. If including fixed factors (bacterial strain) was significant an improvement to model fit (p value < 0.05 in likelihood ratio test), we calculated least square means and confidence intervals using the *difflsmeans* function to calculate differences among strains using the "lmerTest" package version 2.0-3 (Kuznetsova *et al.* 2014). We measured host response to bacterial inoculation by calculating the percent change in trait values ((mean trait value for *Populus* inoculated with bacterial strain – mean non-inoculated trait value) × 100).

To test physiological responses (carboxylase activity, A_{max}, A_{sat}) of plant hosts to bacterial inoculation, we used one-way analysis of variance (ANOVA) using the *Anova* function in the "CAR" package, (version 2.0-22, Fox & Weisberg 2011) because we collected physiology data on only a single sampling date ($n = 3$).

Results

Bacterial strains differ in genomic content

We compared the genomes of *Burkholderia* BT03 and *Pseudomonas* GM30 and GM41 based on predicted enzyme functions using the COG database (Table 1). Overall, our genome comparison demonstrated that the bacterial strains differed in genome size and functional gene content. *Burkholderia* BT03

had a relatively large genome (10.9 Mb) compared to *Pseudomonas* GM30 (6.1 Mb) and *Pseudomonas* GM41 (6.6 Mb) (Table 1). We found all three bacterial strains shared functions that were likely critical for establishment and survival in the plant microbiome including the production of the plant hormone auxin, pili, flagella, chemotaxis, increased signal transduction, and secretion systems. However, we found many functional differences among our strains. The genome of *Burkholderia* encoded multiple pathways predicted to be involved in the metabolism of the plant hormones, salicylate and ethylene (Table 1). Relative to the *Pseudomonas* genomes, the *Burkholderia* genome encoded for numerous secondary metabolite biosynthesis pathways and more carbohydrate and lipid transporters, suggesting increased metabolic capabilities within *Burkholderia* (Table 1).

Even through *Pseudomonas* GM30 and *Pseudomonas* GM41 were classified as the same 16S OTU, their genome size differed as did the predicted functional capabilities of the two strains. The genome of *Pseudomonas* GM41 encoded for phosphorus solubilization and nitrate reduction, which were lacking in the *Pseudomonas* GM30 genome. Additionally, *Pseudomonas* GM41 contained more secondary metabolite biosynthesis elements compared to *Pseudomonas* GM30. We also found that the genome of *Pseudomonas* GM41 contained more genes coding for carbohydrate metabolism, lipid metabolism, and amino acid transport and metabolism, energy production and conversion, suggesting that *Pseudomonas* GM41 may contain more metabolic breadth than *Pseudomonas* GM30 (Table 1). Taken together, our results demonstrated that these three bacterial strains differ in genome size and their functional gene content.

Bacterial colonization of Populus root tissue

All three of the bacterial strains colonized *Populus* hosts. Colony-forming units were enriched in all three bacterial strains relative to the control in the 0.1× and 0.01× dilutions (0.1× dilution $F = 18.77$, $p < 0.0001$; 0.01× dilution $F = 13.78$, $p < 0.0001$, Table 2), although CFU number was variable across dilutions, tissue types, and bacterial strain. However, we found no difference in CFUs among non-inoculated control and *Pseudomonas* GM30, GM41, and *Burkholderia* BT03 inoculated host plants at the 1× dilution ($F = 1.24$, $p = 0.319$ Table 2). Across nearly all tissue types, we found that *Pseudomonas* GM30, *Pseudomonas* GM41, and *Burkholderia* BT03 inoculated plants had 10-10000× more CFUs than did non-inoculated control plants (Table 2). All three bacterial strains colonized leaf and stem tissues, but the highest CFUs across bacterial treatments were consistently observed in roots (Table 2). Inoculated host plants contained 0-28809015 CFU mg-1 in roots, 0-1166273 CFU mg-1 in stems, and 0-73537 CFU mg-1 in leaves compared to 0-400 CFU mg-1 in root tissues, 0 CFU mg-1 in stem tissue, 0-1000 CFU mg-1 in leaf tissue compared to non-inoculated control plants (Table 2).

Plant structure is modified by bacterial inoculation

Overall, we found that plant trait response to bacterial endophytes was strain specific. Specifically, mean root growth rate increased 184% with *Pseudomonas* GM30 colonization (t = 3.84, p = 0.001), however root growth rates were unaffected by *Pseudomonas* GM41 (t = 1.61, p = 0.12), and *Burkholderia* BT03 (t = 1.18, p = 0.25) inoculation (Fig 1). Similarly, mean leaf growth rate increased 114% and 138% with *Pseudomonas* GM30 (t = 2.27, p = 0.03) and *Pseudomonas* GM41 (t = 2.86, p = 0.01) inoculation, but leaf growth rate was unaffected by *Burkholderia* inoculation (t = 1.02, p = 0.32) (Fig 1). Inoculation by *Pseudomonas* GM30 increased leaf number by 36% (t = 3.34, p = 0.003) but leaf number was unaffected by *Pseudomonas* GM41 (t = 0.93, p = 0.36) and *Burkholderia* BT03 (t = 1.418, p = 0.17) inoculation (Fig 1). We observed no differences in stem elongation with bacterial inoculation (χ^2= 0.06, p = 0.97).

Interestingly, we observed no differences in total plant dry mass (χ^2 = 3.27, p = 0.195, Fig 2), root dry mass (χ^2 = 0.00, p = 1.00, Fig 2), root:shoot ratio (χ^2=0.00, p = 1.00) or plant height (χ^2 = 1.99, p = 0.158) with bacterial inoculation. However, *Pseudomonas* GM30 inoculation increased leaf dry biomass by 86% (t = 2.43, p = 0.02) relative to control plants, however leaf biomass was unaffected by *Pseudomonas* GM41 (t = 0.97, p = 0.33) and *Burkholderia* BT03 (t = 1.70, p = 0.10) (Fig 2). We observed no differences in specific leaf area with bacterial inoculation (χ^2= 2.60, p = 0.46). Thus, inoculation of *Pseudomonas* GM30 increased leaf surface area (t = 2.27, p = 0.03) and aboveground dry mass (t = 2.43, p = 0.02), without changing leaf area:mass ratios. We found no significant differences in root length:dry mass (specific root length, χ^2 = 1.06, p = 0.79) with bacterial inoculation. Our results indicate that bacterial strains modify plant resource allocation but not total dry mass production.

Plant physiology is not affected by bacterial inoculation

Bacterial inoculation had no measurable effects on any physiological trait we measured: chlorophyll content (SPAD) (χ^2 = 2.15, p = 0.54), quantum yield of photosynthesis (ϕ) (F = 1.01, p = 0.43), net photosynthesis at saturating light conditions (A_{sat}) (F = 0.76, p = 0.55) or maximum net photosynthesis at saturating light and [CO2] (A_{max}) (F = 1.98, p = 0.19) (Fig 3). In agreement with the total dry mass data, we did not observe significant changes in the measured photosynthetic parameters. Thus, changes in plant structure were not linked with increased photosynthetic capacity, efficiency, or carbon assimilation rates.

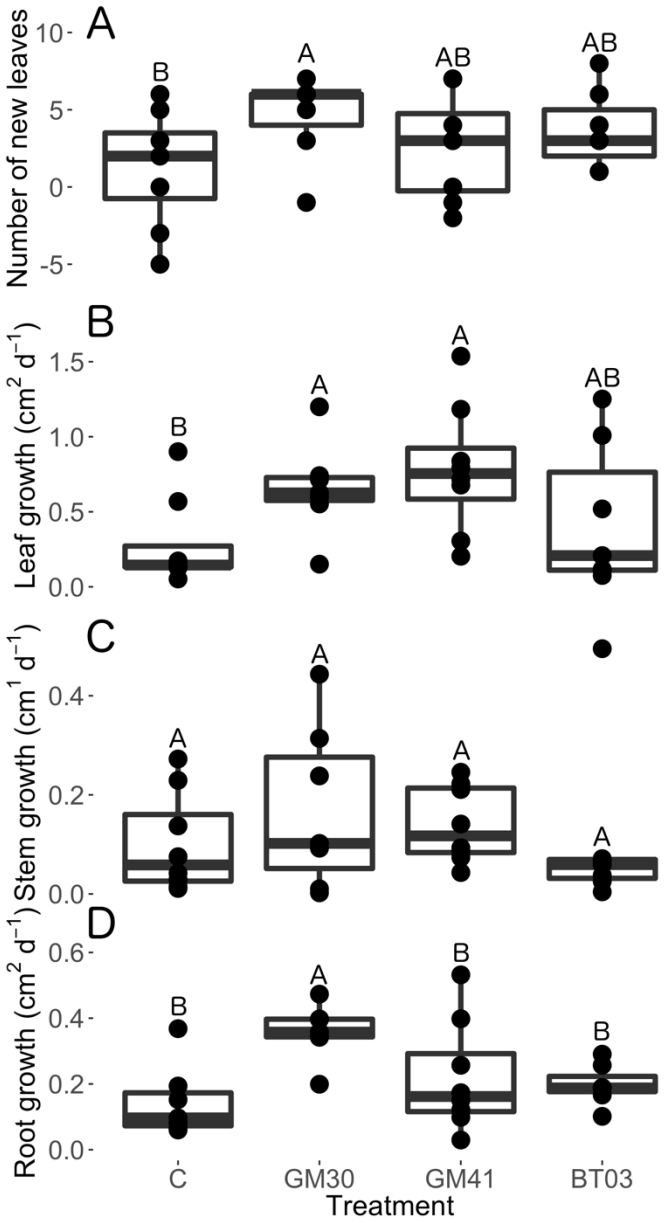

Figure 1. Structural traits of *Populus trichocarpa* that were not inoculated with bacteria (no microbe control) (n = 8), were inoculated with *Pseudomonas* GM30 (n = 7), *Pseudomonas* GM41 (n = 8), or *Burkholderia* BT03 (n = 7). a) Change in leaf number from the first to last day of the experiment. Negative values indicate that leaves senesced during the experiment. b) Leaf surface area growth rates (cm^2 d^{-1}), c) Stem growth rate (cm^1 d^{-1}). d) Root surface area growth rates (cm^2 d^{-1}). Letters represent significant differences of post-hoc least squares means among bacterial treatments. Boxplots display median, first and third quartiles, and vertical lines represent 1.5× inner quartile range of our dataset. The dots represent raw data values.

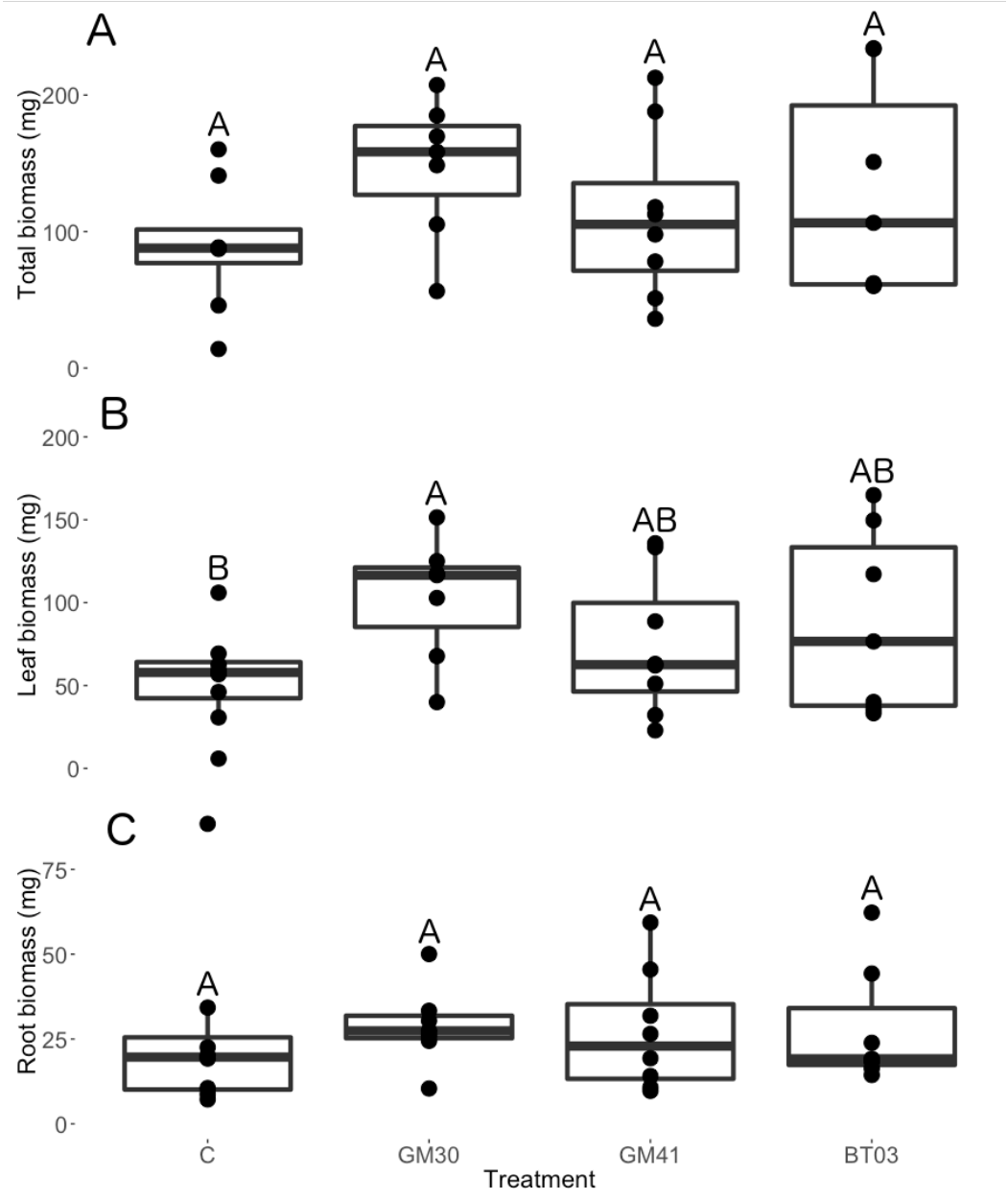

Figure 2. Biomass allocation of *Populus trichocarpa* that were not inoculated with bacteria (no microbe control) (n = 8) , were inoculated with *Pseudomonas* GM30 (n = 7), *Pseudomonas* GM41 (n = 8), or *Burkholderia* BT03 (n = 7). a) Total dry mass (mg). b) Leaf biomass (mg), c) Root biomass (mg). Letters represent significant differences of post-hoc least squares means among bacterial treatments. Boxplots display median, first and third quartiles, and vertical lines represent 1.5×inner quartile range of our dataset. The dots represent raw data values.

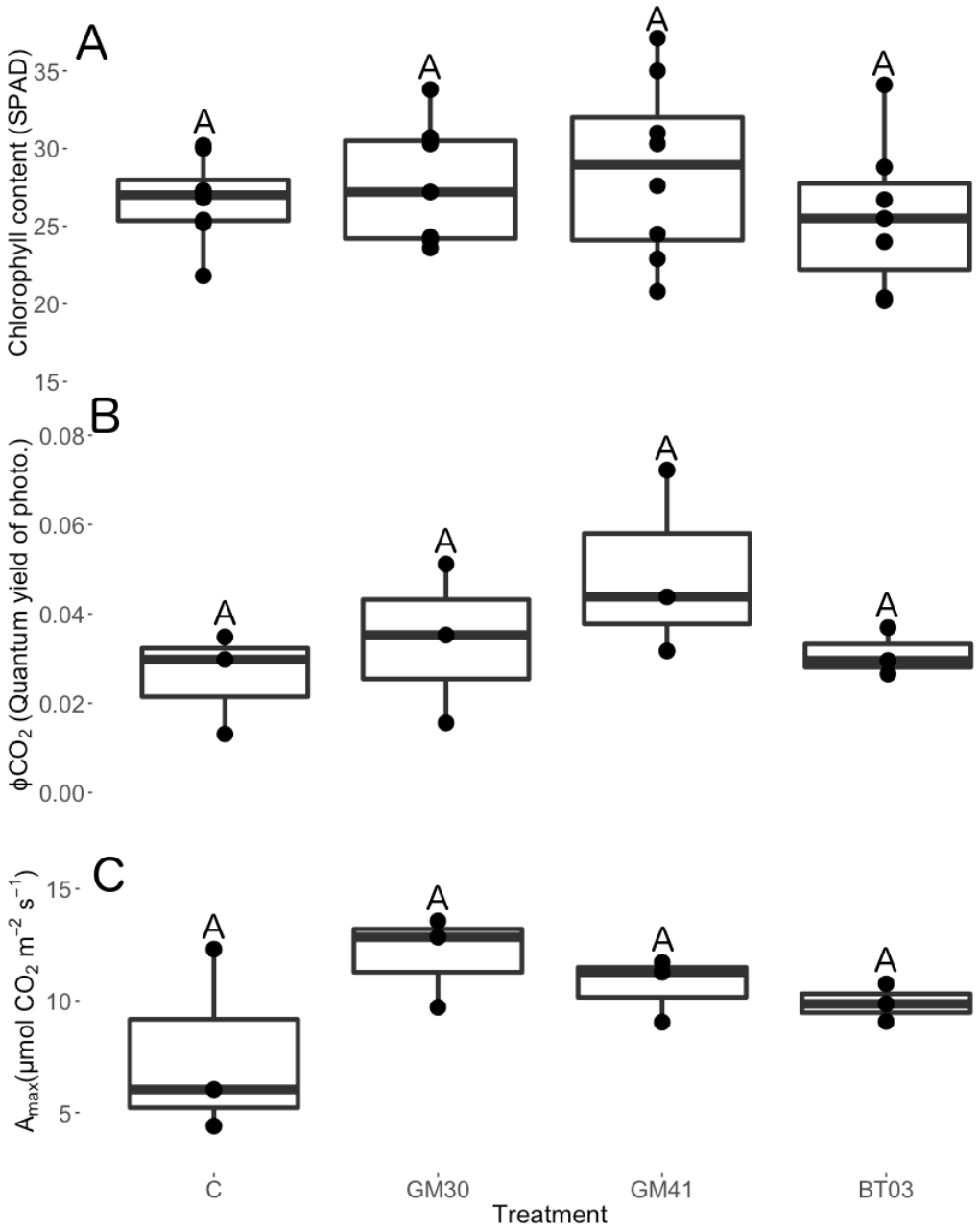

Figure 3. Physiology traits of *Populus trichocarpa* that were not inoculated with bacteria (no microbe control) (n = 8), were inoculated with *Pseudomonas* GM30 (n = 7), *Pseudomonas* GM41 (n = 8), or *Burkholderia* BT03 (n = 7). a) Plant chlorophyll content (SPAD), b) ΦCO_2 (expressed as the slope of carboxylase activity across different light levels), and c) carboxylase activity under maximum light level and CO_2 concentration (A_{max}). Letters represent significant differences of post-hoc least squares means among bacterial treatments. Boxplots display median, first and third quartiles, and vertical lines represent 1.5×inner quartile range of our dataset. The dots represent raw data values**.**

Discussion

The plant root microbiome can have a strong influence on plant production and phenotype (Friesen 2013; Vandenkoornhuyse *et al.*, 2015); yet, less is known about how plant trait expression, production, and physiology are influenced by individual endophytic strains. We explored how plant morphological traits, productivity, and cellular physiology in *Populus trichocarpa* responded to inoculation with three bacterial strains, two closely related *Pseudomonas fluorscens* strains (GM30 & GM41) and a more distantly related *Burkholderia* strain (BT03). We selected bacterial strains that were predicted to differ in metabolic capabilities, plant hormone production and metabolism, and secondary metabolite synthesis in an effort to understand how plant phenotype is influenced by inoculation with different strains of common endophytic bacteria (Table 1, Timm *et al.* 2015; 2016). Our comparative genomic analysis revealed that while all three strains share many common endophytic functions like plant hormone signal disruption, production of plant hormone auxin, pili, flagella, and chemotaxis, strains potentially differed in their ability to perform these functions. Overall, we found that *Burkholderia* and *Pseudomonas* genomes differed in the carbon substrates they were predicted to degrade, plant hormone production and metabolism, and secondary metabolite synthesis, which led us to predict that plant response to bacterial inoculation would lead to different phenotypes between treatments. All three strains could colonize *Populus* roots, leaves, and stems, however CFU number was highest within root tissues in all three strains (Table 2).

Overall, we found root endophyte inoculation altered plant resource allocation patterns without influencing total plant biomass accumulation (Fig 1,2). Additionally, we found that plant trait response and overall phenotype differed across bacterial strains in ways that would not have been predicted from our genome analysis. Specifically, *Burkholderia* BT03 was predicted to produce auxin and to metabolize salicylate and ethylene, three plant hormones crucial to plant growth and development (see Yang & Hoffman 1984; Wasternack & Parthier 1997; Chen *et al.* 2009; Dempsey *et al.* 2011). Additionally, we found the *Burkholderia* genome encoded for multiple transposase elements that degrade poplar-produced aromatics and metabolites (Timm *et al.* 2015; 2016). Despite the predicted ability of *Burkholderia* to manipulate multiple plant hormonal and signaling pathways, we observed no measurable changes in any traits when *Populus* was inoculated with *Burkholderia* (Figs 1,2,3). This was especially surprising since we consistently measured the highest CFU abundance within *Burkholderia* inoculated individuals (Table 2).

In spite of close genetic relatedness and classification under the same 16S OTU profile, our *Pseudomonas* strains differed in key functional capabilities. Specifically, *Pseudomonas* GM41 encoded for phosphate solubilization and denitrification ability, suggesting these two strains may differentially influence host nutrition, although this remains untested. Our genome analysis revealed that

both strains were capable of producing the plant hormone auxin, however another study found that *Pseudomonas* GM41 produced two times more auxin than *Pseudomonas* GM30 (Timm *et al.* 2015). Auxin synthesis by endophytic bacteria can increase root branching and lateral root formation and decrease overall plant height, leaf number, chlorophyll content and photosynthetic efficiency (Romano *et al.* 1993; Fu & Harberd 2003; Weston *et al.* 2012). Thus, we predicted that *Pseudomonas* GM41 would have a strong influence on plant root traits, however we observed no measurable effects of *Pseudomonas* GM41 inoculation on root growth rate or morphology (Fig 1). Belowground, *Pseudomonas* GM30 inoculation increased root surface area growth rate by 184% (Fig 1) without increasing root biomass (Fig 2), suggesting *Pseudomonas* GM30 inoculation may change root morphology, leading to longer, thinner, highly-branched roots with similar biomass, as we predicted. Similar patterns have been observed when *Pseudomonas* GM30 is inoculated on both Arabidopsis (Weston *et al.* 2012) and *Populus deltoides* (Timm *et al.* 2015; 2016). Additionally, inoculation of *Pseudomonas* GM30 increased leaf surface area growth rate by 114% (Fig 1), leaf number by 36% (Fig 1), and aboveground biomass by 86% (Fig 1) but did not influence specific leaf area, whereas closely-related *Pseudomonas* GM41 increased leaf surface area growth rate by 138% (Fig 1) but did not change leaf number (Fig 1) or aboveground biomass (Fig 2). Unlike *Burkholderia*, *Pseudomonas* genomes do not contain the genes to directly metabolize salicylate, however inoculation of *Pseudomonas* GM41 can up-regulate salicylic acid synthesis and degradation in *Populus* (Timm *et al.* 2016). Taken together, our data suggest that predicting plant phenotypic response to bacterial inoculation, even in overly simplified systems using fully sequenced bacterial strains, is extremely complex and difficult.

Contrary to our predictions, leaf physiology (Fig 3), plant height, root:shoot, specific leaf area, specific root length, and total plant dry mass (Fig 2) were not influenced by bacterial inoculation. It is possible that multiple, overlapping plant signaling and gene expression effects induced by bacterial endophyte inoculation may mask a hosts' phenotype response. For example, endophytes simultaneously up- and down-regulate numerous genes and metabolites in plant tissue (see Verhagen *et al.* 2004; Wang *et al.* 2005; Walker *et al.* 2011; Weston *et al.* 2012; Timm *et al.* 2016). Thus, counteracting influences among different gene pathways may conceal plant responses to endophyte inoculation when measuring down-stream phenotype and functional traits (Bashan *et al.* 2004; Timm *et al.*, 2016). Additionally, host physiological response to endophyte inoculation may vary with bacterial strain (Kandasamy *et al.* 2009; Weston *et al.* 2012; Timm *et al.* 2016), plant host (Smith & Goodman 1999), plant ontogeny (Siddiqui & Shaukat 2003), or plant stress (Dimkpa *et al.* 2009; Yang *et al.* 2009; Lau & Lennon 2012). For example, Root colonization by *Pseudomonas* can reduce chlorophyll content and net photosynthesis (A_{sat}) in a variety of plant hosts (Zou *et al*. 2005; Weston *et al.* 2012). However *Pseudomonas* colonization can also increase photosynthetic activity and

chlorophyll content (Kandasamy *et al.* 2009, Timm *et al.* 2016). Thus, biotic and abiotic contexts may drive the phenotypic response of hosts to endophyte inoculation, however this idea requires further testing.

Our study focused on the response of plant functional traits to monoculture associations of common endosphere bacteria, however future studies should focus on exploring how plant phenotype responds to diverse microbiome communities. With a few well-known exceptions (Tan & Tan 1986; Harris *et al.* 1985; Ma *et al.* 2003, Lau & Lennon 2011; 2012), bacterial community composition in roots has been ignored in studies exploring what drives natural variation in plant traits (Friesen *et al.* 2011; Friesen 2013; Timm *et al.* 2016). We propose a multifaceted approach to investigate linkages among the plant microbiome and natural plant trait variation. First, incorporation of microbiome composition into studies that currently investigate host identity/genotype and environmental parameters may be important for finding patterns in natural trait variation – especially when conduced across a variety of environmental gradients. Second, once correlations between microbiomes and plant traits are observed in the field, detailed work constructing communities in the lab and greenhouse would enable a mechanistic understanding of what is underlying the observed patterns. These studies could be especially fruitful when conducted across natural biotic and abiotic environmental gradients in the laboratory, greenhouse, and field settings (Classen *et al.* 2015).

Conclusions

Our study demonstrates that bacteria living in plant roots can influence plant morphological traits. Increasingly, ecologists are using plant functional traits to explore how changing environments might alter plant function (Wright *et al.*, 2004; Reich 2014). Plant traits, such as specific leaf area and specific root length, are often significantly correlated with important plant functions such as carbon fixation and nutrient uptake (Diaz & Cabido 2001). Researchers are using correlations between plant traits and function to extrapolate how plants and ecosystems will respond to global changes (Reich *et al.*, 1999; Wright *et al.*, 2004; Reich 2014). While interactions between plant genotype and environment undoubtedly influence plant phenotypic plasticity (Bradshaw 1965; Schlichting 1986; Sultan 2000; Des Marais *et al.* 2013), phenotype is also heavily influenced by biotic factors, like the microbiome bacterial endophytes (Lau & Lennon 2011; 2012; Wagner *et al.* 2014; Hacquard & Schadt 2015). Given that plant-microbial studies, including ours, have observed strong linkages between microbiome and plant phenotype (reviewed in Friesen *et al.* 2011; Friesen 2013) interactions among global change drivers, plant genotypes, and plant microbiomes, should be considered in trait-based approaches to ecological questions (Classen *et al.* 2015).

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Appendix

Table 1. Predicted plant-interaction pathways in bacterial strains *Burkholderia* sp. BT03*, Pseudomonas fluorescens* GM30, and *Pseudomonas fluorescens* GM41. Genome size, relevant pathways, and COG category statistics were identified using IMG tools. Where applicable, gene loci indicating predicted functions in genomes (individual genes or pathways) were included. NA = not applicable.

	***Burkholderia* BT03**	***Pseudomonas* GM30**	***Pseudomonas* GM41**
Genome size (Mb)	10.9	6.1	6.6
ACC deaminase	PMI06_0002752	PMI25_02765	PMI27_01478
salicylate metabolism	PMI06_001931	*NA*	PMI27_05197
auxin biosynthesis	PMI06_005275	PMI25_03791	PMI27_00952
pili, fimbriae	PMI06_00372-3373	PMI25_00378-0372	*NA*
Flagella	PMI06_009483-9498	PMI25_03624 -3649	PMI27_02843-2866
Chemotaxis	PMI06_009463-9475	PMI25_05665-5658	PMI27_05395-5382
type 2 secretion	PMI06_001352-1341	PMI25_00837-00844	*NA*
type 3 secretion	PMI06_000607-0617	*NA*	*NA*
type 4 secretion	PMI06_009642-9622	*NA*	*NA*
type 6 secretion	PMI06_001813-1833	PMI25_012011220	PMI27_02378-2397
carb. metabolism (# of genes)	582	222	291
secondary metabolite metabolism (# of genes)	337	113	148

Table 2. Colony forming units found in leaf, root, and stem tissue of *Populus* trichocarpa inoculated with *Burkholderia* sp. BT03*, Pseudomonas fluorescens* GM30, and *Pseudomonas fluorescens* GM41 across three dilution factors: 1×, 0.1×, 0.01× orginal concentrations. *Pseudomonas fluorescens* GM41 and *Burkholderia* sp. BT03 data were first published in Timm *et al.* 2016. Tiss. = plant tissue type, Resid. = residual.

Treatment	Tiss.	Dilution	mean CFU	St dev		Df	F	*p*
Control	leaf	1.0E+01	1080.5	1871.5	Bact.	3	1.24	0.319
GM30	leaf	1.0E+01	19574.7	30672.7	Tissue	2	1.92	0.1699
GM41	leaf	1.0E+01	1141.3	1809.3	B × T	6	0.74	0.6264
BT03	leaf	1.0E+01	41175.9	45063.1	Resid.	24		
Control	root	1.0E+01	110.2	131.5				
GM30	root	1.0E+01	170447.1	212977.7				
GM41	root	1.0E+01	2438.9	1563.8				
BT03	root	1.0E+01	309628.0	106958.6				
Control	stem	1.0E+01	0.0	0.0				
GM30	stem	1.0E+01	1166273.0	1872593.0				
GM41	stem	1.0E+01	1510.2	2135.8				
BT03	stem	1.0E+01	654513.2	688365.7				
Control	leaf	1.0E-01	1044.4	1809.0	Bact.	3	18.77	**>0.001**
GM30	leaf	1.0E-01	16643.8	28827.9	Tissue	2	9.21	**0.001**
GM41	leaf	1.0E-01	566.2	980.7	B × T	6	8.91	**>0.001**
BT03	leaf	1.0E-01	60745.9	54910.0	Resid.	24		
Control	root	1.0E-01	402.7	377.6				
GM30	root	1.0E-01	120591.5	111174.4				
GM41	root	1.0E-01	2851.9	3319.7				
BT03	root	1.0E-01	3096279.7	1069585.6				
Control	stem	1.0E-01	0.0	0.0				
GM30	stem	1.0E-01	289189.7	330089.7				
GM41	stem	1.0E-01	0.0	0.0				
BT03	stem	1.0E-01	904314.7	1099508.6				
Control	leaf	1.0E-02	0.0	0.0	Bact.	3	13.78	**>0.001**
GM30	leaf	1.0E-02	0.0	0.0	Tissue	2	11.79	**>0.001**
GM41	leaf	1.0E-02	0.0	0.0	B × T	6	11.47	**>0.001**
BT03	leaf	1.0E-02	73537.1	80004.4	Resid.	24		
Control	root	1.0E-02	0.0	0.0				
GM30	root	1.0E-02	368195.0	510398.0				
GM41	root	1.0E-02	20595.2	35671.9				
BT03	root	1.0E-02	28809015	14126690				
Control	stem	1.0E-02	0.0	0.0				
GM30	stem	1.0E-02	227127.9	252544.5				
GM41	stem	1.0E-02	0.0	0.0				
BT03	stem	1.0E-02	1805855.2	1567125.7				

CHAPTER II
RARE BACTERIAL ENDOPHYTES DETERMINE PLANT RESOURCE ALLOCATION AND PHENOTYPE

The Following section is a slightly modified version of a paper in preparation:

Henning, J.A., D.J. Weston, D.A. Pelletier, C.M. Timm, S.S. Jawdy, A.T. Classen. *In prep*. Rare bacterial endophytes determine plant resource allocation and phenotype

The use of “we” in this chapter refers to my co-authors and me. As the lead author of this article I was responsible for this paper. J.A.H., D.J.W., D.A.P., and A.T.C. designed research, D.A.P. provided microbial strains, J.A.H, C.M.T., S.S.J, performed research, J.A.H. analyzed data, J.A.H. wrote the manuscript, and all other co-authors assisted with writing and revision.

Abstract

- Low abundant community members are assumed to contribute very little to the functioning of the system where they reside, especially in highly diverse communities with high amounts of functional redundancy like plant endophytic bacterial communities where recent evidence suggests that strains shape plant morphology and physiology.
- We conducted two complementary experiments where we constructed endophytic communities on germ-free *Populus deltoides* as single microbe inoculations then as ‘complex’ communities and measured plant biomass allocation and nutrient acquisition. We used single inoculation trials to predict the plant biomass allocation and nutrient acquisition patterns in ‘complex’ communities, which we predicted would match community composition.
- Although all bacterial endophytes had measurable effects on plant phenotype when inoculated alone, in mixed communities we found that low-abundant *Pseudomonas* sp. (GM17, GM30, or GM41) strains disproportionately shaped plant resource allocation and resource acquisition patterns over the dominant strain that produced 98-99% of community relative abundance, *Burkholderia* BT03.
- Our results demonstrate that counter to predictions set out by the mass:ratio hypothesis and assumptions about functional redundancy, subtle shifts among closely-related, low abundance community members can have large consequences in plant resource allocation and acquisition patterns.

Introduction

Low abundance species are assumed to contribute very little to the functioning of the systems they reside within. The mass:ratio hypothesis (Grime 1998) predicts that a species’ effect on ecosystem function is proportional to its relative abundance (Bilá *et al.* 2014). Numerous examples from plant (Grime

1998; Ervin & Wetzel 2002; Smith & Knapp 2003), plankton (Dayton 1971; Norberg 2004), and animal (Dangles & Malmqvist 2004; Larsen *et al.* 2005; Balvanera *et al.* 2005; Bilá *et al.* 2014) communities demonstrate that dominant species shape ecosystem function. However, in many systems, low-abundance (rare) species can exert large effects on ecosystem function (Lyons *et al.* 2005; Zavaleta & Hulvey 2004; Hol *et al.* 2010; Mouillot *et al.* 2013). For instance, in Alaskan shrub wetlands, *Equisetum*, which contributes < 5% of above- and belowground biomass, contributes 55% of phosphorus and 75% calcium to 2-year-old soil nutrient pools (Marsh *et al.* 2000; Lyons *et al.* 2005). In another example, the loss of top predators leads to population explosions of herbivores and loss of native plant diversity (Ceballos & Brown 1995; McShea *et al.* 1997; Shelton *et al.* 2014). Surprisingly, there have been relatively few empirical tests investigating the role of low abundant species in determining the functioning of systems in species rich microbial communities, likely because functional redundancy is assumed to be high (Nannipieri *et al.* 2003). Despite this, recent studies have demonstrated that low abundant microbes can mediate plant herbivore interactions through changes in plant nutritional resources (Hol *et al.* 2010) and can also cause disease symptoms within the human microbiome (Gerristen *et al.* 2011; Gilbert *et al.* 2016; Hedin *et al.* 2017).

The community of microbes that live on plants can influence their morphology as well as their function (Lau & Lennon 2012; Friesen 2013; Wagner *et al.* 2014). A flurry of papers provide mechanistic insight into how bacterial endophytes regulate plant gene expression (Timmusk & Wagner 1999; Verhagen *et al.* 2004), plant hormone pathways (Ryu *et al.* 2003; Overvoorde *et al.* 2010), and plant metabolite concentrations (Walker *et al.* 2011; Timm *et al.* 2016). Changes in plant gene expression and signaling cascade to shape plant physiology and phenotype (Zamioudis *et al.* 2013; Henning *et al.* 2016). Further, even closely-related microbes can have strong differential effects on plant physiology and phenotype (Zamioudis *et al.* 2013; Henning *et al.* 2016). However, the studies cited above have been conducted on single-strain inoculated plants, and there have been limited attempts to quantify “strain-effects” in mixed communities to test the role of dominant versus low abundant strains.

Here, we designed two, complementary experiments to test how microbial community composition influenced *Populus deltoides* morphology and physiology. First, we assessed how individual root endophytic bacteria changed plant phenotype. Next, we constructed intentional 3-member communities to explore how changes in the root bacterial community composition altered plant morphology and physiology. We tested whether we could predict plant phenotype in the mixed communities from phenotype of the single inoculated communities. To test this, we inoculated *Populus* in a factorial design using four endophytic bacterial strains originally isolated from wild *Populus deltoides*, *Burkholderia* sp. BT03, *Pseudomonas* sp. GM17, *Pseudomonas* sp. GM30, and *Pseudomonas* sp. GM41 (Brown *et al.* 2012; Jun *et al.* 2016). Past work with *Burkholderia* and

Pseudomonas strains revealed that all four strains readily colonize *Populus* roots, alter hormone signaling, gene expression pathways and shape above- and below-ground plant phenotypes, (Gottel *et al.* 2011; Weston *et al.* 2012; Timm *et al.* 2015; 2016; Henning *et al.* 2016). However, in single strain inoculation trials, *Burkholderia* typically colonizes root systems at levels that are 10- to 100-fold higher than *Pseudomonas* GM41 or *Pseudomonas* GM30 (Timm *et al.* 2016; Henning *et al.* 2016). Thus, we hypothesized that *Burkholderia* would contribute heavily to community composition and function of our mixed communities. To explore composition changes among closely-related but functionally different (Jun *et al.* 2016; Timm *et al.* 2015) *Pseudomonas* strains, we included 3 Pseudomonad strains that differed in their influence on plant phenotype during single inoculation trials (Timm *et al.* 2015; Henning *et al.* 2016; Weston *et al.* 2013). We hypothesized that single strains would differentially influence plant phenotype, however, "strain effects" would be contingent on the relative abundance in the community, conforming to predictions of the mass-ratio hypothesis.

Methods

Plant propagation

We propagated *Populus deltoides* in tissue culture following standard procedures (see Henning *et al.* 2016). Briefly, surface-sterilized shoot tips were cultured in a magenta box (Sigma-Aldrich, St. Louis, MO) containing 80 mL of tissue media (1× Murashige & Skoog (MS) basal medium (Murashige & Skoog 1962) supplemented with MS vitamins (Caisson Labs, North Logan, UT, USA), 0.05% 2-(*N*-morpholino) ethanesulfonic acid (MES hydrate) (Sigma-Aldrich, St. Louis, MO, USA), 3% sucrose, 0.1% PPM™ (plant protective mixture) (Plant Cell Technology, Washington, DC, USA), 0.5% activated charcoal (Sigma-Aldrich, St. Louis, MO, USA), and 0.15% Gelzan (Plantmedia, bioWORLD, Dublin, OH, USA). Plants were sub-cultured until we determined the plants were axenic using microscopy and colony formation units with an R2A medium. Plant cultures were maintained in a growth room at 25 °C under a 16 h photoperiod. After root establishment, we selected plants that were similar in size and at a similar development stage to use in experiments. To account for variation in initial plant size, we weighed each individual plant prior to establishing experimental treatment.

Experimental design

We constructed closed mesocosms by interlocking two sterile Magenta boxes (Sigma-Aldrich, St. Louis, MO, USA) with a coupler (Sigma-Aldrich, St. Louis, MO, USA). We added 150 ml calcined clay (Pro's choice Sports Field Products, Chicago, IL, USA) and 70 ml of 1× Hoagland's nutrient solution (Sigma-Aldrich, St. Louis, MO, USA) into each mesocosm (Fig 1). We drilled two 7 mm holes on adjacent sides of the upper magenta box to allow air flow into and

out of the mesocosm. We covered the air holes with adhesive microfiltration discs to prevent microbial contamination of the mesocosm (Tissue Quick Plant Laboratories, Hampshire, United Kingdom). Prior to bacterial addition, we double sterilized each closed mesocosm by autoclaving on a 60 m dry cycle on consecutive days. We grew the bacterial strains in isolation and at a constant temperature, 25 °C, in 5 ml of R2A medium. After growing the strains overnight we pelleted and re-suspended them in sterile water to an OD600 of 0.01 (~1.0×E7 cells ml^{-1}).

Bacterial isolates

Pseudomonas sp. strains (GM17, GM30, and GM41) and *Burkholderia* sp. (BT03), hereafter termed *Pseudomonas* GM17, *Pseudomonas* GM30, *Pseudomonas* GM41, and *Burkholderia* BT03 were isolated from *Populus* endospheres (for isolate descriptions, see Brown *et al.*, 2012; Weston *et al.*, 2012). Comparative genomics of *Pseudomonas* GM30, GM41, GM17 and *Burkholderia* BT03 strains can be found in (Brown *et al.* 2012; Timm *et al.* 2015; 2016; Henning *et al.* 2016).

Experiment 1: Individual bacterial impacts on plant traits

We grew *Populus* individuals in isolated mesocosms. Each mesocosm was inoculated with individual *Pseudomonas* GM30, *Pseudomonas* GM41, or *Burkholderia* BT03. There was also a bacteria-free plant only control treatment. In total, there were 32 mesocosms with four treatments (n = 8). In each plant-free mesocosm controls, we added 10 ml of the bacterial strain (10^7 $cells^1$ ml^{-1}) to the clay substrate and stirred the slurry for 30 s to distribute the bacteria. We then transferred an individual *Populus* into each mesocosm. We grew each experimental block for five weeks under a 16 hr photoperiod, at 21°C, and at 80% relative humidity.

After 5 weeks of growth, plants were harvested and measured for leaf chlorophyll content. Individual plants were submerged in sterile deionized H_2O to remove clay from the root system and were scanned with a portable scanner (VuPoint Solutions Inc., City of Industry, CA, USA) at 600 × 600 dpi and were then dried for 48 hours at 70°C to determine leaf, stem, and root biomass. Scans were analyzed with WinRhizo to determine final root surface area, total root length, stem length, and leaf surface area and divided by leaf and root biomass to determine specific root length (cm mg^{-1}), specific root area (cm^2 d^{-1}), and specific leaf area (cm^2 d^{-1}). We also measured plant resource allocation strategies by calculating leaf, root, and stem mass fractions (leaf, root, or stem weight (mg) / total biomass (mg) ×100).

Experiment 2: Assembling 'complex' bacterial communities

Using a similar experimental design, we explored how shifts in bacterial community composition influenced host phenotype. However, we inoculated 'complex' three-member bacterial communities using *Pseudomonas* GM17,

Pseudomonas GM30, *Pseudomonas* GM41, *Burkholderia* BT03 strains, in a factorial design. Our experimental design resulted in four treatment combinations: (1) *Pseudomonas* GM17 + *Pseudomonas* GM30 + *Pseudomonas* GM41, (2) *Pseudomonas* GM17 + *Pseudomonas* GM30 + *Burkholderia* BT03, (3) *Pseudomonas* GM17 + *Pseudomonas* GM41 + *Burkholderia* BT03, and (4) *Pseudomonas* GM30 + *Pseudomonas* GM41 + *Burkholderia* BT03. In each mesocosm, we pipetted 10 ml of the assigned bacterial community (3.33 ml of each strain diluted to 10^7 cells^1 ml^{-1}) and stirred the clay for 30 s to evenly distribute the bacteria. We then transferred plants into the community treatments and mesocosms were maintained as described above. After 28 days of growth, we measured chlorophyll content, final leaf, stem, and root biomass, leaf, stem, and root mass fraction as described above.

To measure total Kjeldahl N and phosphorus (P) content by mass of the dried tissue samples (both leaf and root; Pérez-Harguindeguy *et al.* 2013), we used a modified micro-Kjeldahl digestion (Parkinson & Allen 1975). First, we first ground the tissue samples with a mortar and pestle. We weighed 75 mg, of the ground sample and folded it into a piece of adhesive-free cigarette paper. We digested the sample for 5 h at 350° C in 5 mL H_2SO_4 in a Kjeldatherm digestion block (Gerhardt, Königswinter, Germany). After the digests cooled, we added 45 mL deionized water to each one. We used a SmartChem 200 discrete analyser (Unity Scientific, Brookfield, CT, USA) to measure total Kjeldahl N and P, expressed as a proportion of total tissue mass.

Bacterial community composition

To measure bacterial community composition, we collected a random ~3g subsample of root tissue and flash frozen in liquid N_2. Next, we extracted DNA from 50 mg of each sample using the PowerPlant kit (MOBIO). We quantified each bacterial strain in our community using qPCR primers designed to detect *Pseudomonas* GM17 (forward 5'-TGTCACTATTATCAGCCATTGTAGA-3' and reverse 5'-AACAGTGGATGAGGTCTAATAACAA-3'), *Pseudomonas* GM30 (forward 5′-ATCCGTACCATTTATGTTGATGAGT-3′ and reverse 5′-GAAACACATCCTCTTCGTTCTGTAT-3′) *Pseudomonas* GM41 (forward 5′-ATCCGTACCATTTATGTTGATGAGT-3′ and reverse 5′-GAAACACATCCTCTTCGTTCTGTAT-3′) and *Burkholderia* BT03 (forward 5′-AGACTTCTTTGATTGAGGTGAAGTA-3′ and reverse 5′-CATATAGTCGAGATGGTCATTTAGG-3′). We performed qPCR with the iTaq Supermix (Biorad, Hercules, CA, USA) on a Biorad CFX96 instrument using gDNA as a standard according to manufacturer's instructions. We corrected qPCR results to account for the initial wet root weight and corrected by dry biomass to convert our measurements to bacterial cells g^{-1} of dry root biomass.

Predicting plant phenotype from community structure and trait means

To predict plant phenotype of *Populus* inoculated with mixed communities of bacteria, we used the mean trait values of *Populus* inoculated with single

bacteria, and added the strain effects, which we corrected for bacterial relative abundance. First, mean biomass allocation to roots or to shoots was calculated for each strain in experiment 1. Next, the mean root biomass allocation and shoot biomass allocation for each strain was weighted by qPCR community composition data which was converted to relative abundance values (predicted allocation value = ∑ (mean trait value × relative abundance). To understand differences in predicted values and actual phenotype, we calculated residual variation (residual = actual allocation value – predicted allocation value).

Statistical analysis

All statistical analyses were conducted in R version 3.3.2 (R development core team 2016) and RStudio version 1.0.44 (RStudio 2016), with packages cited where applicable. We assessed how single bacterial strains and communities altered plant resource allocation, nutrient content, and chlorophyll content, using linear mixed-effect models. We tested for normality using the *normalTest* function in the fBasics package (version 3011.87, R metrics core team 2016). If the data were not normally distributed, we performed log or square-root transformations to satisfy the normality assumptions of ANOVA. Bacterial strain identities were treated as a fixed effect in the model; and the experimental blocks (three establishment dates) were treated as random factors using the lme4 package (Bates *et al.* 2014). Additionally, we incorporated initial plant size as covariates. In each case, we fit a full model with fixed and random effects included, then fit subsets of the model with and without fixed effects and covariates and determined best fit models by comparing AIC scores. If bacterial strains were retained as predictors within the best-fit model, we calculated the least-square means and confidence intervals using the *difflsmeans* function in the "lmerTest" package (version 2.0-3, Kuznetsova *et al.* 2014).

We explored how bacterial community composition influenced plant biomass allocation patterns, chlorophyll content, and leaf nutrient content we analyzed data using one-way ANOVAs. We then ran a principal components analysis (PCA) including significant or trending traits identified from trait ANOVAs. Our PCA included the following traits: total plant biomass, shoot biomass, root biomass, leaf area growth rate, stem growth rate, specific root length, specific root area, root:shoot and was performed using the *rda* function in the "vegan" package (Oksanen *et al.* 2015). We included the following traits in our PCA: root:shoot, specific leaf area, chlorophyll content, specific root length, specific root area, root biomass, stem height, whole plant biomass, leaf growth rate, stem growth rate, shoot biomass. To visually understand how different bacterial communities influenced overall plant phenotype, we constructed a PCA ordination using: percent biomass allocated toward roots, stems, and leaves, as well as total Kjeldahl leaf nitrogen and phosphorus as factors. We tested for differences in dispersion among the different bacterial strains with the *betadisper* function in the "vegan" package (Oksanen *et al.* 2015).

We assessed differences in the number of cells g^{-1} of dry root biomass and the relative abundance of bacterial strains within our community experiment using ANOVA. We visualized differences in bacterial community composition with PCA ordination using cells g^{-1} of dry root biomass for all four bacterial strains as factors.

To predict plant biomass allocation patterns in *Populus deltoides* using plant biomass allocation patterns from single-strain inoculated *Populus* plants corrected for the relative abundance of our microbial community qPCR, we performed a one-sample t-test on our residuals (actual allocation – predicted allocation) with each bacterial community to measure deviations from zero. Significant deviations from zero suggested mismatches in predicted and observed values.

We performed a symmetrical co-inertia analysis to test for linkages between plant biomass allocation patterns, nutrient content, and bacterial community composition using the *coinertia* function in the "ade4" package (Dray & Dufour 2007). Briefly, this method calculates a covariance matrix from the two data tables used to construct plant phenotype PCA and bacterial community PCA, projected in a common ordination space of coinertia axes. We conducted a Monte-Carlo test with 999 iterations, using the *RV.test* function in the "ade4" package to explore the relationship between our PCAs (RV coefficient) and strength of the co-inertia test.

Results

Experiment 1: single bacterial strain influence on plant function.

To understand strain influence on plant phenotype, we first inoculated strains individually on *P. deltoides* and measured chlorophyll content and plant allocation patterns. Overall, we found that inoculation with individual bacterial strains altered plant host growth and function, but host response to bacterial colonization was strain specific (Fig 4). For instance, we found that inoculation of *Pseudomonas* GM30 lead to 9% more biomass being allocated to leaves (t = 2.78, p = 0.01), and 2% reduction in stem allocation (t = -1.77, p = 0.09) and a 7% reduction root allocation (t = -2.12, p = 0.04). Additionally, we observed a 17% decrease in chlorophyll content (t = -2.29, p = 0.03) and a 19% reduction in specific root length (t = -2.06, p = 0.05), suggesting stouter, wider diameter roots. Inoculation with closely related *Pseudomonas* GM41 decreased root mass allocation by 9% (t = -3.28, p = 0.003), without changing root functional traits (SRL t = -0.66, p = 0.51, SRA t = -0.91, p = 0.374). Aboveground, inoculation with *Pseudomonas* GM41 led to 71% taller plants (t = 2.13, p = 0.04) and a 9% increase in leaf biomass allocation (t = 3.20, p = 0.004) without changing stem biomass allocation (t = -1.02, p = 0.32), chlorophyll content (t = -0.91, p = 0.37),

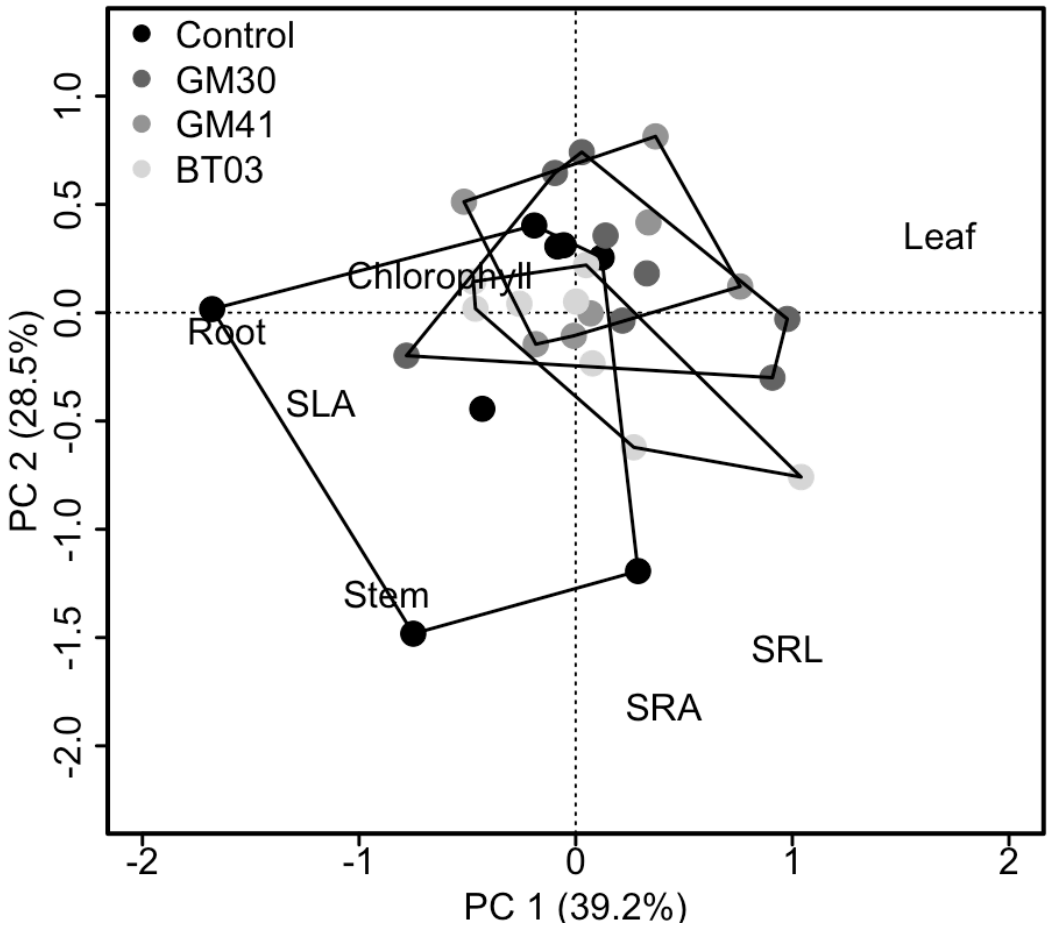

Figure 4. Principle component analysis of plant phenotype influenced by single microbe plant inoculation (Experiment 1). The PC1 axis explains approximately 41% of data variation within plant phenotype (SRL = specific root length, SLA = specific root area, leaf growth = leaf growth rate (cm^2 d^{-1}), shoot biomass = shoot biomass (mg), total biomass = total plant biomass (mg), stem growth = stem growth rate (cm d^{-1}), root biomass = root biomass (mg), root:shoot = root biomass (mg) / shoot biomass (mg)) while the PC2 axis explains 23% of phenotypic variation. In this PCA, GM30 plants are most different from control plants. GM30 plans have more biomass (root, shoot, and total), stem height, and leaf surface area then control plants. Control plants have a higher chlorophyll content and a higher root:shoot then all of the inoculated plants. There was no difference in dispersion among treatments (Betadisper, p = 0.10).

or specific leaf area (t = 0.30, p = 0.77). Inoculation with *Burkholderia* BT03 led to an 11% increase in leaf biomass allocation (t = 3.71, p = 0.001), and a 2% decrease in stem biomass allocation (t = -1.83, p = 0.07) and 9% decrease in root allocation (t = -3.17, p = 0.004). The decrease in root biomass allocation was matched with an 8% reduction in root tissue density (specific root surface area, t = -2.12, p = 0.05), however the increase in leaf biomass allocation did not result in any change in chlorophyll content (t = 0.82, p = 0.42) or specific leaf area (t = 0.42, p = 0.68). Thus, although strains may influence biomass allocation and functional traits differently, all bacterial strains generally lead to an increase in leaf biomass allocation at the expense of root allocation.

To understand whole-plant phenotype, we performed an ordination using plant biomass allocation patterns, leaf and root traits, as well as chlorophyll content with a principal components analysis. Overall, we found that root versus leaf allocation patterns between non-inoculated and inoculated plants drove about 39.2% of the variation along the first principal component and differences in leaf allocation and chlorophyll content versus root trait values explained 28.5% of the variation along the 2nd principal component (Fig 4).

Experiment 2: bacterial community composition influence on host function.

In mixed communities, we found bacterial community composition did not influence total plant biomass (f = 2.50, p = 0.08, Fig 5) in *P. deltoides*, however strain composition influenced the allocation of biomass toward leaves (f = 7.24, p = 0.001), roots (f = 7.05, p = 0001), or stems (f = 6.75, p = 0.002). For example, we found a 10% increase in leaf biomass allocation (t = 4.40, p = 0.001, Fig 5) and an 11% reduction in root biomass allocation (t = -4.68, p < 0.001, Fig 5), when comparing communities composed of *Pseudomonas* GM30, *Burkholderia* BT03, and *Pseudomonas* GM17 versus *Pseudomonas* GM30, *Burkholderia* BT03, and *Pseudomonas* GM41. Additionally, we found a 2% increase in stem biomass allocation (t = 3.61, p = 0.01, Fig 5) and an 8% reduction in root allocation (t = -3.04, p = 0.03, Fig 5) when communities were composed of *Pseudomonas* GM30, GM41, and GM17 compared to *Pseudomonas* GM30, GM41, and *Burkholderia* BT03. Taken together, this suggests bacterial community composition may be crucial to understanding plant resource acquisition strategies. Additionally, we found no difference in chlorophyll content (f = 1.38, p = 0.28) among our different bacterial community compositions, suggesting bacterial community composition had little influence on physiological traits of *Populus*.

Overall, we found bacterial community composition altered the nutrient acquisition ability of *P. deltoides*. Specifically, we found 19% higher shoot nitrogen content (mg N g^{-1}, t = 3.08, p = 0.03, Fig 6) and 6% higher root nitrogen content (mg N g^{-1}, t = 2.97, p = 0.05, Fig 6) when communities containing *Pseudomonas* GM41, *Burkholderia* BT03, and *Pseudomonas* GM17 were compared to communities composed of *Pseudomonas* GM41, *Burkholderia* BT03, and *Pseudomonas* GM30. Additionally, we found 7% higher root nitrogen

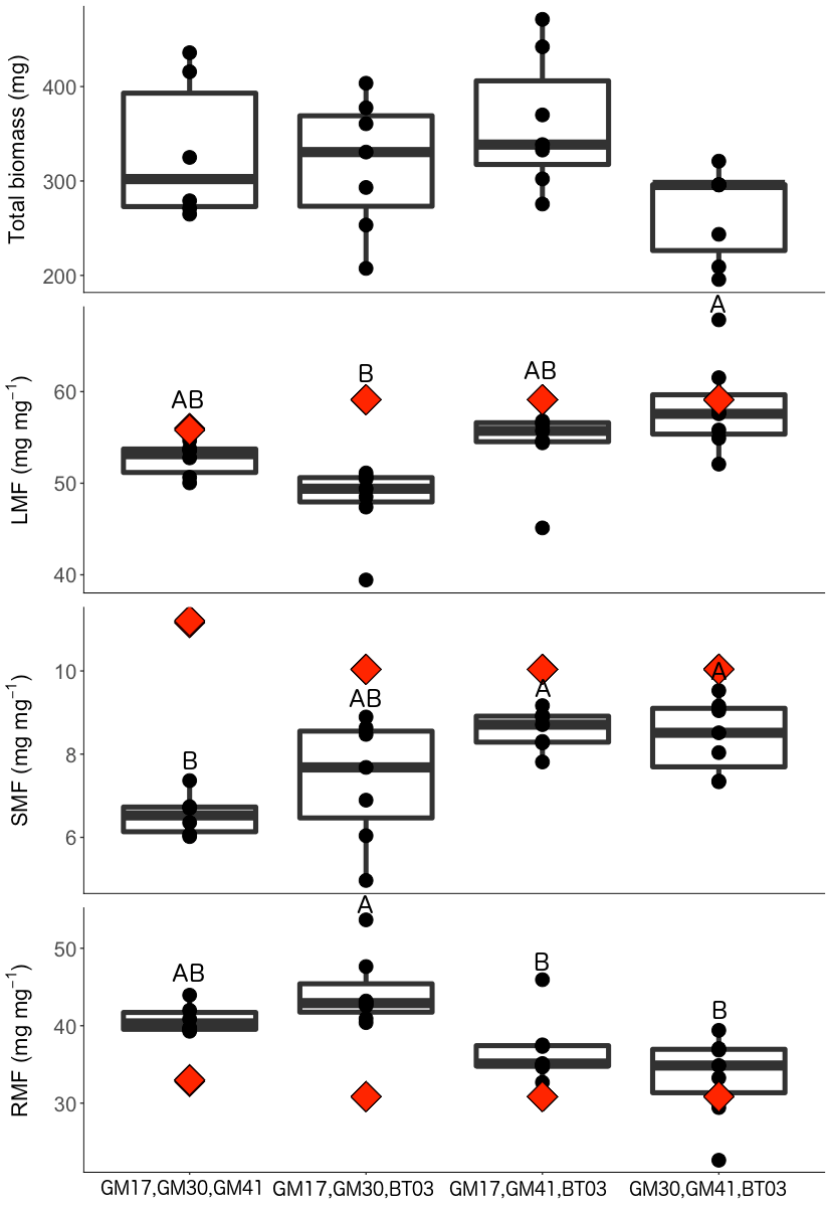

Figure 5. Changes in *Populus* morphology inoculated with four unique, 3-member endophyte communities composed of 4 different bacterial strains (17 = *Pseudomonas* GM17, 30 = *Pseudomonas* GM30, 41 = *Pseudomonas* GM41, Bt = *Burkholderia* BT03). (A) Total biomass production (mg). Plant biomass allocation patterns to leaves, stems, and roots displayed as leaf mass fraction (LMF) (B), stem mass fraction (SMF) (C), and root mass fraction (RMF) (D), calculated by leaf, stem or root biomass (mg) / total plant biomass (mg) × 100.

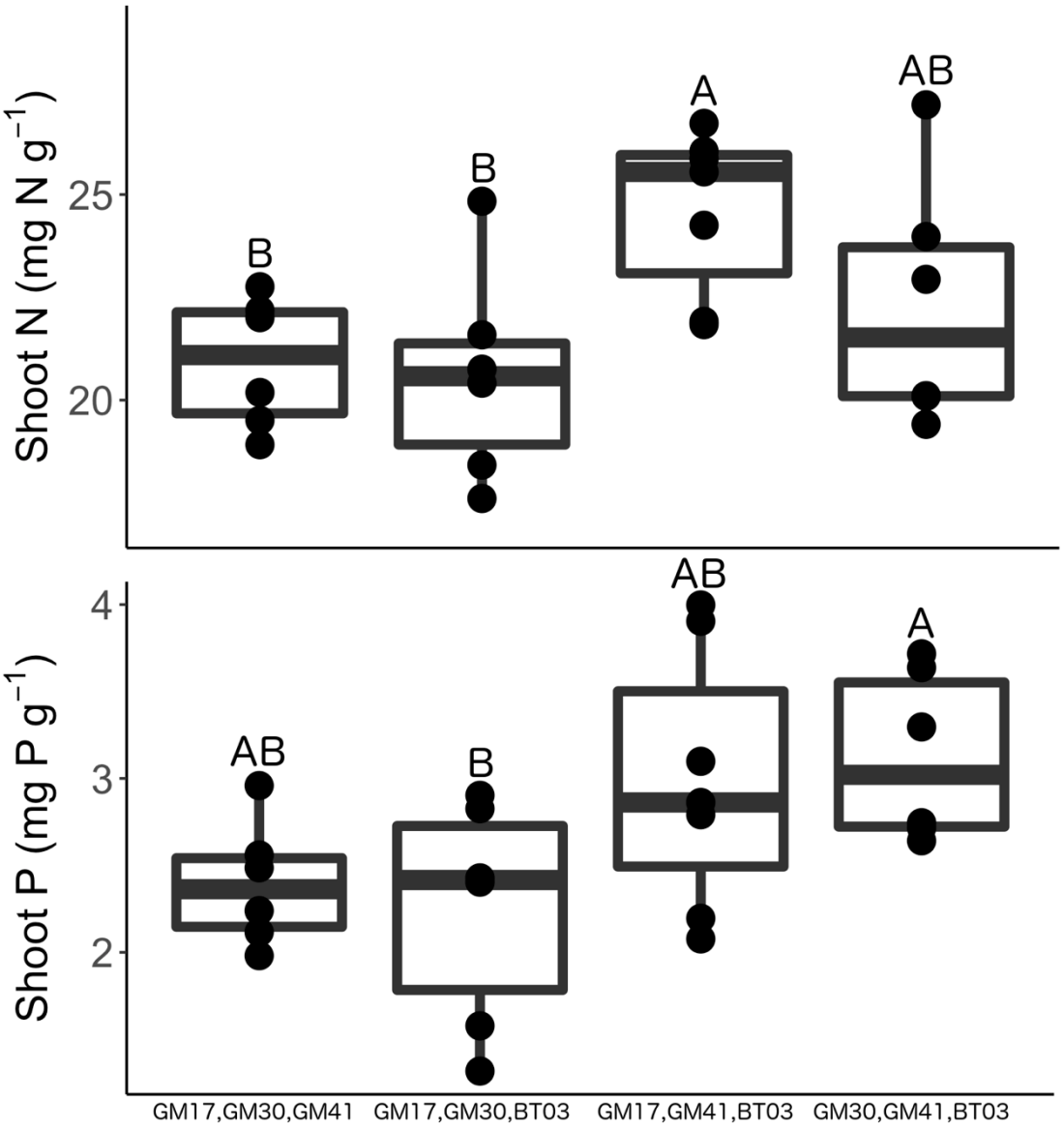

Figure 6. Total Kjeldahl nitrogen (A) and phosphorus (B) content in Populus dried green leaf tissue inoculated with four unique, 3-member endophyte communities composed of 4 different bacterial strains (GM17 = *Pseudomonas* GM17, GM30 = *Pseudomonas* GM30, GM41 = *Pseudomonas* GM41, Bt03 = *Burkholderia* BT03).

content and 32% higher shoot phosphorus content (mg P g^{-1}, $t = 2.59$, $p = 0.07$) when communities containing *Pseudomonas* GM30, *Burkholderia* BT03, and *Pseudomonas* GM17 were compared to communities composed of *Pseudomonas* GM30, *Burkholderia* BT03, and *Pseudomonas* GM41 (Fig 6). Although community composition influenced nitrogen and phosphorus content of both shoots and roots, we observed no differences in shoot ($f = 1.38$, $p = 0.28$) or root ($f = 0.42$, $p = 0.74$) nitrogen: phosphorus across different bacterial communities.

To understand overall plant phenotype across *Populus* individuals inoculated with different bacterial communities, we conducted a principal components analysis combining measures of plant biomass allocation toward leaves, stems, or roots, with shoot nitrogen and phosphorus content. Overall, we found our first principal component explained over 57% of variation in data and was associated with belowground versus aboveground biomass allocation patterns. Our second principal component explained 19% of the remaining data variation and was associated with a tradeoff between aboveground biomass allocation and tissue nutrient concentrations (Fig 7). We found that communities containing both *Pseudomonas* GM17 and GM30 clustered more closely together and were positively correlated with root biomass allocation, independent of whether the third community member was *Pseudomonas* GM41 or *Burkholderia* BT03. However, we found that when communities contained both *Burkholderia* BT03 and *Pseudomonas* GM41, independent of whether the third community member was *Pseudomonas* GM17 or GM30, were positively correlated with stem and leaf biomass allocation and shoot nitrogen and phosphorus content (Fig 7). This suggests that microbe-microbe interactions may be important for determining overall plant resource allocation and nutrient acquisition patterns.

Bacterial community composition

Overall, we found all four bacterial strains colonized *Populus* roots. Surprisingly, we found that strain abundance and relative abundance was not contingent on the other community members, and strains existed at similar abundance levels regardless of community composition. This suggests no competitive interactions among any bacterial strains in our constructed communities. However, bacterial strains colonized root tissues at different abundances. As predicted from single strain inoculations, *Burkholderia* BT03 colonized *Populus* roots greater than 2 orders of magnitude higher relative to any of the *Pseudomonas* strains. *Burkholderia* BT03 accounted for 96.98%, 99.79%, and 95.74% of the bacterial community relative abundance and differences in *Burkholderia* BT03 and *Pseudomonas* GM41 and GM17 explained 31% of the community variation our ordination (Fig 8). The second principal component (PC2) explained ~27% of the community variation and was driven by abundance changes between *Pseudomonas* GM30 and *Pseudomonas* GM17 (Fig 8).

To understand if changes in bacterial community composition were correlated to changes in plant biomass allocation and nutrient concentration we

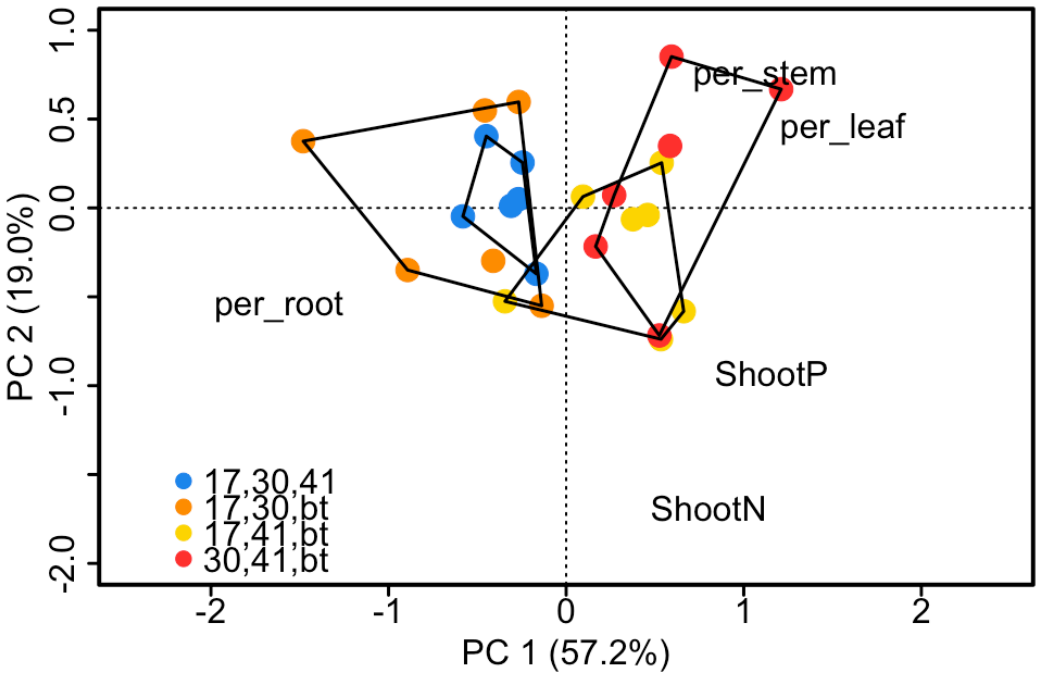

Figure 7. Populus deltoides phenotype principal component analysis. Axes composted of (per_stem = stem biomass (mg) / total biomass (mg) × 100, per_leaf = leaf biomass (mg) / total biomass (mg) × 100, per_root = root biomass (mg) / total biomass (mg) × 100, ShootP = Total Kjeldahl phosphorus (mg P g^{-1} leaf tissue), ShootN = Total Kjeldahl nitrogen (mg N g^{-1} leaf tissue) influenced by four unique bacterial treatments, composed of four bacterial strains (17 = *Pseudomonas* GM17, 30 = *Pseudomonas* GM30, 41 = *Pseudomonas* GM41, Bt = *Burkholderia* BT03).

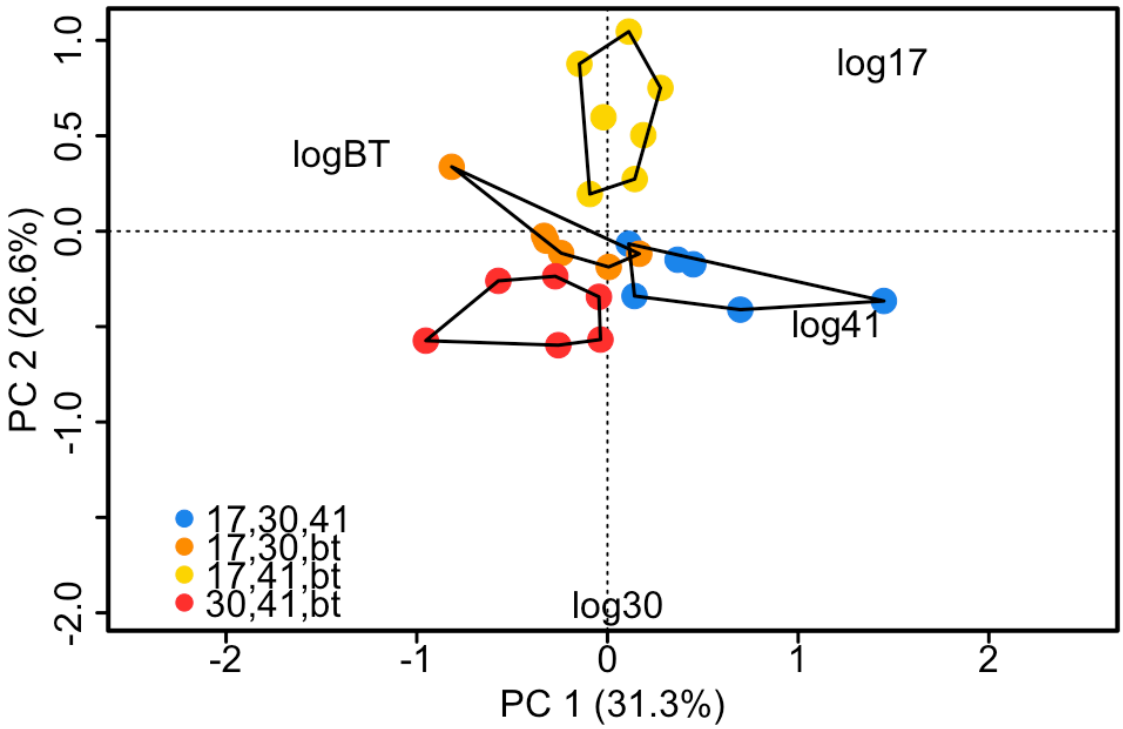

Figure 8. Principle component analysis of bacterial community composition composed of (log17 = log *Pseudomonas* GM17 cells mg^{-1} dry root material, log30= log *Pseudomonas* GM30 cells mg^{-1} dry root material, log41 = log *Pseudomonas* GM41 cells mg^{-1} dry root material, logBT= log *Burkholderia* BT03 cells mg^{-1} dry root material) across four unique bacterial treatments, composed of four bacterial strains (17 = *Pseudomonas* GM17, 30 = *Pseudomonas* GM30, 41 = *Pseudomonas* GM41, Bt = *Burkholderia* BT03).

performed a co-inertia analysis. Overall, we found little correlation between raw bacterial abundance numbers and plant phenotype (Total inertia = 0.0012, RV coefficient = 0.0032) (Fig 9). We conducted a Monte Carlo test to follow up our co-inertia analysis with 999 iterations and our results confirm the findings of the initial co-inertia analysis, with a weak relationship between bacterial community composition and plant phenotype (999 iterations, $p = 0.33$).

Predicting plant phenotype based on community composition

We used trait means from single-strain inoculated *Populus* plants in experiment 1 to predict the plant biomass allocation toward leaves, stems, or roots of mixed communities in experiment 2 using the qPCR relative abundance of bacterial communities. Overall, we found very little overlap in predicted allocation patterns and actual allocation patterns (Fig 5, Table 3). For example, in all communities except *Pseudomonas* GM30, GM41, and *Burkholderia* Bt03, we significantly over-predicted leaf and stem biomass allocation and under-predicted root allocation (Table 3). However, in communities containing *Pseudomonas* GM30, GM41, and *Burkholderia* Bt03, we over-predicted stem allocation ($t = -4.88$, $p = 0.003$), however we observed no difference in predicted versus observed values in leaf ($t = -0.46$, $p = 0.66$) and root ($t = 1.16$, $p = 0.28$) allocation (Table 3).

Discussion

Contrary to the expectations of the mass-ratio hypothesis, we found that subtle changes among low-abundant, closely-related root bacterial community members drove biomass allocation (Fig 5) and nutrient acquisition (Fig 6) patterns. We found almost no overlap between our predicted relative abundance-weighted biomass allocation patterns. For instance, our qPCR community quantification revealed that *Burkholderia* BT03 made up 97-99% in every community it was inoculated into. Thus, we predicted that biomass allocation patterns would resemble biomass allocation patterns of *Burkholderia* BT03 grown alone. However, we found that all three communities that contained *Burkholderia* BT03 had distinct plant biomass allocation and nutrient acquisition patterns (Fig 5). Our predicted values for biomass allocation consistently under-predicted root biomass allocation and over-predicted leaf and stem biomass allocation, although the prediction fits varied greatly among the bacterial communities (Fig 5). For instance, communities composed of *Pseudomonas* GM17, GM30 and *Burkholderia* BT03, we under-predicted root allocation by over 12%, and over predicted stem and leaf allocation by over 2% and 10%, respectively (Fig 5). However, in communities of *Pseudomonas* GM17, GM30, and GM41, root allocation was under predicted by only 8%, while stem allocation was over predicted by over 4% and leaf allocation was only over predicted by 3% (Fig 5). Taken together, our results suggest that plant biomass and nutrient

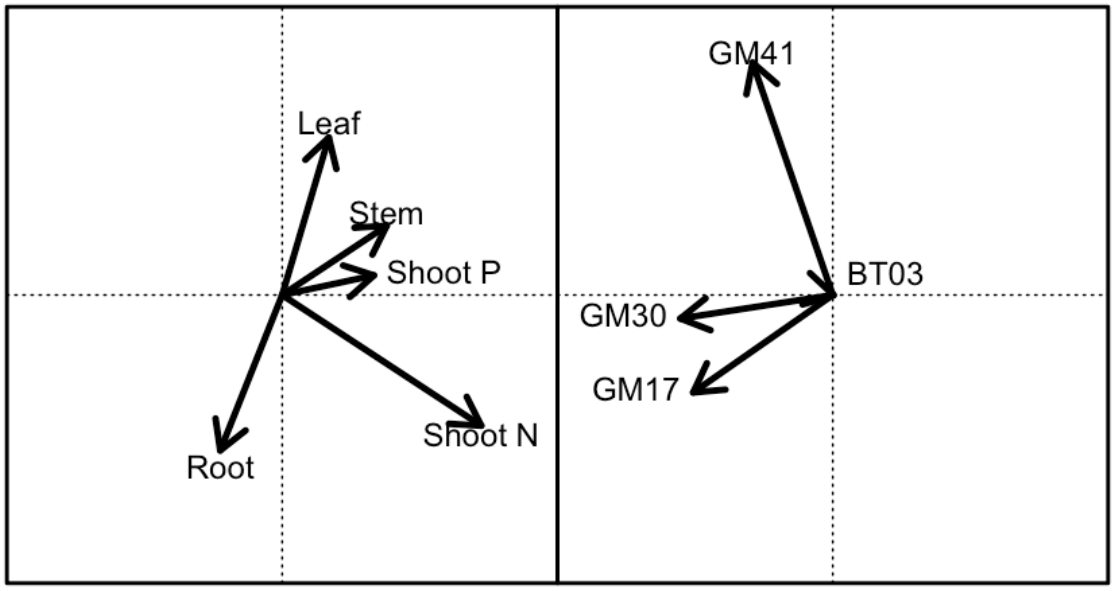

Figure 9. Co-inertia plot comparing how differences in the bacterial community influence plant trait expression. On the left, measures of plant phenotype (stem (stem mass fraction), leaf (leaf mass fraction), root (root mass fraction), Shoot P (mg P g^{-1}), Shoot N (mg N g^{-1}) are displayed in co-intertia space. On the right, microbial community composition: GM17 (log *Pseudomonas* GM17 cells mg^{-1}), GM30 (log *Pseudomonas* GM30 cells mg^{-1}), GM41 (log *Pseudomonas* GM41 cells mg^{-1}), BT03 (log *Burkholderia* BT03 cells mg^{-1}) are displayed on co-inertia space. (Total inertia = 0.0012, RV coefficient = 0.0032.

acquisition were influenced by the combination and interactions between closely-related, low abundant *Pseudomonas* strains.

Our results provide strong evidence in the role of 1) low-abundant and 2) closely-related bacterial endophytes in driving plant phenotype and function, challenging two long-standing assumptions in community ecology. In microbial communities, the high diversity and assumed, high functional redundancy, have limited experimental tests of the importance of low abundant or rare and closely-related organisms. However, several recent studies have challenged assumptions of functional redundancy among closely-related bacterial strains (Zamioudis *et al.* 2013; Henning *et al.* 2016; Timm *et al.* 2015) and the role low abundant organisms in system functioning (Clay & Holah 1999; Hol *et al.* 2010; 2015; Mouillot *et al.* 2013; Gilbert *et al.* 2016; Hausmann *et al.* 2016; Hedin *et al.* 2017). For instance, closely-related *Pseudomonas* GM17, GM30, and GM41 strains which are classified under the same 16S OTU profile (Brown *et al.* 2012), differ in their metabolic capabilities and their influence on plant phenotype (Timm *et al.* 2015; Henning *et al.* 2016). This suggests that caution must be given when linking phylogenetic relatedness to functional overlap in microbial communities. Additionally, although empirical tests may be limited, low abundant microbes can have important impacts on plant performance (Hol *et al.* 2010; 2015). In one prominent study Hol and colleagues (2010) found that the removal of low abundant soil microbes resulted in increased plant biomass but decreased tissue nutrient quality, which negatively influenced plant herbivores. However, methodological limitations prevented the authors from identifying the specific strains that were involved in phenotypic changes. Here, we provide a direct tests of mass-ratio hypothesis within plant endophyte communities to link endophyte community composition to plant function.

Our results suggest that microbe-microbe interactions may be more important than absolute relative abundances in driving plant function and that low abundant strains may disproportionately influence plant function. The next step is to explore the mechanisms underlying microbe-microbe interactions that shape plant phenotype. For instance, microbe-microbe cross-communication (Davis *et al.* 2008), syntrophic (Orphan 2009), or antagonistic or competitive interactions (de Boer *et al.* 2007) may result in non-additive phenotypic effects (Foster & Bell 2012; Pande *et al.* 2013; Hussa & Goodrich-Blair 2013; Kemen 2014). Additionally, unique functional capabilities (Hong & Rhee 2014; Hol *et al.* 2015) or micro-niche differences among strains may allow *Pseudomonas* strains occurring at low abundance to exert a strong influence on plant phenotype (Ofek-Lalzar *et al.* 2014; Lynch & Neufield 2015).

Our results reinforce the necessity to incorporate community dynamics and species interactions in to plant-microbe studies. A recent flurry of papers that have demonstrated the ability of plant endophytes to control plant gene expression, immune response, and overall functioning however are conducted in single-microbe systems and ignore the role of species interactions in diverse communities. Likewise, large-scale correlative studies exploring the relationship

between endophyte communities and plant phenotype cannot determine how specific strains or their interactions influence plant function. To truly dissect the linkages between endophytes and plant function will require a combined approach in which controlled, constructed community approaches can be used to test mechanisms from patterns that have been observed in natural ecosystems. Our study provides one of the first explicit tests of the influence of low-abundant bacterial endophytes in driving biomass allocation patterns and nutrient concentrations in *Populus* hosts. We have provided strong evidence that microbe-microbe interactions and not simple presence/absence of single strains are important drivers of plant phenotype, even though the mechanisms underlying these patterns remain unknown.

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Appendix

Table 3. Residual differences in observed and predicted leaf, stem, and root biomass allocation in response of 4 different bacterial community composition values analyzed by one-sample t-tests.

Tissue	Treatment	mean	95% conf int	t	df	p value
Leaf	17,30,41	-3.303	(-5.24, -1.36)	-4.376	5	0.007
Leaf	17,30,bt	-10.960	(-14.72, -7.20)	-7.131	6	0.0003
Leaf	17,41,bt	-4.590	(-8.64, -0.54)	-2.772	6	0.03
Leaf	30,41,bt	-0.895	(-5.65, -3.85)	-0.461	6	0.66
Stem	17,30,41	-4.647	(-5.19, -4.10)	-21.870	5	>0.0001
Stem	17,30,bt	-2.669	(-4.03, -1.31)	-4.800	6	0.003
Stem	17,41,bt	-1.453	(-1.89, -1.02)	-8.125	6	0.0002
Stem	30,41,bt	-1.616	(-2.43, -0.81)	-4.879	6	0.003
Root	17,30,41	7.949	(6.06, 9.84)	10.820	5	0.0001
Root	17,30,bt	13.630	(9.30, 17.96)	7.703	6	0.0002
Root	17,41,bt	6.043	(2.04, 10.04)	3.698	6	0.01
Root	30,41,bt	2.510	(-2.76, 7.79)	1.165	6	0.28

CHAPTER III
NITROGEN, NEIGHBORS, AND THE GHOSTS OF NEIGHBORS PAST SHAPE THE COLONIZATION PATTERNS OF CO-OCCURRING ROOT-COLONIZING FUNGAL SYMBIONTS

The Following section is a slightly modified version of a paper in preparation:

Henning, J.A., Read, Q.D., Sanders, N.J., A.T. Classen. *In prep*. Nitrogen, neighbors, and the ghosts of neighbors past shape the colonization patterns and plant-soil feedbacks of co-occurring root-colonizing fungal symbionts.

The use of "we" in this chapter refers to my co-authors and me. As the lead author of this article I was responsible for this paper. Q.D.R., A.T.C. and N.J.S. designed and established field experiment, J.A.H and A.T.C. designed the fungal sampling, J.A.H. analyzed data, J.A.H, N.J.S., and A.T.C. designed the greenhouse experiment, and J.A.H. conducted the greenhouse experiment, J.A.H. wrote the manuscript, and all other co-authors assisted with writing and revision.

Abstract

1. Root-associated fungal symbionts, including arbuscular mycorrhizal fungi (AMF) and dark septate endophytes (DSE), regulate plant productivity, access to soil nutrients, and plant competition. Thus, root-associated fungal communities will mediate ecosystem responses to global change drivers like nitrogen (N) deposition and non-random species extinctions.

2. Here, we tested how three years of N addition and removal of a dominant plant species, *Festuca thurberi*, independently and interactively influence fungal colonization patterns in co-dominant plant species, *Helianthella quinquenervis.* Overall, we found that distance from *Festuca* but not N addition or *Festuca* removal predicted *Helianthella* fungal colonization. Specifically, DSE colonization decreased by 1% for each 1 cm increase in distance from *Festuca*, although AMF colonization was consistently high (~78%) independent of distance from *Festuca*. Thus, neighbors and legacy of past neighbors are strong determinants of fungal community composition.

3. As a follow-up, we conducted a plant-soil-feedback experiment to explore whether neighbor-mediated alteration of fungal communities had any tradeoffs for plant performance. We found a positive relationship between seedling survival and distance from *Festuca* in the greenhouse. Surprisingly, we found that if *Helianthella* survived in soils that originated near *Festuca*, there were no apparent performance tradeoffs in the field or in greenhouse-grown *Helianthella*.

4. Synthesis. We found that contemporary as well as the ghosts of neighbors past can shape fungal communities associated with focal plant species. Further, changes in fungal communities can alter survival of focal species, which may have important implications for plant community composition and the resistance and resilience of ecosystems facing ongoing global change.

Introduction

Decades of research in plant community have demonstrated that soil resource availability and dominant species both independently and interactively influence the structure and function of plant communities (Suding *et al.* 2004; McLaren & Turkington 2010; Lavoral 2013; Souza *et al.* 2015). For example, nitrogen (N) addition typically increases plant productivity as resource limitations are relaxed (Elser *et al.* 2007; LeBauer & Treseder 2008; Read *et al.* 2017). However, the response of community composition to N addition varies among ecosystem types and likely depends on dominant and subordinate plant species as well as soil and climate properties (Foster & Gross 1998; Elser *et al.* 2007; Johnson *et al.* 2015; Farrer & Suding 2016). However, few of these studies have simultaneously investigated the role of root-associated fungi in mediating these idiosyncratic outcomes (Urcelay *et al.* 2009; Farrer & Suding 2016). Because root-associated fungal communities, which are composed of fungi from multiple functional groups like arbuscular mycorrhizal fungi (AMF) and dark septate endophytes (DSE), regulate plant productivity (van der Heijden *et al.* 1998), access to soil nutrient resources (Smith & Read 2009), and plant-plant competitive interactions (Scheublin *et al.* 2007), root-associated fungi will likely influence the response of plant community composition and ecosystem function to global change drivers such as nitrogen (N) deposition and non-random species extinctions.

The composition and function of root-associated fungal communities are influenced by soil nutrient availability (Egerton-Warburton & Allen 2000; Johnson *et al.* 2003; Henning *et al.* in review), plant host identity (Bever *et al.* 1996; Vandenkoornhuyse *et al.* 2002; 2003; Verbruggen *et al.* 2012), and plant neighbors (Hausmann and Hawkes 2009). For example, N addition leads to predictable losses of conservative, beneficial fungal strains with increases in less-beneficial, weedy mycorrhizal species (Egerton-Warburton *et al.* 2007; Henning *et al.* in review). Additionally, N addition typically reduces plant carbon allocation toward roots and fungal associates, leading to higher competition among fungi for plant resources (Högberg *et al.* 2003; Kennedy 2010). However, plant and fungal response to nitrogen addition are contingent on ecosystem properties and plant community composition (Johnson *et al.* 2003; Egerton-Warburton *et al.* 2007). Additionally, plant roots can be colonized by multiple fungal functional groups, and N addition or shifts in plant composition may shift competitive interactions between fungal groups. For instance, in many ecosystems AMF co-occur with DSE, often co-colonizing roots (Zubek *et al.* 2009; Chaudry *et al.* 2009). Changes in plant community composition and nutrient availability may shift the composition and function of fungal endophyte communities, since AMF and DSE differ in their functional capabilities (Mandyam and Jumpponen 2014; Caldwell *et al.*2000), services they provide to plant host (Jumpponen 2001; Newsham 2011), host preferences (Haselwandter & Read 1982; Jumpponen 2001), and physiological tolerances (Mandyam *et al.* 2012;

Alberton *et al.* 2010). Shifts in fungal community composition and function can feedback to further influence plant community dynamics and ecosystem function (Johnson *et al.* 2004; Wardle & Zackrisson 2005; van der Heijden *et al.* 1998). However, direct tests of these feedbacks have been limited (Ureclay *et al.* 2009; Marshall *et al.* 2011).

Here, we tested how N addition and removal of a dominant plant species, Festuca thurberi, both independently and interactively influence fungal colonization patterns in a co-dominant plant species, Helianthella quinquenervis. Second, we tested whether changes in fungal colonization patterns feedback to shape the performance of Helianthella in the field and in the greenhouse. First, we measured fungal colonization in Helianthella within a three-year N addition × dominant plant species (Festuca thurberi) removal experiment near Gothic, Colorado (Read *et al.* 2017). Second, to understand how N addition and dominant removal fed back to alter Helianthella performance in the field and in the greenhouse, we measured Helianthella height in the field and used field-collected soils in which we measured Helianthella field colonization patterns as inoculum for a greenhouse plant-soil feedback experiment. In this study, we asked the following questions:

- Do organic and inorganic N fertilization differ in their effects on the AMF and DSE colonization patterns of Helianthella quinquenervis in a montane meadow?
- Does the presence of a dominant tussock grass species, Festuca thurberi, affect the AMF and DSE colonization patterns of co-dominant Helianthella quinquenervis?
- Are the effects of dominant species on Helianthella colonization patterns contingent on soil N or distance from Festuca?
- Do changes in fungal colonization patterns feedback to mediate Helianthella performance

Materials & methods

N addition & Festuca removal experiment

The experimental design and site characteristics are outlined in Read *et al.* (2017). Briefly, in the summer of 2012, we established 36 permanent 1.5 × 1.5 m plots in Maxfield Meadow, a montane meadow on the property of the Rocky Mountain Biological Laboratory (RMBL), Gothic, Colorado, USA (38°56′58″, 107°59′34″W) at 2910 m above sea level. The two most abundant plant species found in the meadow are Festuca, thurberi, a large, DSE-associated, perennial tussock grass (Poaceae) and *Helianthella quinquenervis* (Asteraceae). Other abundant species include *Erigeron speciosus* (Asteraceae), *Heliomeris multifleris* (Asteraceae), and *Bromopsis inermis* (Poaceae).

Within the 36 plots, we established a full factorial cross (n = 4) of N addition and dominant species removal. We had three levels of N treatment in which we added 10 g organic N m^{-2} y^{-1} (as 21.7 g urea, $(NH_2)_2$), addition of 10 g inorganic

N m^{-2} y^{-1} (as 29.4 g ammonium nitrate, NH_4NO_3), and no N addition. Overlaid on the N addition was a three-factor dominant species (*Festuca thurberi*) removal, control, or random biomass removal. In the *Festuca* removal treatment, we removed all aboveground *Festuca* biomass within the 1.5 × 1.5 m plot. After *Festuca* removal, we applied a dilute mixture of glyphosate herbicide to kill the remaining belowground biomass. *Festuca* removal was conducted yearly to remove any new *Festuca* from removal plots. In the control treatment plots, we removed no biomass. In the random biomass removal plots, we first calculated percent cover of *Festuca*, then removed an equivalent amount of vegetative cover was randomly selected from among all species in the plot, including *Festuca*, and removed. We sampled only the control plots and the *Festuca* removal plots giving us a total of 24 plots.

Helianthella root sampling

Within each plot, we identified all individual *Helianthella* and *Festuca* plants and haphazardly selected three individual *Helianthella* plants of similar size growing within the plot. Prior to sampling, we measured the distance from each *Helianthella* to the closest *Festuca* individual (either intact or removed). Additionally, we haphazardly selected one *Festuca* individual in each plot to measure background *Festuca* colonization patterns. Under each selected *Festuca* and *Helianthella* individual, we collected a single soil core (2.5-cm × 15-cm) from directly under individual host plant. Soil cores were transported back to Rocky Mountain Biological Laboratory on ice, and stored at 4° C until processed.

Mycorrhizal colonization

To measure mycorrhizal colonization, soil cores were dry-sieved at 2mm; to remove all soil from the alive intact-roots and the remaining soil was frozen to use as inoculum for the plant-soil feedback experiment. The root system of each plant was clipped into 2-cm lengths and placed in Fisherbrand Histosette II tissue cassettes (Thermo Fisher Scientific, Waltham, MA, USA). We then cleared root samples in a 10% KOH solution, followed by an acidification in a 2% HCl solution, and stained roots using a 0.05% trypan blue solution (Koske & Gemma 1989, Henning *et al.* in review). After staining, root segments were mounted on glass microscope slides using Polyvinyl-lacto-glycerol (PVLG) (INVAM 2013). We assessed AMF and DSE colonization using the magnified intersection method (McGonigle *et al.* 1990). A minimum of 50 random root intersections were inspected for each slide and % AMF and % DSE colonization was determined as number of root segments with AMF hyphae, arbuscules, and vesicles, or DSE hyphae. Additionally, we monitored root samples for presence of possible pathogens.

Plant height

To assess differences in *Helianthella* performance in response to N addition and *Festuca* removal, we measured height (cm) of the tallest stem of each

Helianthella individual we sampled to assess mycorrhizal colonization.

Plant-soil feedback experiment

To explore differences in *Helianthella* performance in response to removal, N addition and distance from *Festuca*, we used the field-collected soils from our colonization study as inoculum to establish a greenhouse plant-soil feedback experiment. Each soil sample was split into four equal parts (~50ml of inoculum). Two of the parts were sterilized to remove soil microbes, while the two remaining soil samples were treated as "live" control soils. Additionally, one sterile soil sample and one live soil sample were randomly selected to receive a *Festuca* pre-treatment, while the remaining sterile and live inoculums only received a *Helianthella* seedling. These four soil treatments allowed us to mechanistically test how plant neighbors shape fungal communities. For instance, contrasting *Helianthella* growth in live soil with and without *Festuca* pre-treatment allowed us to test if *Festuca* actively primed soil communities. Next, we tested the role of soil community in *Festuca* priming of soils by contrasting live versus sterilized *Festuca* pre-treated soils. Contrasting live and sterile non-*Festuca* pre-treated sols allowed us to test the role of legacy soil communities shaping *Helianthella* growth. We measured explore the role of non-microbial *Festuca*-effects on *Helianthella* by contrasting growth in sterile soils with and without *Festuca* pre-treatment.

To test for differences in *Festuca* priming effects and differences in inoculum potential between field-collected soils, 750 ml of twice-autoclaved field-collected soil was added to 1L pots, followed by 50 ml of inoculum, then 200 ml of twice-autoclaved field-collected soil to reduce inoculum-inoculum contamination. Next, pre-germinated *Festuca* seedlings were transplanted in our *Festuca*-conditioned live and sterile soils and grown in greenhouses at the University of Tennessee for three months. After 3-months, we clipped all *Festuca* aboveground biomass from the pot and transplanted a pre-germinated *Helianthella* seedling into each pot. All removed *Festuca* biomass was oven-dried and weighed. The second set of sterile and live soils received only the *Helianthella* addition. *Helianthella* seedlings were grown in all pots for 4 months. We measured plant height and leaf number bi-weekly to assess for growth differences among soil treatments. After 4 months of growth, we harvested *Helianthella* plants and measured aboveground, belowground biomass, and fungal colonization were measured using the methods described above.

Statistical analysis

All statistical analyses were conducted in R version 3.3.2 (R development core team, 2016) and RStudio version 1.0.44 (RStudio 2016), with packages cited where applicable. To explore the influence of N addition, *Festuca* removal, and the distance from *Festuca* on the AMF colonization, DSE colonization, and *Helianthella* plant height, we constructed linear mixed-effect models using the "lme4" package (Bates *et al.* 2014). Within our mixed models, we tested for

differences in DSE colonization, AMF colonization, and plant height response variables and modeled N addition and *Festuca* removal as fixed effect with distance from *Festuca* modeled as a random factor. To test for significance of N addition and *Festuca* removal (fixed effects), we constructed models with and without fixed effects and performed a likelihood ratio test and compared AIC scores. If including fixed factors (N and removal) significantly improved model fit (p value < 0.05 in likelihood ratio test), we calculated least square means and 95% confidence intervals using the *difflsmeans* function in the "lmerTest" package (version 2.0-3, Kuznetsova *et al.* 2014).

To test for differences in AMF structures, vesicles, spores, arbuscules, and potential root pathogens, oomycotans among N addition, distance from *Festuca*, and *Festuca* removal treatments, we constructed generalized linear models using a Poisson distribution. N addition, distance from *Festuca*, and *Festuca* removal were all incorporated as predictor variables.

To understand the role of soil legacies and soil sterilization shaping the growth of our *Festuca* pre-treatment plants, we constructed linear mixed-effects models with N addition, *Festuca* removal, and soil sterilization modeled as fixed effects and the distance of our inoculum from *Festuca* in the field modeled as a random factor, similar to the above model. We constructed a full model that incorporated soil sterilization, N addition, and *Festuca* removal, as well as simplified models removing fixed factors. We selected the best fit model as described above. Overall, we found that our best fit model only retained soil sterilization as the sole fixed factor (AIC=287.85, SI Table X). Overall, *Festuca* plants in live soil were ~19% larger than *Festuca* grown in sterile soil (χ^2 = 6.1877 p = 0.013). Because we found no influence of N addition or *Festuca* removal in field measurements of fungal colonization, *Helianthella* field height, or greenhouse *Festuca* biomass, we did not include N or removal treatments in the remaining statistical models of greenhouse survival, growth, or soil feedbacks. We also tested for a relationship between pre-treatment *Festuca* biomass and *Helianthella* biomass, with Pearson's correlation test because increased *Festuca* growth may have reduced nutrient availability for *Helianthella*. However, we found no relationship between aboveground biomass of pre-treatment *Festuca* growth (conditioning phase) and total *Helianthella* biomass (r^2 = 0.02, p = 0.1928)

To understand how soil treatment influenced *Helianthella* survival over the duration of the experiment, we first transformed growth data into binary survival data (0 = dead, 1 = alive) and fit a generalized linear mixed model using the binomial distribution. We modelled *Festuca* pre-treatment (2 levels, *Festuca* pre-treatment or control) and soil activity (2 levels, live or sterile), and sampling date as fixed factors and modeled inoculum ID nested within the plot from which the inoculum originated as random effects. We constructed the full model described above, as well as models removing each of the fixed effects individually, and a null model with no fixed factors. Models were constructed using the *glmer* function with the "lme4" package (Bates *et al.* 2015) and we used AIC scores to determine the best fit model.

To test how *Festuca* pre-treatment, soil sterilization, and distance from *Festuca* shape the growth of *Helianthella*, we analyzed our bi-weekly leaf number and tallest leaf height data with a repeated-measures ANOVA. For both leaf number and tallest leaf length models, we included soil sterilization, *Festuca* pre-treatment, and inoculum distance as predictor variables and calculated error terms as soil treatment (sterile soil with *Festuca* pre-treatment; sterile soil without *Festuca* pre-treatment; live soil with *Festuca* pre-treatment; live soil without *Festuca* pre-treatment) nested within sampling date. To test for differences among the soil treatments, we calculated post-hoc pairwise t-tests with Bonferroni corrections.

To explore how soil sterilization, *Festuca* pre-treatment, and distance from *Festuca* shaped *Helianthella* traits and biomass, we constructed linear models with predictors of *Festuca* pre-treatment, soil sterilization, and distance from *Festuca*, to explore differences in total *Helianthella* biomass, green leaf biomass, belowground biomass, and biomass root:shoot. We tested for differences in biomass across treatments using the *Anova* function in the "car*"* package (Fox *et al.* 2011).

To test for potential mechanisms shaping plant-plant-mycorrhizal interactions, we performed pairwise feedback contrasts, feedback = Treatment A[i] / Treatment B[i] (pairwise), paired by soil inoculum origin (van der Putten *et al.* 1993; Brinkman *et al.* 2010) across our different soil treatments. Our first contrast tested our live soil inoculum treatments with and without *Festuca* pre-treatment. Observed deviations from 1, tested if *Festuca* actively primed soil communities. Our second contrast tested the role of the soil community in *Festuca* priming of soils by contrasting live versus sterilized *Festuca* pre-treated soils. Our third contrast compared our non-*Festuca* pre-treated live versus sterilized soils to test the role of legacy soil communities shaping *Helianthella* growth. The fourth and final contrast tested non-microbial *Festuca*-effects on *Helianthella* growth by contrasting *Helianthella* growth in sterile soils with and without *Festuca* pre-treatment. After feedback calculations were conducted, we then binned the feedback values based on the distance from *Festuca* in three categories, near = 0-10cm, mid = 11-20cm, and far = +21cm. Next, we conducted a 1-sample t-test on each of our binned feedback scores to test for deviations from 1. For example, a deviation greater than 1, in our first contrast, would suggest that *Festuca* positively primed soil communities for neighboring *Helianthella*. However, a negative deviation from 1 would suggest that *Festuca* primed a soil community that negatively influences *Helianthella* growth.

Results

Field experiment

Surprisingly, neither N addition nor *Festuca* removal altered AMF (N addition: $\chi 2 = 0.0$, $p = 1.0$, *Festuca* removal: $\chi 2 = 0.0$, $p = 1.0$) or DSE (N addition: $\chi 2 = 3.35$, $p = 0.07$, *Festuca* removal: $\chi 2 = 0.50$, $p = 0.48$) colonization

patterns in *Helianthella* (Fig 10). However, distance from *Festuca* was retained in the best model to predict DSE colonization patterns within *Helianthella*. Distance from *Festuca* explained 31% of the variation in DSE colonization in *Helianthella*. *Helianthella* growing near *Festuca* had relatively high DSE colonization rate (~70%), but colonization dropped by 1% for each additional 1 cm of distance from *Festuca* ($r^2 = 0.31$, $p < 0.001$, Fig 10). The positive effect of distance from *Festuca* on DSE colonization, combined with the lack of *Festuca* removal effect, suggests that there is a strong legacy of *Festuca* on DSE colonization patterns. Arbuscular mycorrhizal fungal colonization of *Helianthella* was unaffected by distance from *Festuca* ($r^2 = 1.9e^{-6}$, $p = 0.99$, Fig 10). AMF colonization was consistently high (~80%) in *Helianthella* roots independent of N addition, removal, or distance from *Festuca* (Fig 10). Additionally, we found no effect of N addition, *Festuca* removal, or distance from *Festuca* on any AMF structures (vesicles: N addition $\chi^2 = 0.027$, $p = 0.99$, removal $\chi^2 = 0.014$, $p = 0.91$, distance from *Festuca* $\chi^2 = 0.028$, $p = 0.87$; coils: N addition $\chi^2 = 0.001$, $p = 0.99$, removal $\chi^2 = 0.037$, $p = 0.85$, distance from *Festuca* $\chi^2 = 0.011$, $p = 0.92$), or potential root pathogens, oomycotans (N addition $\chi^2 = 0.024$, $p = 0.99$, removal: $\chi^2 = 0.039$, $p = 0.84$, distance from *Festuca*: $\chi^2 = 0.200$, $p = 0.65$) (Table 4). Surprisingly, we found no differences in *Helianthella* height in response to N addition ($\chi^2 = 1.59$, $p = 0.21$), *Festuca* removal ($\chi^2 = 0.80$, $p = 0.37$), or distance from *Festuca* ($r^2 = 0.01$, $p = 0.46$) (Fig 11). We found that *Helianthella* height was highly variable between treatments and distances from *Festuca* which likely reflects differences in plant age or microsite variation.

Plant-soil feedback experiment

We found that sampling date and *Festuca* conditioning were retained in our best model to predict *Helianthella* survival. As expected, we found that *Helianthella* survival declined over time ($z = -4.930$, $p < 0.0001$). We found that *Festuca* pre-treatment increased *Helianthella* survival by about 10% relative to *Helianthella* grown without *Festuca* pretreatment ($z = 4.983$, $p < 0.0001$). Surprisingly, the activity of soil communities (live or sterile) was not retained in our best fit model.

To explore how soil legacy shaped plant survival at the time of harvest, we modelled *Helianthella* survival and included sterilization, *Festuca* pre-treatment, the distance from *Festuca*, as well as their interactions as predictors. Overall, we found a significant interaction in *Helianthella* survival between soil sterilization and distance from *Festuca* ($z = -2.09$, $p = 0.037$). In sterilized soils, *Helianthella* survival was highest in soils closest to *Festuca* and survival was reduced with increasing distance to *Festuca*, however in live soils, *Helianthella* survival

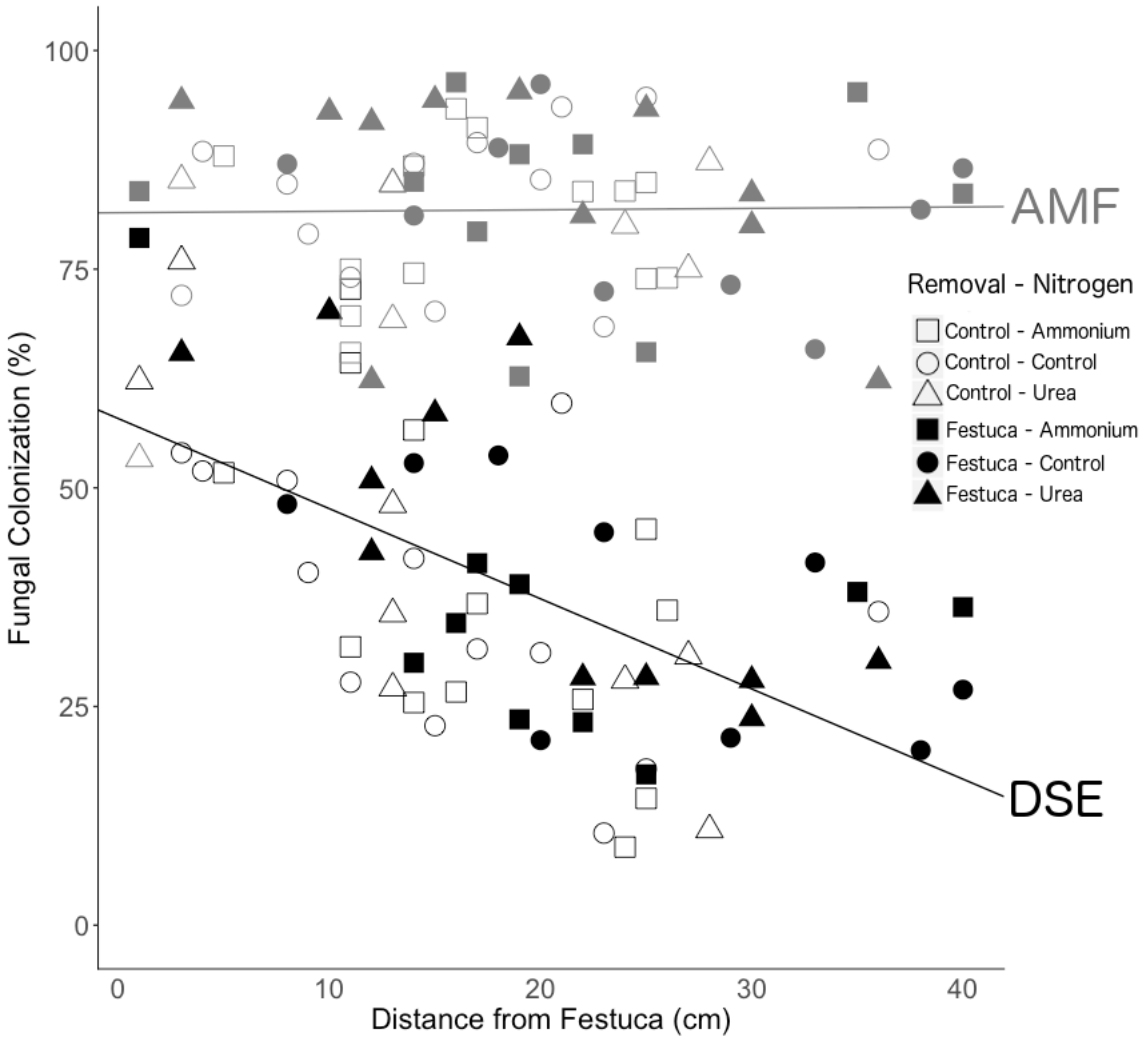

Figure 10. AMF (gray) and DSE (black) colonization of *Helianthella quinquenervis* across different distances from *Fesutca thurberi* in a nitrogen addition and Festuca removal.

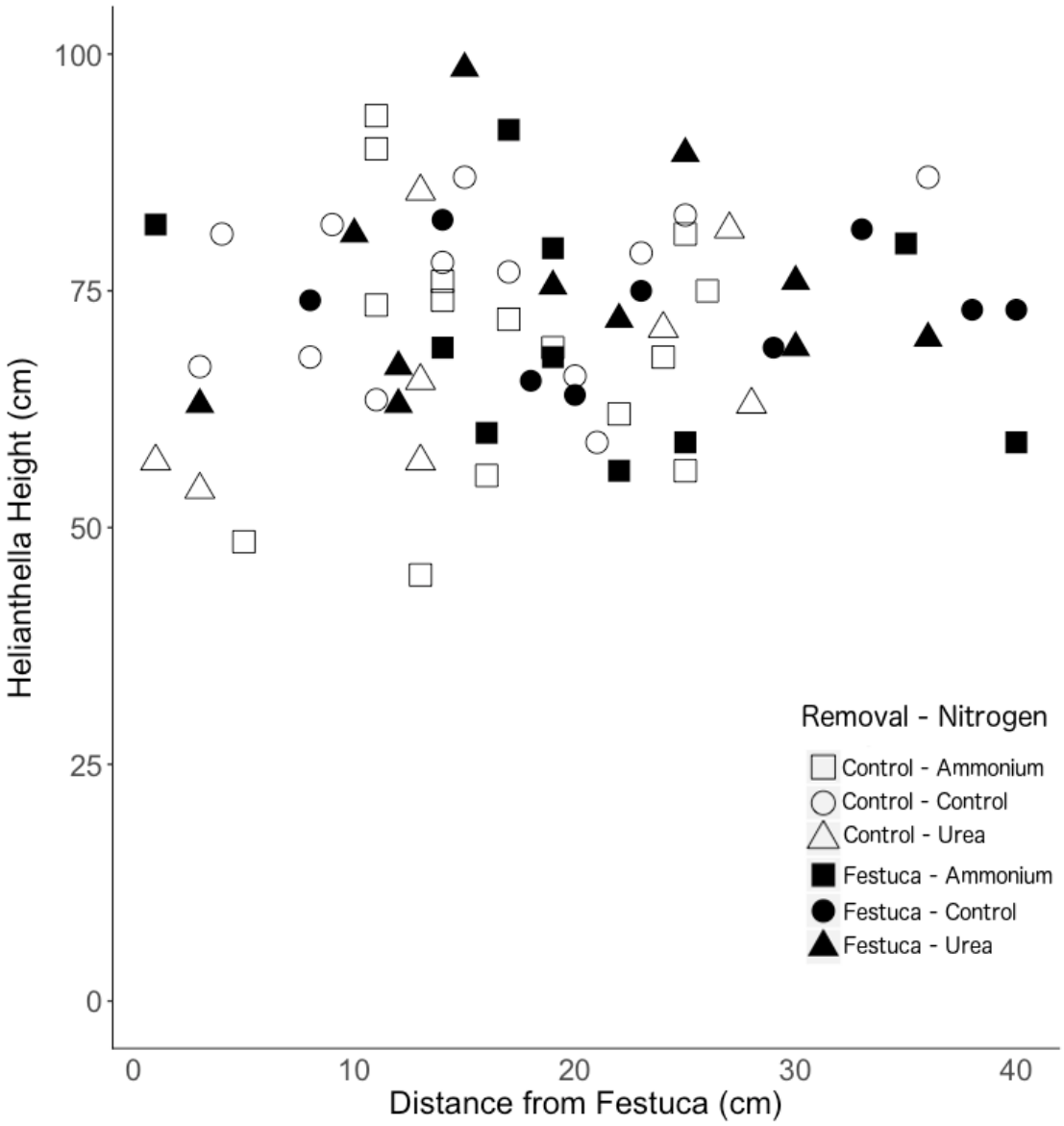

Figure 11. Plant height of *Helianthella quinquenervis* at various distances from *Festuca thurberi* in response to Festuca removal (solid) and nitrogen addition (shape).

increased with increasing distance from *Festuca* (Fig 12). Although the overall pattern we observed was driven by the interaction between sterilization and distance, we also found that sterilization increased *Helianthella* survival (z = 2.22, $p = 0.026$), and a marginal increase in survival with distance from *Festuca* (z = 1.90, $p = 0.06$). Counter to the patterns observed in control soils, we found *Festuca* pre-treatment stabilized *Helianthella* survivorship at about 70%, independent of distance from *Festuca* and activity of soil community (Pre-treatment x distance interaction z = 0.144, $p = 0.13$, Fig 12).

Although *Festuca* pre-treatment stabilizes survivorship in *Helianthella*, *Helianthella* seedlings grown following *Festuca* pre-treatment produced 98% smaller leaves ($F_{(1,116)} = 457.99$, $p = 0.029$) and 66% fewer leaves ($F_{(1,116)} = 382.82$, $p = 0.033$). We found marginal increases in *Helianthella* leaf size ($F_{(1,116)} = 153.73$, $p = 0.051$), and leaf number ($F_{(1,116)} = 104.60$, $p = 0.062$), with soil sterilization. However, we observed no differences in leaf size ($F_{(1,116)} = 1.19$, $p = 0.47$) or leaf number ($F_{(1,116)} = 21.73$, $p = 0.134$) with distance from *Festuca*. Accordingly, we found no relationship between distance from *Festuca* and *Helianthella* total biomass ($F_{(1, 157)} = 0.409$, $p = 0.52$), green leaf biomass ($F_{(1, 122)} = 0.227$, $p = 0.63$), root biomass ($F_{(1, 164)} = 0.001$, $p = 0.99$) or root:shoot ($F_{(1, 155)} = 0.001$, $p = 0.97$) (Fig 13). In agreement with the growth data, we found that soil sterilization increased 112.7% and *Festuca* condition decreased 97.3% total *Helianthella* biomass (Sterilization: $F_{(1, 157)} = 4.04$, $p = 0.046$) ; *Festuca* pretreatment: $F_{(1, 157)} = 4.94$, $p = 0.027$) (Fig 13). We found that soil sterilization lead to an 132% increase in root biomass ($F_{(1, 164)} = 3.19$, $p = 0.07$), 110% increase in green leaf biomass ($F_{(1, 122)} = 7.97$, $p = 0.006$), and a 107% increase in root allocation ($F_{(1, 155)} = 5.131$, $p = 0.025$) in *Helianthella*, however *Festuca* pre-treatment did not (root biomass - $F_{(1, 164)} = 1.84$, $p = 0.17$; green leaf biomass - $F_{(1, 123)} = 2.304$, $p = 0.13$; root allocation - $F_{(1, 155)} = 0.841$, $p = 0.36$) (Fig 13). Thus, soil sterilization increased root allocation, root biomass, green leaf biomass, and total biomass, but did not influence total leaf number or leaf size. Whereas *Festuca* pre-treatment reduced leaf number, leaf size, and belowground biomass without influencing plant biomass allocation patterns or aboveground biomass, while distance from *Festuca* had no measurable effects on any growth or biomass measurements.

To understand mechanisms involved in neighbor-mycorrhizal interactions, we performed feedback contrasts between our four soil treatments. Surprisingly, we observed no difference in plant-soil feedbacks in any of our four contrasts, or between the near or far distance comparisons (Table 5, Fig 14).

Discussion

Overall, we found that distance from *Festuca* was a strong predictor of DSE, but not AMF colonization of *Helianthella* roots. AMF colonization was consistently high (~78%) in *Helianthella* roots at all distances from *Festuca*.

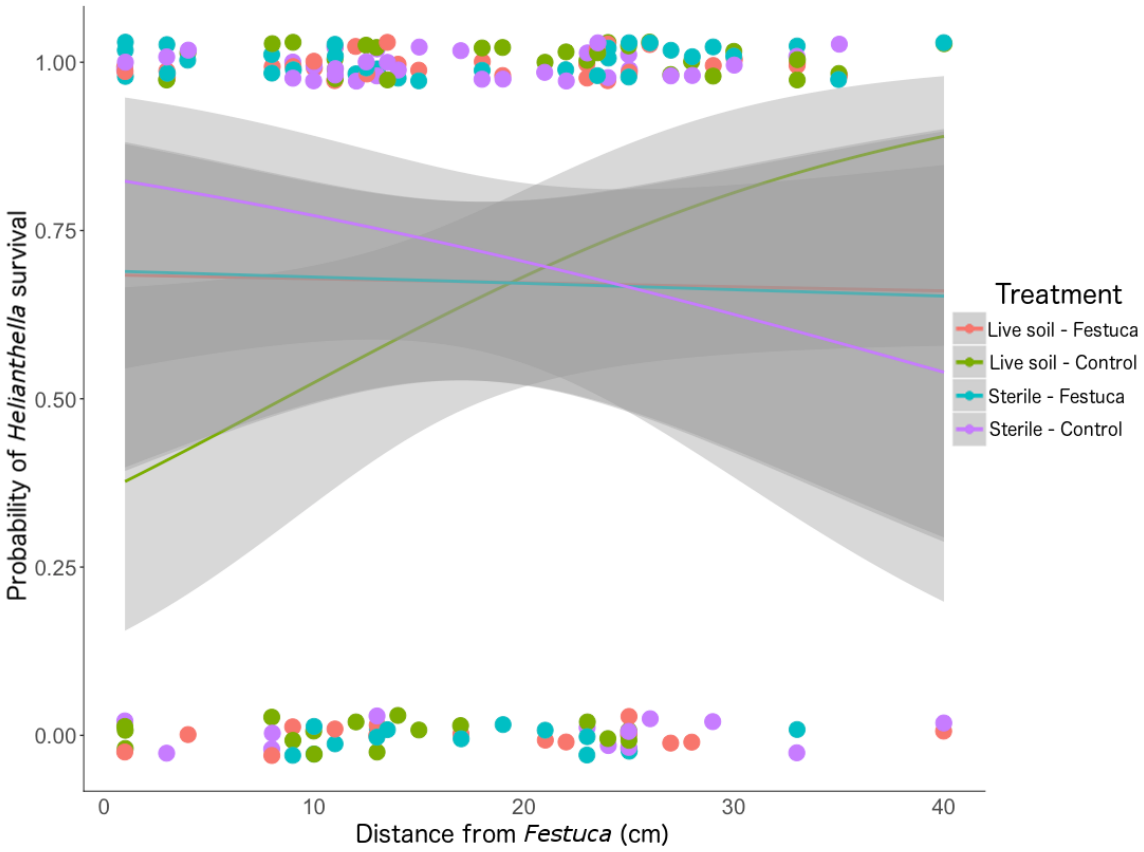

Figure 12. Probability of *Helianthella quinquenervis* seedling survival at the conclusion of our plant-soil feedback experiment grown in inoculum that originated from variable spatial distances from *Fesuca*. Inoculum treatments differed by the activity of soil community (live, pink, green; sterile, blue, purple) and whether or not the soils received a Festuca pre-treatment (with Festuca pre-treatment (pink, blue) or not (green, purple).

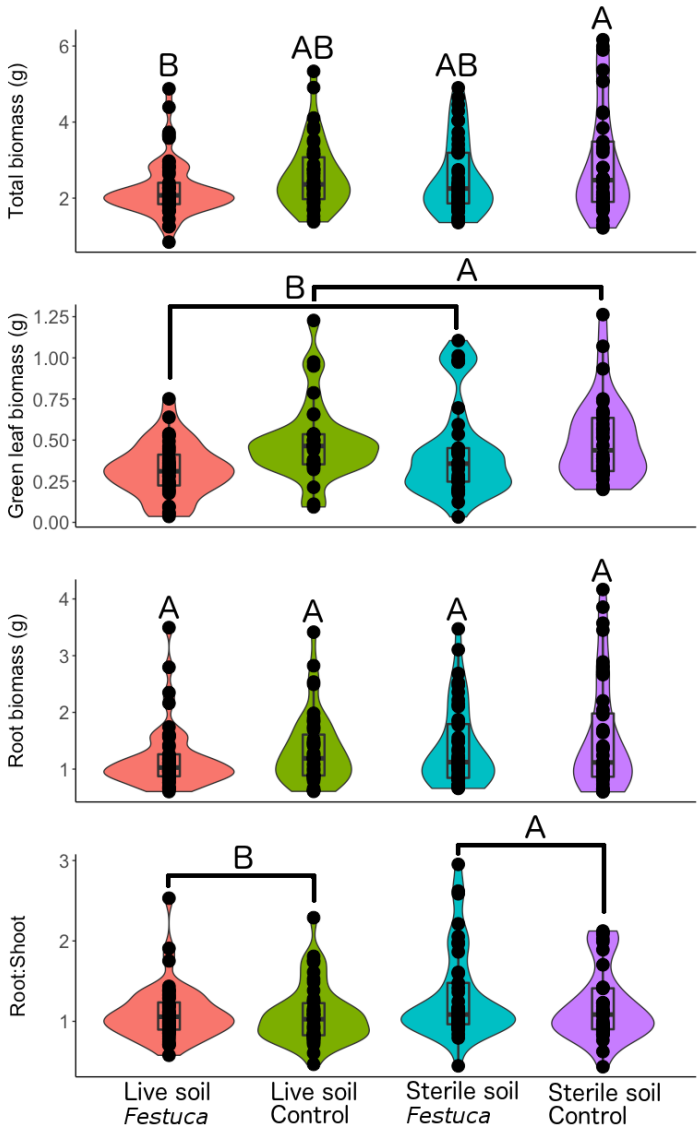

Figure 13. Total *Helianthella quinquenervis* biomass (A), green leaf biomass (B), root biomass (C), and root:shoot (D) across our 4 different treatments in our plant soil feedback experiments. Live soil *Festuca* = unsterilized soils that were first planted with *Festuca* for 7 weeks prior to *Helianthella* transplant, Live soil control = unsterilized soils that were not pre-treated with *Festuca*, sterile soil *Festuca* = sterilized soil inoculum that was pre-treated with *Festuca*, Sterile soil control = sterilized soils that were not pre-treated with *Festuca*. Letters indicated significant differences.

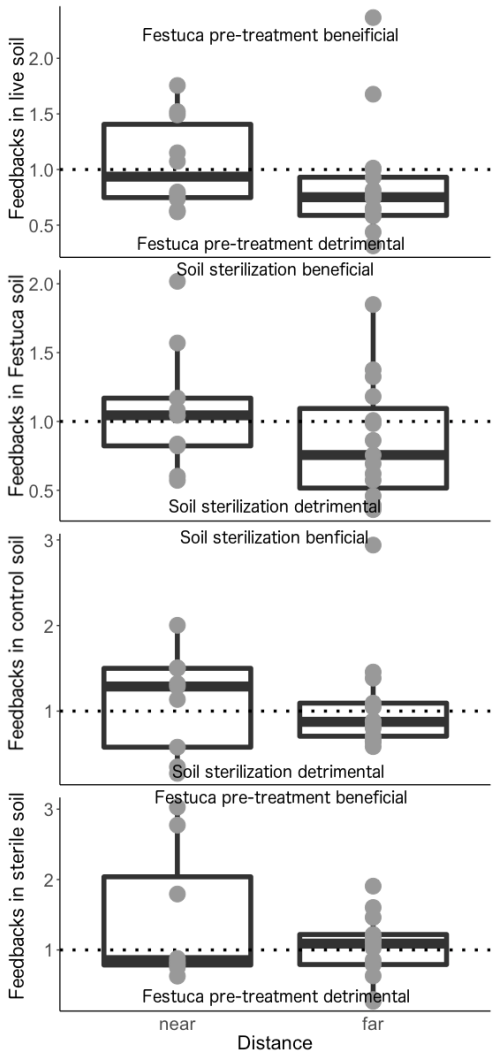

Figure 14. Plant soil feedbacks in *Helianthella* grouped by inoculum collected near (0-10 cm) or far (+20 cm) from *Festuca*. Each plant soil feedback tested potential mechanisms: (A) contrasts in *Helianthella* biomass in live soils either with or without *Festuca* pre-treatment, deviations from 1 suggest *Festuca* priming of microbial communities; (B) contrasts of *Helianthella* biomass in *Festuca* pre-treated soils that were either live or sterile, deviations from 1 explore the role of microbial community in driving neighbor effects; (C) contrasts of *Helianthella* biomass in soils without *Festuca* pre-treatment either sterilized or non-sterilized, deviations from 1 explore the benefit of soil microbes to *Helianthella* growth; (D) contrasts of *Helianthella* biomass in sterilized soils with or without *Festuca* pre-treatment, deviations from zero explore non-microbial mediated influences of *Festuca* on the growth of *Helianthella*. Although, none of our contrasts deviated significantly from 1 (one-sample t-test).

Surprisingly, neither N addition nor the removal of dominant, DSE-associated, *Festuca thurberi* had any influence on AMF and DSE colonization patterns in *Helianthella quinquenervis*. We found that greenhouse survivorship of *Helianthella* was positively correlated with the distance from *Festuca* in live soils, but survival was negatively correlated with distance in sterile soils, suggesting that maintenance of a higher DSE colonization comes at a cost of increased mortality. Surprisingly, if individuals *Helianthella* survived in soils that originated near *Festuca*, there were no differences between growth or total biomass and distance from *Festuca* in the greenhouse. This corresponds to the observations made in the field, of no relationship between plant height and distance from *Festuca.* Additionally, we found that *Helianthella* growth and biomass was highest in sterilized soils. We concluded that both AMF and DSE had negative growth effects or soil pathogens out-weigh the growth benefits from AMF and DSE in this system, but this requires additionally testing.

Ghosts of neighbors past

The negative correlation between distance from *Festuca* and DSE colonization was found consistently in plots where *Festuca* was intact and where *Festuca* was removed. Additionally, we found a positive correlation between *Helianthella* survival and distance from *Festuca* in our greenhouse control soils that did not receive a *Festuca* pre-treatment. However, when Helianthella received *Festuca* pre-treatment, Helianthella survival stabilized across distance from Festuca. Taken together, this suggests that the influence of plants on fungal community in both surrounding soil and neighboring plant species persists after death, which we term, the ghost of neighbors past. However, the duration, spatial extent, and persistence of neighbor legacies in an ecosystem is currently unknown. Evidence from other systems suggest that soil legacies can reside in soils for more than 40 years following plant removal (Wardle *et al.* 2008; Urcelay *et al.* 2009). Further, the legacies of past neighbors on fungal communities suggests that fungal community composition may be resilient to some non-random plant species losses in an ecosystem. If fungal communities can maintain composition following changes in plant community composition, fungal communities may mediate resilience and resistance of plant community composition and ecosystem function to disturbance events (Read *et al.* 2017). However, this needs to explicitly be tested. To tackle this question, researchers could take an integrated approach measuring simultaneous changes in fungal communities and plant communities following a disturbance event or removal treatment and monitoring the response of aboveground and belowground communities over time.

Mechanisms driving plant-plant-fungal interactions

Plant-neighbor interactions may drive fungal community composition directly by priming microbial communities and shifting competitive ability of microbial taxa, passively by altering microbial inoculum potential, directly

suppressing the surrounding fungal communities (Stinson *et al.* 2006; Callaway *et al.* 2008; Lankau 2012), or changing nutrient availability which indirectly shift fungal communities (Lilleskov *et al.* 2002; Leff *et al.* 2015). Our plant-soil feedback experiment directly tested these potential mechanisms. However, because of low total biomass differences among our treatments and high non-random mortality within live soils across inoculum origin distance, we were unable to elucidate underlying mechanisms in our system (Fig 14). We hypothesize that either 1) growth differences and feedbacks between plant and fungal communities are relatively weak or 2) multiple mechanisms may be occurring simultaneously, clouding overall patterns; however, this is an obvious research gap to be filled. To fill this gap, studies will need to focus on the mechanisms controlling plant community response to disturbance events or removal treatments, and integrating how biotic and abiotic factors may shape these responses.

Interactions between multiple fungal types

To our knowledge, this is the first report to demonstrate strong plant-plant interactions shaping fungal communities involving multiple fungal functional types. Shifts among belowground functional groups may have ecosystem-level consequences for carbon storage and nutrient cycling because fungal functional groups differ in their functional capabilities, growth benefits for hosts, and physiological tolerance (Smith & Read 2009, Phillips *et al.* 2013). Shifts among functional groups may impact ecosystems to a much greater extent than the fine-tuning of species identities within a single fungal group that have been described in other studies (Hausmann & Hawkes 2009). For example, DSE produce suites of carbon-degrading enzymes, which AMF lack (Mandyam and Jumpponen 2014; Caldwell *et al.*2000). Additionally, AMF and DSE may differ in the nutrient resources they obtain from soil, nutrient resources they exchange with host plant, the host plants they support, and their physiological tolerances (Jumpponen 2001; Upson *et al.* 2009; Newsham 2011; Mandyam *et al.* 2012; Alberton *et al.* 2010). Thus, changes in the relative abundance of AMF/DSE in a community may alter nutrient availability, carbon storage, abiotic stress tolerance of the plant host, and potentially lead to turnover of plant species.

Studies investigating AMF and DSE co-occurrence are still rare. Thus, our understanding of co-existence between AMF and DSE in root space is in its infancy. Root colonization by AMF and DSE differs greatly between plant functional groups, with grasses commonly supporting a high colonization of DSE relative to AMF, whereas legumes and other forbs are typically more heavily colonized by AMF (Ranelli *et al.* 2015; Newsham 2011; Mandyam & Jumpponen 2014). Site characteristics and climate also heavily structure AMF and DSE co-existence (Kauppinen *et al.* 2014; Schmidt *et al.* 2008; Ranelli *et al.* 2015), as DSE are typically more prevalent in cold, wet habitats (Jumpponen 2001). Previous work would suggest that a combination of plant hosts, climate, and site characteristics likely structure co-existence between AMF and DSE. However,

we provide clear evidence that plant-plant interactions can have a greater influence on co-colonization of AMF and DSE than nutrient availability or plant community composition in both field and the greenhouse.

Conclusions

In conclusion, we found that plant neighbors and the ghosts of neighbors past can outweigh soil nutrient availability in determining mycorrhizal community structure. Although our study was not able to test the duration of these legacies, other authors suggest the legacies of plant removals may persist for decades. We hypothesized neighbor interactions would feedback to influence the performance of focal plant species. As we hypothesized, plant neighbor interactions influenced survivorship of focal seedlings, but did not affect total biomass, growth rates in the greenhouse, or plant height in the field. Plant-plant interactions may affect associated fungal communities in a number of different ways; our plant-soil feedback experiment to test for possible mechanisms suggests that multiple drivers may be acting simultaneously and perhaps acted in the past.

Acknowledgments

We would like to thank Peter Meidl, Krissy Dominguez, Kenna Rewcastle, Ben Miller, Courtney Patterson, and Helen Law for assistance in the field and in the greenhouse. Additionally, we would like to thank discussions with the Bailey-Schweitzer lab group and Classen lab group in project development. We would also like to thank the staff of the Rocky Mountain Biological Laboratory (RMBL) for their support, and the organizations that funded this research: the Department of Ecology and Evolutionary Biology, University of Tennessee (Q.D.R., J.A.H.), the RMBL Dr Jean Langenheim Fellowship (Q.D.R.), the RMBL Dr Lee R. G. Snyder Memorial Fellowship (J.A.H.), and the Fran Hunter Fellowship (J.A.H.) in part, by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research, Terrestrial Ecosystem Sciences Program under Award Number DE-SC0010562.

Appendix

Table 4. ANOVA table for fungal colonization (AMF or DSE), AMF vesicles, AMF coils, DSE microsclerotia, and Oomycotans in response to distance from focal neighbor (Festuca), fungal group (AMF or DSE), or removal and nitrogen (N) treatments (Removed Festuca no nutrient addition, removed Festuca with ammonium nitrate addition, removed Festuca with urea addition, Festuca intact with no nutrient addition, Festuca intact with ammonium nitrate addition, Festuca intact with urea addition. No effect of treatment, but a significant interaction between Fungal group and distance. AMF and DSE colonization were modeled with linear models, AMF vesicles, coils, DSE microsclerotia, and Oomycotans were modeled as Poisson distributions.

	Effect	F value	P value
AMF and DSE Colonization	**Distance**	**17.67**	**>0.0001**
	Fungi	**422.8**	**>0.0001**
	Removal + N	1.744	0.13
	Distance*Fungi	**18.19**	**>0.0001**
	Distance*R+N	1.834	0.11
	Fungi*R+N	0.773	0.57
	Distance*Fungi*R+N	1.389	0.23
	Effect	$\chi 2$	P value
AMF Vesicle	Distance	0.012	0.91
	N.tx	0.028	0.99
	Removal.tx	0.011	0.92
AMF Coil	Distance	0.017	0.90
	N.tx	0.002	0.99
	Removal.tx	0.015	0.90
DSE Microsclerotia	Distance	0.242	0.63
	N.tx	0.054	0.97
	Removal.tx	0.032	0.86
Oomycota	Distance	0.170	0.68
	N.tx	0.006	0.99
	Removal.tx	0.021	0.89

Table 5. Plant soil feedback contrasts. Plant soil feedbacks in *Helianthella* grouped by soil inoculum that was collected near (0-10 cm) or far (+20 cm) from field-*Festuca*. Each plant soil feedback tested potential mechanisms. Contrast 1 tests *Helianthella* biomass in live soils either with or without *Festuca* pre-treatment, deviations from 1 suggest *Festuca* priming of microbial communities; Contrast 2 tests *Helianthella* biomass in *Festuca* pre-treated soils that were either live or sterile, deviations from 1 explore the role of microbial community in driving neighbor effects; Contrast 3 tests *Helianthella* biomass in soils without *Festuca* pre-treatment either sterilized or non-sterilized, deviations from 1 explore the benefit of soil microbes to *Helianthella* growth; Contrast 4 tests *Helianthella* biomass in sterilized soils with or without *Festuca* pre-treatment, deviations from zero explore non-microbial mediated influences of *Festuca* on the growth of *Helianthella*. Although, none of our contrasts deviated significantly from 1 (one-sample t-test), trends would suggest that feedbacks may differ across distance and that our four mechanisms may be occurring simultaneously.

Feedback Contrast	Distance	mean	95% conf int	t	df	p value
1	Near	1.055	(0.76, 1.35)	0.427	9	0.68
1	Far	0.845	(0.59, 1.10)	-1.276	16	0.22
2	Near	1.079	(0.72, 1.43)	0.511	8	0.62
2	Far	0.855	(0.61, 1.09)	-1.295	14	0.22
3	Near	1.110	(0.66, 1.56)	0.541	8	0.60
3	Far	1.068	(0.69, 1.45)	0.395	12	0.70
4	Near	1.435	(0.62, 2.25)	1.263	7	0.25
4	Far	1.035	(0.75, 1.32)	0.264	12	0.80

CHAPTER IV
FUNGI ON MOUNTAINSIDES: LINKING GLOBAL PATTERNS TO EXPERIMENTS TO EXPLORE MECHANISMS OF FUNGAL DISTRIBUTIONS

The Following section is a slightly modified version of a paper in preparation:

Henning, J.A., Read, Q.D., Sundqvist, M., He, J.-S., Rewcastle, K.E., Newman, G., Chisholm, C., Ottinger, S., Sanders, N.J., A.T. Classen. *In prep*. Fungi on Mountainsides: Linking global patterns to experiments to explore mechanisms of fungal distributions.

The use of “we” in this chapter refers to my co-authors and me. As the lead author of this article I was responsible for this paper. J.A.H., A.T.C., and N.J.S. designed experiment, J.A.H., Q.D.R., M.S., K.E.R., G.N., C.C., obtained fungal samples, J.A.H., N.J.S., and A.T.C. designed follow-up experiments, J.A.H., K.E.R., and S.O. conducted follow-up experiments, J.A.H. analyzed data, J.A.H. wrote the manuscript, and all other co-authors assisted with writing and revision.

Abstract

Soil communities are hyper diverse, are difficult to observe, and influence many community and ecosystem processes such as nutrient mineralization and plant-coexistence. The operating assumption has been that plant-associated fungal communities will match aboveground plant hosts, however, recent studies have questioned this assumption. Here, we take a combined approach, conducting a large-scale global synthesis combining fungal colonization data from 12 mountain sites. Colonization data from multiple plant species were combined with publically available, WorldClim data and soil properties, to explore global patterns of fungal communities and to generate testable hypotheses on the biotic and abiotic factors structuring fungal communities. Next, we tested these hypotheses at a single representative gradient site near Rocky Mountain Biological Laboratory, Colorado, USA. Generally, we found AMF colonization is higher at low elevation and DSE colonization is generally higher at high elevation sites, although we found exceptions to this generalization. However, we found that AMF and DSE differed in the combination of factors that correlated with colonization patterns. For instance, obligate plant-associated AMF correlated strongly with plant host functional group and seasonal temperature variation, compared to facultative-associated DSE which was structured strongly by soil pH and mean annual temperature, although both functional groups may be performing semi-analogous functions. As a follow-up, we tested several of these hypotheses with observational and manipulative experiments at a single, representative gradient site in Colorado, USA. Overall, we found little agreement between the hypotheses that were generated by our global synthesis and the observational and mechanistic experiments to test them. For instance, our global synthesis predicted that AMF colonization would be higher in hot, dry, sites and DSE colonization would be higher in cool, wet sites, however, we found no link between temperature and soil moisture and fungal colonization patterns in an observational field experiment across growing season or a manipulative soil moisture experiment. This suggests that temperature and soil moisture across an

elevational gradient co-vary with true drivers of fungal communities, that the regional and local factors structuring fungal communities may differ, or the response of fungal colonization is contingent on plant hosts, which are fruitful avenues of future research.

Introduction

Soil communities are hyper diverse, are difficult to observe, and influence many community and ecosystem processes such as nutrient mineralization and plant-coexistence (Tedersoo *et al.* 2014; Jing *et al.* 2015; Jiang *et al.* 2017). Thus, understanding what biotic and abiotic factors influence microbial diversity patterns at local and global scales is an important (Hendershot *et al.* in press; Sundqvist *et al.* 2013; Classen *et al.* 2015). Based on the accumulated evidence for the diversity of life aboveground (Rahbek 1995; Hillebrand 2004; Westgate *et al.* 2014), the operating assumption has been that that diversity belowground would increase toward the equator and peak at intermediate elevations (Sundqvist *et al.* 2013). However, several recent studies have cast doubt on these assumptions (Fierer *et al.* 2011; Tedersoo *et al.* 2014, Beck *et al.* 2015, Hendershot *et al.* in review). Collectively, these studies demonstrate there is very little correlation between belowground diversity and aboveground organisms (Fierer *et al.* 2011; Tedersoo *et al.* 2014, Beck *et al.* 2015, Prober *et al.* 2015; Hendershot *et al.* in press). Comparison across these global studies reveals very few consistent patterns of belowground communities across elevational or latitudinal gradients (Hendershot *et al.* in press).

However, these global studies have numerous limitations that impair mechanistic understanding of belowground communities. First, many studies lump free-living and plant-associated microbial communities (Hendershot *et al.* 2017; Prober *et al.* 2015), however we might predict that plant-associated microbial communities would mirror their plant host, whereas free-living communities may be structured heavily by climatic and soil properties (Martiny *et al.* 2006; Fuhrman *et al.* 2008). Second, studies are typically limited by the biotic and abiotic factors that are measured, which are limited to factors deemed important by the researcher or research group. Along natural gradients, several biotic and abiotic factors co-vary, confounding or misrepresenting distribution patterns when one attributes patterns to single factors (He & Callaway 2009; Sundqvist *et al.* 2013). For instance, many studies investigate community composition changes in response to publically available temperature or precipitation data (Bahram *et al.* 2012; Serna-Chavez *et al.* 2013; Tederasoo *et al.* 2014), without physically collecting data on other factors that may co-vary with elevation, for example soil moisture, nutrient availability, plant community composition, soil texture, pH, just to name a few (Fierer and Jackson 2006, Koyama *et al.* 2014; Ma *et al.* 2015). Physically collecting biotic and abiotic factors at each gradient site may create logistical and financial limitations to conduct research across many sites. Typically, studies collecting a wealth of

biotic and abiotic are limited to single or few sites, which inhibit synthesis of broad-scale patterns (Bryant *et al.* 2008; Ranelli *et al.* 2015). Finally, along elevational gradients, multiple belowground functional groups may perform analogous functions, but can be spatially structured by biotic and abiotic factors, analogous to plant communities. Across most elevational gradient sites, arbuscular mycorrhizal fungi (AMF), ectomycorrhizal fungi (Ecto), ericoid mycorrhizal fungi (ERM), and dark-septate endophytes (DSE) often co-occur although few studies have documented the distributions of the "community" of functional groups across elevational gradients.

To overcome these logistical challenges, we combined experimental approaches to conduct a multi-site synthesis of plant-associated fungal colonization patterns along elevational gradients, which provided testable hypotheses for a series of observational and manipulative experiments. By focusing on only plant-associated fungal communities, we narrowed the scope of our investigation from broad microbial communities of mixed and unknown function to focus on multiple functionally-analogous groups of fungi that we hypothesized would be under similar selection to track their plant hosts. Thus, we hypothesized that plant host would be the strongest factor structuring plant-associated fungal communities, relative to climatic and soil properties. To test this hypothesis, we conducted an in-depth analysis of fungal colonization patterns at four sites (Colorado, USA, Abisko, Sweden, Davos, Switzerland, and Tibetan Plateau, China). At each of these sites we collected root samples from >85% of the plant community and collected a suite of climatic and soil properties. Data from these four sites were combined with previously published datasets (Haselwandter 1987; Väre *et al.* 1997; Gonzalo-Turpin *et al.* 2010; Pérez & Frangi 2000; Schmidt *et al.* 2008; Lugo *et al.* in review; Zubek *et al.* 2009; Routsalainen *et al.* 2004; Vohník & Albrechtová 2011) that documented fungal colonization in multiple functional groups across an elevational gradient. To conduct our synthesis, we combined colonization data with publically available temperature and precipitation data and reported data on soil properties like pH, C:N, and N:P to explore global patterns in fungal distributions. We conducted our global synthesis to generate testable hypotheses on the biotic and abiotic factors structuring fungal communities along environmental gradients. Next, we tested these hypotheses at a single representative gradient site near Rocky Mountain Biological Laboratory, Colorado, USA.

Methods

Gradient work

To explore the distribution of root-associated fungal communities along elevational gradients, we collected 3-5 root samples from 10-15 of the most common plant species at low and high elevation sites at Colorado Rocky Mountains, USA (38.9915833333, -107.0665556), the Tibetan Plateau, China (37.7074666, 101.372), the Swiss Alps (46.774113, 9.856959), and Abisko,

Sweden (68.29354945, 19.09915095). Our goal was to measure at least 85% of the total aboveground cover at each site, in order to calculated community-weighted mean colonization patterns. At each site, we identified each plant species within eight 1.5-m^2 radiuses randomly selected around each site. We used percentage aboveground cover of each plant species, estimated visually (to within 1% if ≤ 10% and within 5% if greater) as a proxy for abundance in the plant community composition measurements. This included plant species from four functional groups grasses, forbs, legumes, and shrubs. We collected a single 2.5cm × 10cm soil core from beneath each host plant and the soil cores were stored on ice while transported back to the laboratory. Cores from China, Switzerland, and Sweden were frozen prior to root extraction; however, roots were extracted from cores collected in Colorado within 2 days following sample collection. After we extracted roots from each soil core, we placed roots into tissue cassettes (Fisher Brand Catalog #22-272; Thermo Fisher Scientific, Pittsburg, Pennsylvannia, USA), cleared with a 10% KOH, followed by a 2% HCl solution and we stained fungal tissues using trypan blue (Koske and Gemma 1989). We mounted stained roots horizontally on glass microscope slides using polyvinyl-lacto-glycerol (PVLG) and assessed AMF, DSE, and ERM colonization using the magnified intersection method (McGonigle *et al.* 1990). We assessed a minimum of 50 random root intersection per slide and determined percent colonization of both AMF and DSE as the proportion of intersections containing AMF hyphae, arbuscules, and vesicles, and DSE hyphae, microsclerotia and any potential soil pathogens.

Global synthesis

To synthesize AMF, ERM, DSE colonization patterns across global elevational gradients, we combined the results from our sites in North America, Asia, and Europe with previously published datasets that measured AMF, DSE, and ERM. In October of 2015, we conducted a literature search on ISI Web of Science using the terms “colonization”, “altitudinal gradient”, and “elevational gradient”. The search yielded ~400 studies, which we screened for measurements of AMF and DSE co-colonization under different plant species along an elevational gradient. Data from seven additional previously published studies (cite all the studies here), which included data from 8 mountain sites distributed on all continents except Australia and Antarctica (Fig 1). We extracted temperature and precipitation (MAP) values for all sites across all the studies using the SRTM (Jarvis *et al.* 2008) and Bioclim (Hijmans *et al.* 2005) datasets. To simplify the 11 temperature and 7 precipitation variables from Bioclim databases, we created composite temperature and precipitation variables by performing a principal components analysis (PCA) grouped by temperature and

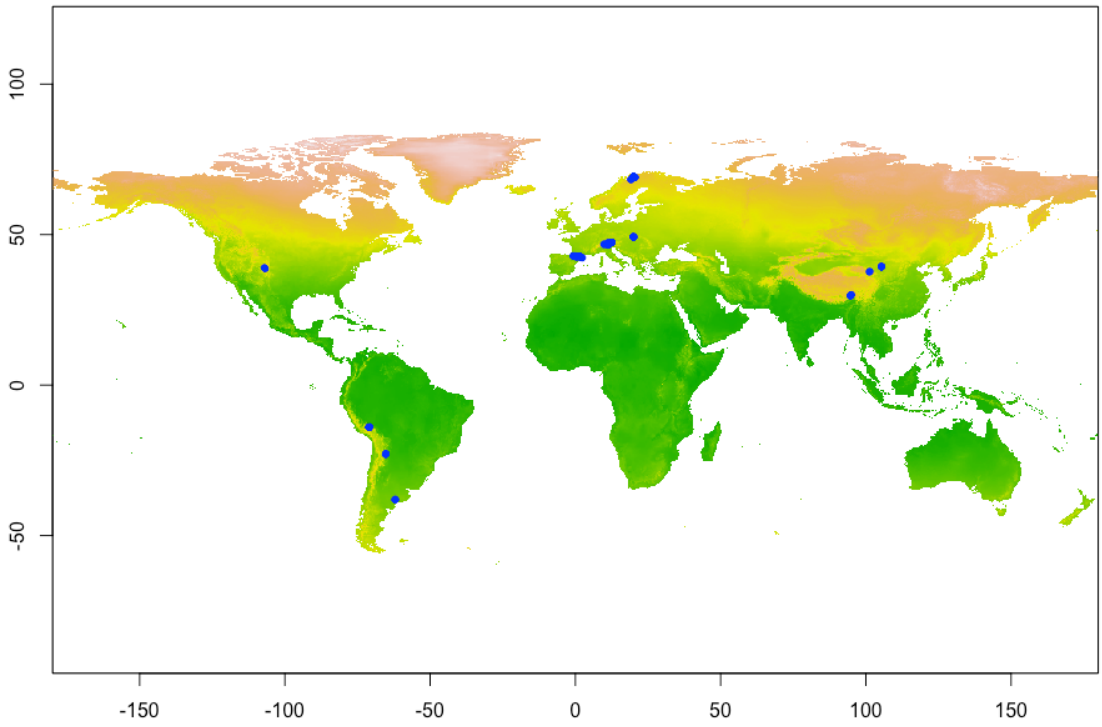

Figure 15. Locations from 12 elevational gradient sites that contributed to our global synthesis. Sites in Colorado, USA, Qinghai Province, China, Abisko, Sweden, and Davos, Switzerland were collected as part of this study. Data from other sites were gathered from: Haselwandter 1987; Väre *et al.* 1997; Gonzalo-Turpin *et al.* 2010; Pérez & Frangi 2000; Schmidt *et al.* 2008; Lugo *et al.* in review; Zubek *et al.* 2009; Routsalainen *et al.* 2004; Vohník & Albrechtová 2011.

precipitation variables. This provided us with two composite temperature variables and two composite precipitation variables that we constructed from the extracted values of the first and second principal components of each PCA. For sites in Colorado, Sweden, Switzerland, China, we measured soil pH by making a 3:1 slurry of 30ml 0.01 M $CaCl_2$ with 10ml air dried soil which was shaken for 5 minutes and allowed to settle for 30 minutes prior to measurement on a Mettler-Toledo Seven (Mettler-Toledo, LLC, Columbus, OH, USA) compact pH meter. We measured total carbon and nitrogen by dry combustion on a LECO elemental analyzer (LECO Corp., Saint Joseph, MI, USA). Total phosphorus was measured following a H_2SO^4+Se wet digestion and colorimetric analysis was done on a spectrophotometer. Soil properties were extracted from original publications when those were provided.

To explore the mechanisms shaping AMF and DSE colonization in detail, we used an elevational gradient site near the Rocky Mountain Biological Laboratory (RMBL) (38.9915833333, -107.0665556). We chose this gradient because it is a well-studied gradient so there were preexisting data we could use (Bryant *et al.* 2008; Ranelli *et al.* 2015; Read *et al.* 2017). At this single gradient site, we are able to explore seasonal patterns of AMF and DSE colonization, as well as AMF and DSE response to changes in soil moisture and soil nutrient availability.

Fungal seasonality

To explore the response of AMF and DSE colonization to simultaneous changes in soil moisture and temperature, we measured colonization in a single, widely-distributed plant host, *Potentilla gracilis,* multiple times during the 2014 growing season at three different elevations. We haphazardly sampled 7 individuals within a pre-established 10 × 10 observational plot at low (2700m), medium (3200m) and high (3500m) elevation near the Rocky Mountain Biological Laboratory (38.9915833333, -107.0665556). Samples were collected at the low-elevation site on May 28th, 2014, June 14th, 2014, and June 29th, 2014. At mid-elevation sites, we collected samples June 2nd, 2014, June 11th, 2014, June 24th, 2014, and September 25th, 2014 and at high elevation, we collected samples July 12th, 2014, July 23rd, 2014, August 15th, 2014, and September 26th, 2014. At mid- and high-elevation sites, our first plant samples were collected at snow melt, we sampled the low-elevation site several weeks after snowmelt but prior to *P, gracilis* flowering. We collected a single 2.5cm × 10cm soil core from beneath each *P. gracilis* and measured colonization as described above. We flagged sampled individuals to prevent resampling the same individual.

Soil moisture manipulation

To test the response of AMF and DSE colonization to soil moisture, we established a pot experiment at the RMBL in Gothic Colorado. We collected 30 individuals of *P. gracilis* from a nearby mid-elevation field site (3200m) and transplanted individuals in to 1L pots filled with field-collected soils. We selected

mid-elevation individuals because previous work demonstrated that *P. gracilis* at this site was colonized by 50% AMF and 50% DSE (Henning unpublished). At the time of transplant, we collected a small subsample (~0.5 g) of root material to measure initial AMF and DSE colonization as described above. We found no difference in pre-treatment colonization levels of AMF (One-way ANOVA, $F_{(2,1)}$ = 0.843, p = 0.44) or DSE (One-way ANOVA, $F_{(2,1)}$ = 0.416, p = 0.66). We randomly selected individual 1L pots and placed pots into 0.69 L containers (Rubbermaid Commercial Products LLC, Winchester, VA, USA) with 1.59 mm holes drilled into 1) the bottom, 2) the sides, or 3) left undrilled, to achieve soil moisture levels of 10%, 20% and 25% respectively (n = 10). We grew *P. gracilis* for 7 weeks under ambient conditions and measured soil moisture, plant height, and leaf number every five days to explore plant response to soil moisture treatments. After 7 weeks, we harvested each pot, aboveground biomass was harvested and dried for 5 days at 70℃ and weighed, roots were extracted from the soil using 0.5cm sieve and we subsampled plant roots to assess for fungal colonization, and the remainder of root material was dried for 5 days at 70℃ and weighed.

Soil nutrient manipulation

To understand how AMF and DSE colonization responds to changes in soil nutrient availability, we grew field-collected seeds of *Poa alpina* and *Juncus drummondi* in field-collected soils with amended nitrogen and phosphorus availability. Field-collected soils from near Rocky Mountain Biological Laboratory (elevation 3700m) were transported back to the University of Tennessee where they were autoclaved. We mixed the autoclaved field soil with autoclaved potting mix at 1:1 ratio. We added 100 ml of fresh-collected field soil within 1L pots over the top of 750 ml of the sterilized soil mix and then topped of pots with sterilized soil (~150 ml). During experimental setup, we mixed our autoclaved soils with either: +10 g N m^{-2}yr−1, +10g P $m^{-2}yr^{-1}$, +10 g N $m^{-2}yr^{-1}$ and +10 g P $m^{-2}yr^{-1}$, +50 g carbon $m^{-2}yr^{-1}$, or left ambient nutrient conditions (n = 10). A single pre-germinated individual of *P. alpina* or *J. drummondi* were added to each pot. Every 4 weeks, we applied an additional dose of +20 mg carbon, +10.4 mg nitrogen, +10.2 mg phosphorus, or +10.4 mg nitrogen and +10.2 mg phosphorus to account for nutrients that had washed out of the pots with watering. After 25 weeks of growth, we harvested plants, subsampled roots for AMF and DSE colonization, and measured aboveground and belowground biomass.

Statistical analysis

All statistical analyses were performed in for R version 3.3.2 (R development core team 2016) and RStudio version 1.0.136 (RStudio 2016) with packages listed where appropriate.

Synthesis

To search for ubiquitous patterns in AMF, DSE, ERM colonization across our 12 mountain sites, we first constructed simple linear models to explore AMF and DSE colonization across elevation and site (low, medium, and high). To explore the biotic and abiotic drivers of fungal communities that contributed to the "elevational" pattern, we constructed multiple regression models to predict AMF and DSE colonization incorporating plant functional types, the first and second axes of temperature and precipitation PCAs. Next, we performed a "forward" and "backward" model selection to select the best fit model, the lowest AIC score, using the *step* function from the "MASS" package in R. We found that for AMF, ERM, and DSE forward and backward model selection converged on the same model. We tested for multi-collinearity among model parameters using the *vif* function and found all best-fit model components had AIC under 1.5. To explore interactions among the factors of our best-fit model, we conducted linear regression including all possible interaction terms, using the *Anova* function in the "CAR" package (Fox *et al.* 2011). To understand the variation explained by each of the predictors in our best fit models, we conducted a commonality analysis on both AMF and DSE best fit models, using the *regr* function in the "yhat*"* package (Kraha *et al.* 2012).

We chose to conduct separate analyses on our soil properties instead of incorporating them into our multiple regression models because soil pH (6 gradients), C:N (5 gradients), and N:P (4 gradients) were collected in so few studies. We analyzed the relationships between soil properties and AMF and DSE colonization using linear models. At sites where we collected plant community composition and had samples from over 85% of the aboveground plant cover (Colorado, China, Sweden, and Switzerland) we calculated plant community-weighted site-level colonization patterns, because soil pH, C:N, and N:P were collected as a 'site-level' measurement. In data extracted from previously published data, we calculated mean colonization across reported species, assuming equal distributions.

Fungal Seasonality

We tested the variation in AMF and DSE colonization within a single host, *P. gracilis*, at multiple points in the growing season at three locations along an elevational gradient. We analyzed colonization by constructing linear models that included: fungal type (AMF or DSE), site (low, medium, high elevation), and sampling date (days since snow melt) as predictor variables. We performed Tukey's post-hoc test to explore the differences among site and sampling dates.

Soil Moisture Experiment

To test if soil moisture differed across our experimental treatments, we performed a repeated measures Analysis of Variance (ANOVA) to account for soil moisture fluctuations over the course of the experiment. We constructed our model of our measured soil moisture values from across the duration of the

experiment and included the experimental soil moisture treatment (low, medium, high) as a predictor and calculated error terms from treatment nested within sampling date. To explore how soil moisture influenced plant growth we analyzed both plant height and leaf number data using a repeated measures ANOVA. We constructed models of height and leaf number with soil moisture treatment predictors and calculated error terms as described above. To test for differences among the soil moisture treatments, we calculated post-hoc pairwise t-tests with Bonferroni corrections. To test how soil moisture treatments influence *P. gracilis* aboveground, fine root, total root, and total plant biomass, we constructed one-way ANOVA models with initial plant height as a predictor variable, so we could assess treatment influence on biomass while accounting for differences in initial plant size.

We assessed the influence of soil moisture on AMF, DSE, associated AMF vesicles, AMF spores, and DSE microsclerotia colonization by constructing one-way ANOVA models with and without initial fungal colonization and soil moisture as model predictors. We calculated and compared AIC values of models constructed with and without initial colonization values and soil moisture to understand how treatments were influenced by standing fungal colonization.

Soil nutrient manipulation

To understand how nutrient availability shape AMF and DSE colonization patterns, we constructed ANOVA models for both AMF and DSE incorporating nutrient addition treatment as predictor variable. We tested for differences among nutrient addition treatments using the “Anova” function, if significant differences were observed among treatments, we conducted a Tukey HSD post-hoc test.

Results

Fungal distributions

We combined fungal colonization data from four elevational gradient sites (Colorado, USA, Abisko, Sweden, Davos, Switzerland, and Qinghai Province, China) with 7 previously published studies that have investigated AMF, DSE, and ERM colonization to explore large scale patterns in fungal distributions along elevational gradients. This analysis combined gradient sites from South America (Argentina, Peru), North America (Niwot Ridge, Colorado, USA), Europe (Austria, Norway, Poland, France, and Finland) (Fig 15). We are presenting only AMF and DSE results within our synthesis because ericoid and ectomycorrhizal plants occurred only at 4 sites. Generally, average root colonization rates of AMF decrease 16%($F_{(2, 755)} = 33.538$, $p < 0.0001$) and DSE increase by 19% ($F_{(2, 729)} = 39.71$, $p < 0.0001$) from what studies designate as their “low” to “high” elevation sites (Fig 16). However, there are exceptions to this generalization. We observed greater AMF at high elevation sites and greater DSE colonization at low elevation at sites in Sweden and Switzerland (Fig 16). Additionally, at a gradient site in

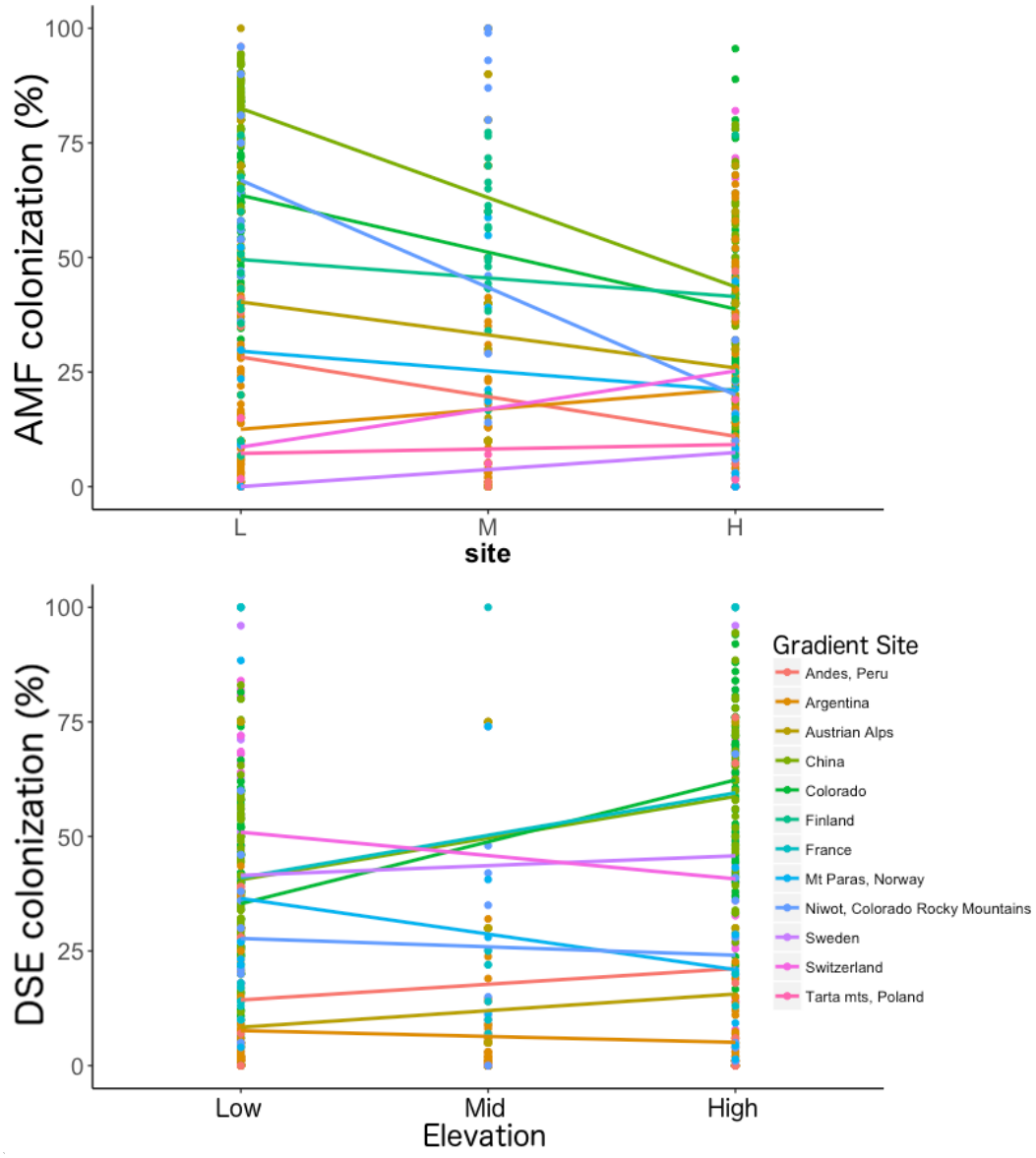

Figure 16. AMF and DSE colonization across elevation.

Argentina, we observed no change in AMF or DSE colonization across the elevations.

To explore potential drivers of fungal colonization patterns we incorporated information on site temperature and precipitation data using WorldClim databases. We condensed all the temperature and precipitation variables into four composite variables, to represent spatial variability in temperature and precipitation without over-parameterizing our models. To create composite variables, we exported the first and second axes of two principal component analyses constructed from all the WorldClim temperature and precipitation variables. Overall, we found that the first axis of our temperature PCA, which explained 52.3% of temperature variation, was associated almost solely with annual mean temperature (Fig 17). Higher composite values represent higher mean annual temperature. The second axis of the temperature PCA explained 28.5% of the variation and represented seasonal variation in temperature. High PC2 values corresponded with higher mean diurnal temperatures, higher summer temperatures and low values corresponding to greater temperatures in cold months and higher temperature seasonality (Fig 17). We found that the first axis of our precipitation composite variable, which explained 75% of the variation, was driven almost entirely by mean annual precipitation (Fig 18). The second axis of the precipitation PCA was driven by seasonal differences in precipitation and explained 22.8% of the data variation. (Fig 18). Lower PC2 values corresponded with more precipitation in cold months and higher PC2 values corresponded to higher precipitation during warmer months.

To explore how temperature, precipitation, and plant host shape colonization patterns of AMF and DSE, we constructed multiple regression models that incorporated our composite temperature and precipitation variables, plant functional group (grass, legume, forb, shrub) and used model selection to determine the best-fit model of biotic and abiotic factors shaping fungal colonization patterns. To predict AMF colonization, our best fit model retained plant functional group, both temperature composite variables, and the 2nd precipitation variable (AIC = 4873.6, $F_{(15,750)} = 48.68$, $p > 0.0001$, $r^2 = 0.483$). Generally, plant functional group ($F_{(1, 750)} = 26.10$, $p < 0.0001$) accounted for 17.6% of AMF colonization variation. AMF colonization is higher in forbs and legumes relative to grasses and shrubs. Generally, AMF colonization was higher in areas of higher diurnal temperature ranges and hotter summer temperatures and was lower in areas with warmer winter temperatures. The relationship of AMF colonization with temperature PC2 explained 39.5% of the variation explained by our model (temperature PC2 $F_{(1, 750)} = 90.17$, $p < 0.0001$, Fig 19). Additionally, we observed a significant interaction between plant functional group and seasonal temperature patterns (temperature PC2 $F_{(1, 750)} = 9.13$, $p = 0.003$) which accounted for 28.4% of explained model variation. Specifically, AMF colonization in grasses and shrubs is much lower relative to forbs and legumes in areas with warmer winter temperatures; however AMF colonization in grasses is

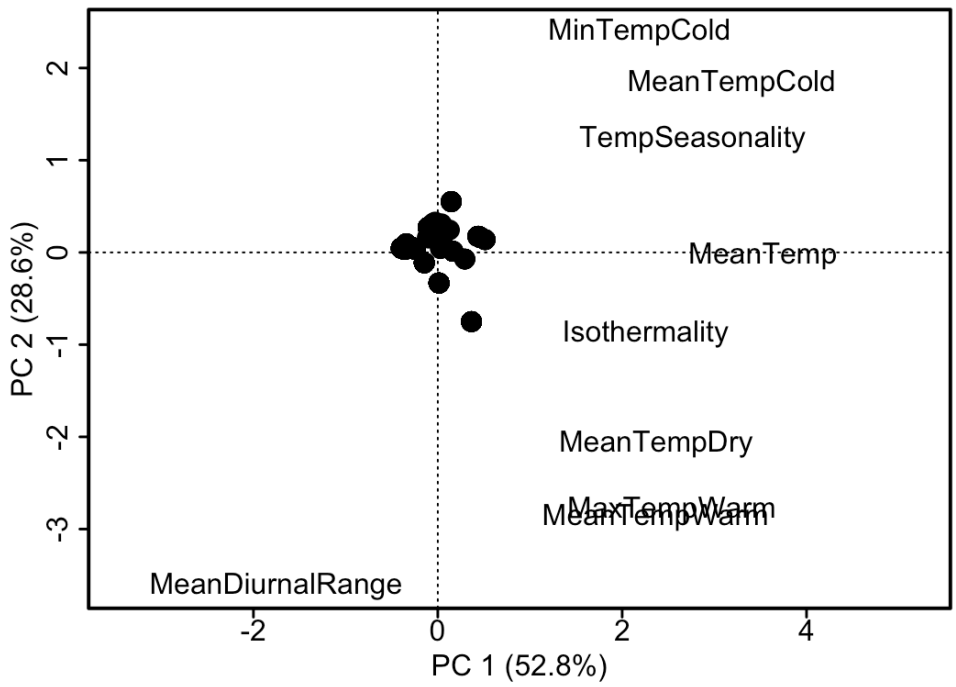

Figure 17. Principal component analysis of Worldclim temperature variables. Each dot represents a study site.

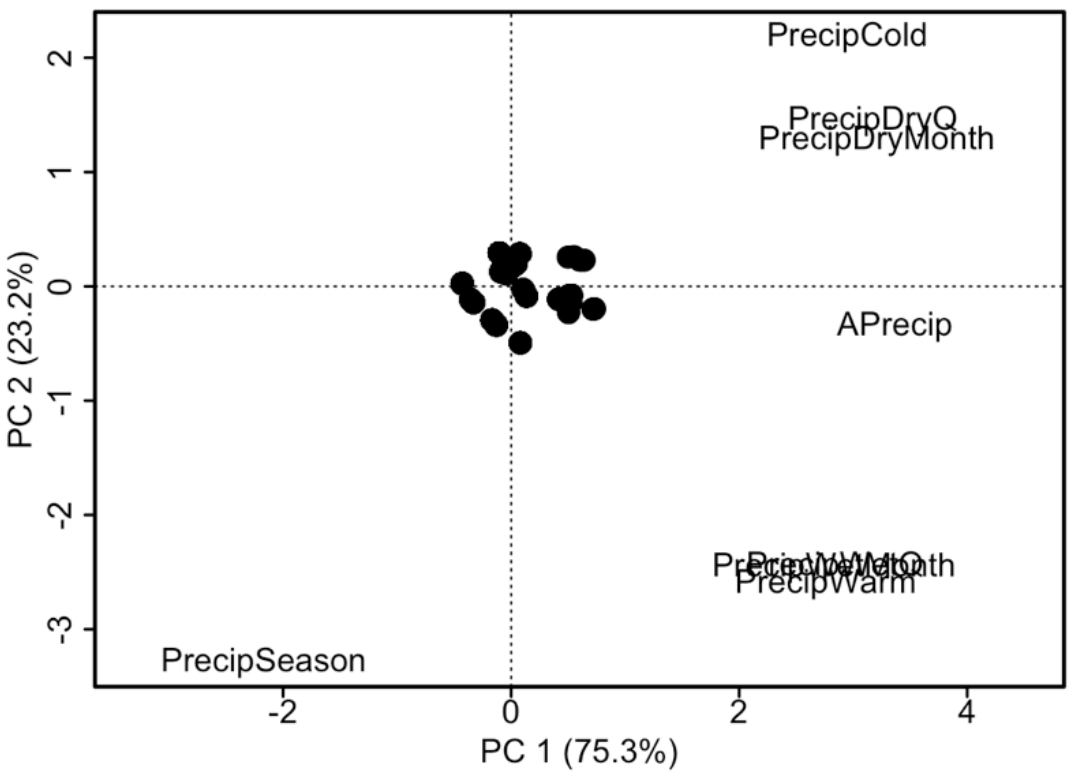

Figure 18. Principal component analysis of Worldclim precipitation variables. Each dot represents a study site.

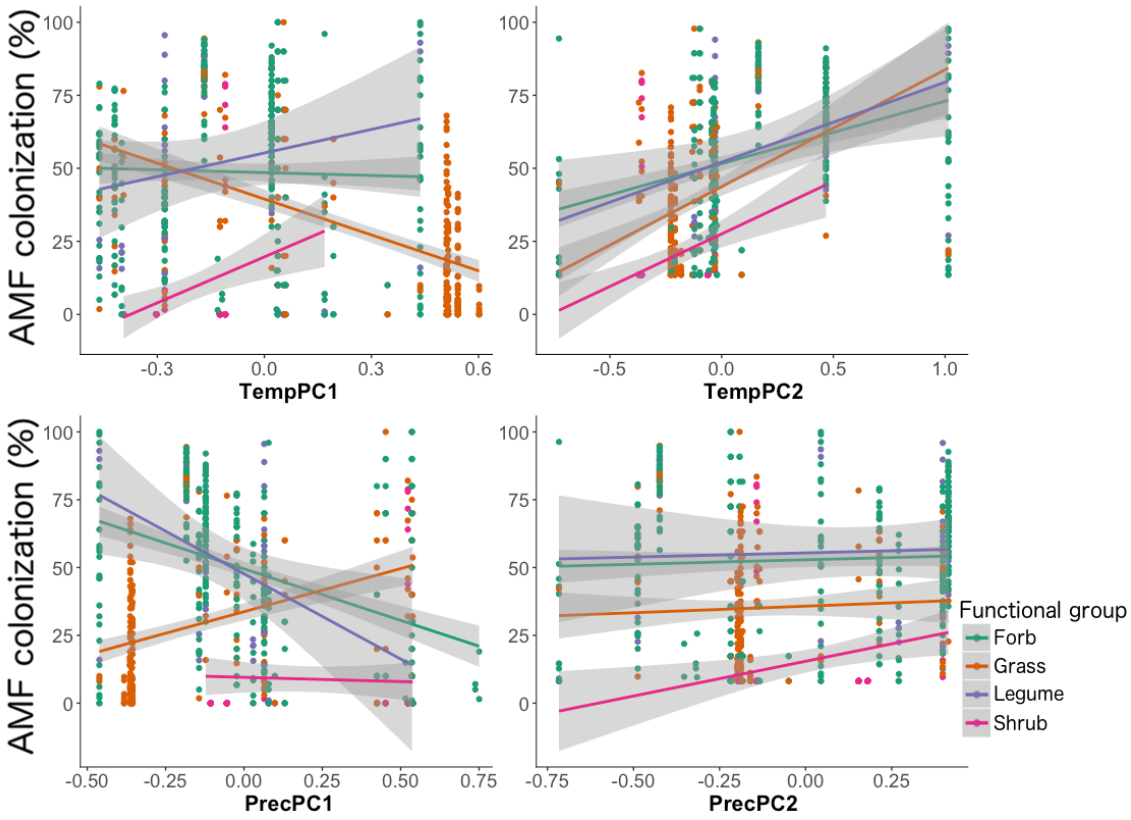

Figure 19. AMF colonization in response to composite temperature and precipitation variables. A) mean annual temperature, B) seasonality in temperature patterns, C) mean annual precipitation, D) seasonality in precipitation patterns, across 4 functional types forbs (green), grass (orange), legume (purple), and shrub (pink).

not distinguishable from colonization in forbs and legumes in areas with higher diurnal ranges and warmer summer temperatures (Fig 19).

Mean annual temperature explained 19.2% of AMF variation (temperature PC1 $F_{(1, 750)} = 102.70$, $p < 0.0001$, Fig 19). However, the response of AMF colonization to increasing mean annual temperature differed across plant functional type (Fig 19). We found a significant interaction between plant functional group and MAT (temperature PC1 $F_{(1, 750)} = 13.73$, $p = 0.0002$). Specifically, AMF colonization in grasses decreased with increasing mean annual temperature, whereas shrub and forb colonization increased with increasing mean annual temperature (Fig 19). Surprisingly, AMF colonization in legumes was unaffected by changes in mean annual temperature (Fig 19). However, the interaction between plant functional group and mean annual temperature explained only 0.43% of our model variation. For instance, precipitation was retained in our best fit AMF model ($F_{(1, 750)} = 56.99$, $p < 0.0001$), explaining 6% of the total model variation. Generally, AMF colonization increased in all plant functional groups that had higher cold-season precipitation and colonization was lower in areas that had higher rates of warm-season precipitation and greater precipitation seasonality (precipitation PC2, Fig 19). We also found a significant interaction between plant functional groups, precipitation, and mean annual temperature ($F_{(1, 750)} = 17.91$, $p < 0.0001$) however these interactions explained very little model variation. Additionally, we observed significant interactions between precipitation and temperature (precipitation PC1 × temperature PC1 $F_{(1, 750)} = 9.77$, $p = 0.002$, precipitation PC1 × temperature PC2 $F_{(1, 750)} = 9.12$, $p = 0.002$) and our two temperature composite variables ($F_{(1, 750)} = 102.58$, $p < 0.0001$). However, precipitation-temperature and temperature-temperature interactions explained very little of the overall model variation.

Our best fit DSE model included plant functional group identity, both precipitation variables and the first temperature composite variable (AIC = 4595.8, $F_{(15, 724)} = 39.74$, $p > 0.0001$, $r^2 = 0.440$); however, retaining plant functional group ($F_{(1, 724)} = 5.06$, p = 0.025) had only marginally increased the AIC score of the best fit model (AIC = 4595.8 with plant functional group; AIC = 4596.2 without plant functional group) and accounted for only 0.26% of DSE variation. The strongest predictor of DSE colonization rate was MAT (Temp PC1, $F_{(1, 724)} = 243.65$, $p < 0.0001$), accounting for 73.9% of colonization variation (Fig 20). Overall, we found that colonization rates of DSE dropped 2% per 5℃ increase in MAT (Fig 20). We observed no interaction between plant functional type and temperature ($F_{(1, 724)} = 0.321$, $p = 0.57$), suggesting decrease in DSE colonization with increased temperature is a ubiquitous pattern for DSE, or at least not linked to host plant identity.

We also found that precipitation was a significant predictor of DSE colonization (precipitation PC1, $F_{(1, 724)} = 243.65$, $p < 0.0001$, Fig 20; precipitation PC2, $F_{(1, 724)} = 243.65$, $p < 0.0001$, Fig 20), and accounted for 4.7% and 7.73% of data variation. The influence of MAP (precipitation PC1) on DSE colonization was contingent on plant functional type (plant functional group × Temp PC1 $F_{(1,}$

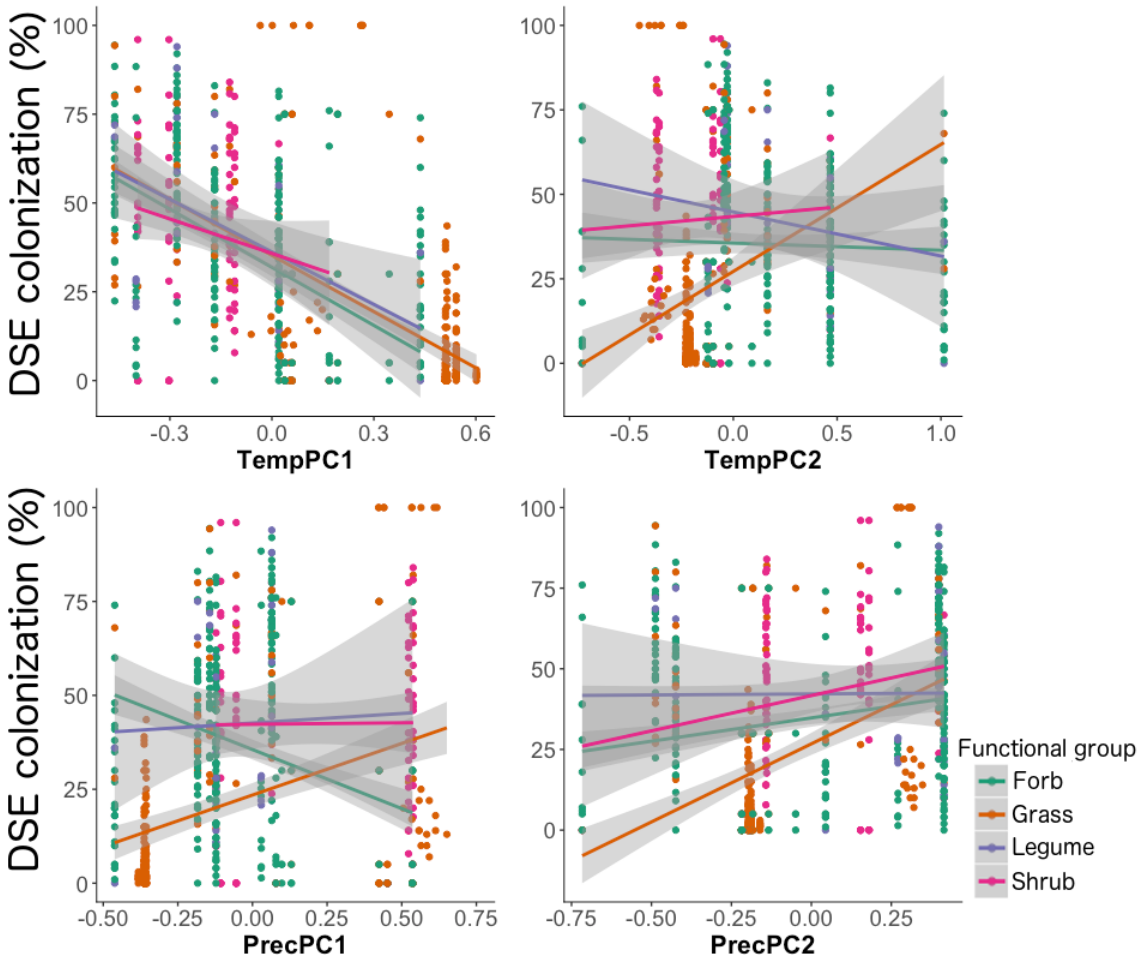

Figure 20. DSE colonization in response to composite temperature and precipitation variables. A) mean annual temperature, B), seasonality in temperature patterns, C) mean annual precipitation, D) Seasonality in precipitation patterns across 4 functional types forbs (green), grass (orange), legume (purple), and shrub (pink).

$_{724)}$ = 17.27, p < 0.0001, Fig 20). Specifically, we found areas of low MAP, DSE colonization in grasses was extremely low, however DSE colonization of grasses was positively correlated with MAP. In forbs, the opposite trend was observed, where increasing MAP decreased DSE colonization and we found MAP had no influence on DSE colonization of shrubs and legumes (Fig 20). However, the interaction between MAP and plant functional type explains only 0.18% of the total model variation. Generally, DSE colonization increased in areas that had higher cold-season precipitation and decreased in areas that had higher rates of warm-season precipitation and greater precipitation seasonality (precipitation PC2, Fig 20), and this pattern was ubiquitous across plant host functional group ($F_{(1, 724)}$ = 0.037, p = 0.85). Additionally, we observed significant interactions between our precipitation composite variables (precipitation PC1 × precipitation PC2 $F_{(1, 750)}$ = 32.41, p < 0.0001), plant functional group, MAT, and Precipitation PC2 $F_{(1, 750)}$ = 16.39, p < 0.0001), precipitation PC1, PC2, and MAT ($F_{(1, 750)}$ = 3.92, p = 0.04), however, these interactions explain very little of the overall variation in DSE colonization.

Soil properties

To understand how soil pH, carbon:nitrogen (C:N), and nitrogen:phosphorus (N:P) ratios shape fungal colonization rates, we tested for relationships between site-level soil properties and site-level plant community-weighted colonization rates. We conducted these analyses separately from the multiple regression synthesis work above because only six gradient sites reported soil pH, five sites measured C:N, and only four sites measured N:P. Overall, we found no relationship between AMF colonization rates and soil pH ($F_{(1,21)}$ = 0.478, p=0.50, Figure 5a), however we found negative correlation between soil pH and DSE colonization ($F_{(1,12)}$ = 15.068, p = 0.0022, Fig 21). Surprisingly, we found no relationship between C:N or N:P and colonization of AMF (C:N, $F_{(1,12)}$ = 2.62, p = 0.13; N:P, $F_{(1,6)}$ = 0.475, p = 0.52) and DSE (C:N, $F_{(1,6)}$ = 0.91, p =0.38; N:P, $F_{(1,6)}$ = 0.233, p = 0.65), although soil nutrient measurements were limited by sample size (Fig 21).

Fungal seasonality

Our global synthesis predicted that dark-septate endophyte colonization would be highest in areas that are cold and wet, while AMF colonization is predicted to increase in areas that are hot and dry, suggesting co-variance in temperature and precipitation. To test this hypothesis, we measured AMF and DSE colonization patterns in, widely-distributed forb, *Potentilla gracilis*, across low, medium, and high elevation sites, bi-weekly through the growing season, in our gradient site near RMBL, Colorado, USA. Measuring at multiple points across the growing season allowed us to measure the transition from cool, wet soils shortly after snowmelt to warmer and drier soils characteristic of this gradient site. Additionally, by measuring at low, medium, and high elevation sites, we could measure these patterns in sites that differed in background fungal

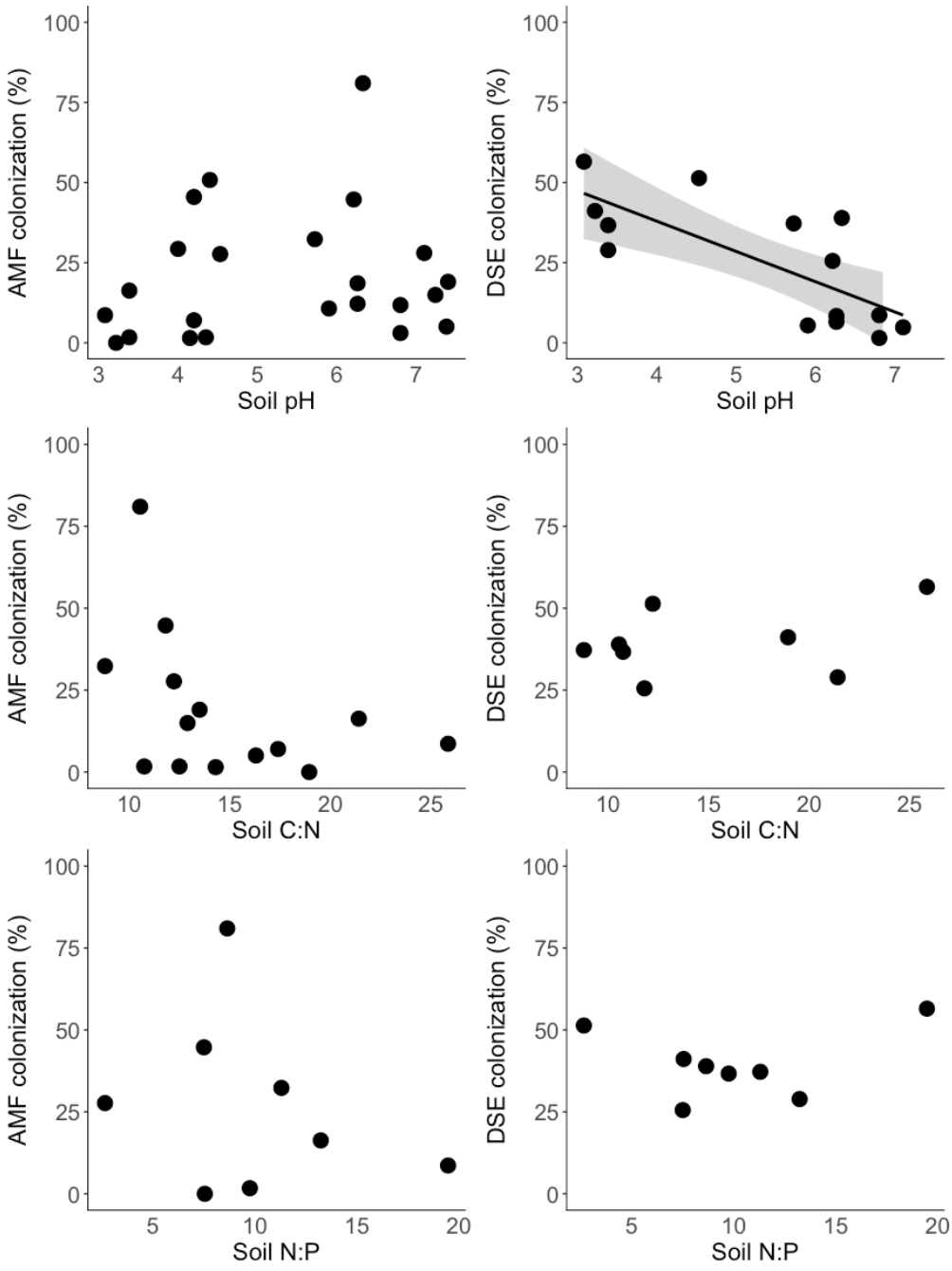

Figure 21. Community-weighted AMF and DSE colonization in response to soil pH, carbon:nitrogen, and nitrogen:phosphorus.

colonization rates and climatic properties. We predicted if DSE were responding positively to cold, wet conditions and AMF were responding positively to warm, dry conditions, then early in the season, we would observe higher DSE colonization relative to AMF, however as the growing season progresses, AMF colonization would increase and DSE colonization would decline. Contrary to these predictions, we found no consistent change in fungal colonization patterns across the growing season between fungal groups ($F_{(1, 110)} = 0.321$, $p = 0.57$), across sites ($F_{(2, 110)} = 0.625$ $p = 0.54$), or across sampling day ($F_{(1, 110)} = 0.328$, $p = 0.54$). However, we observed a significant fungi × site interaction ($F_{(2, 110)} = 103.90$, $p < 0.0001$), which matched the original observation of higher AMF abundance at low elevation sites and higher DSE abundance at high elevation sites (Fig 22). Additionally, we observed a significant fungi × site × sampling day interaction ($F_{(2, 110)} = 13.49$, $p < 0.0001$), suggesting that the response of fungal response across the growing season is contingent on local site conditions (Fig 22). For instance, at low elevation sites, we found AMF decrease through the growing season and DSE increased through the growing season. However, at medium and high elevation sits, there is no consistent response in AMF or DSE across the growing season (Fig 22). Thus, we observed no consistent pattern in AMF and DSE in response to changes in co-varying soil moisture and temperature. To build upon these results, we conducted a more explicit test of soil moisture as a potential driver of fungal colonization.

Soil moisture manipulation

To understand how soil moisture shapes fungal colonization patterns, we grew *Potentilla gracilis* in a common soil and fungal community but manipulated soil moisture between our pots. Based on the predictions of our synthesis, we predicted that AMF and DSE colonization would be negatively correlated with soil moisture. Overall, we found that mean soil moisture differed across our treatments with mean moisture levels of 9%, 14%, and 24% ($F_{(2,12)}= 252.9$, $p < 0.0001$). Surprisingly, we found that soil moisture had no influence on the colonization of AMF ($F_{(2,25)} = 0.07$, $p = 0.93$, Fig 23), DSE ($F_{(2,25)} = 0.23$, $p = 0.79$, Fig 23), the ratio of AMF to DSE ($F_{(2,25)} = 0.04$, $p = 0.96$), or any AMF or DSE structures. Comparing the initial to final AMF and DSE colonization rates, we found that across all soil moisture treatments, AMF colonization declined by 51% ($t_{(38)} = -13.52$, $p < 0.0001$) and DSE colonization declined by 24.7% ($t_{(38)} = -5.96$, $p < 0.0001$). We observed the overall decline in colonization that we expected, however it not was linked to changes in soil moisture. Thus, we conclude changing soil moisture alone likely may not shift fungal colonization levels in soils.

Overall, we found that soil moisture was positively correlated with plant height ($F_{(2,20)}=173$, $p < 0.0001$) and leaf number ($F_{(2,20)}=8.371$, $p = 0.002$) (Figure S4). Overall, we found that the high soil moisture treatment consistently increased plant height (low moisture –high moisture contrast, mean = 1.385, 95% confidence interval: 0.962, 1.809; $p < 0.0001$) and leaf number (low moisture –

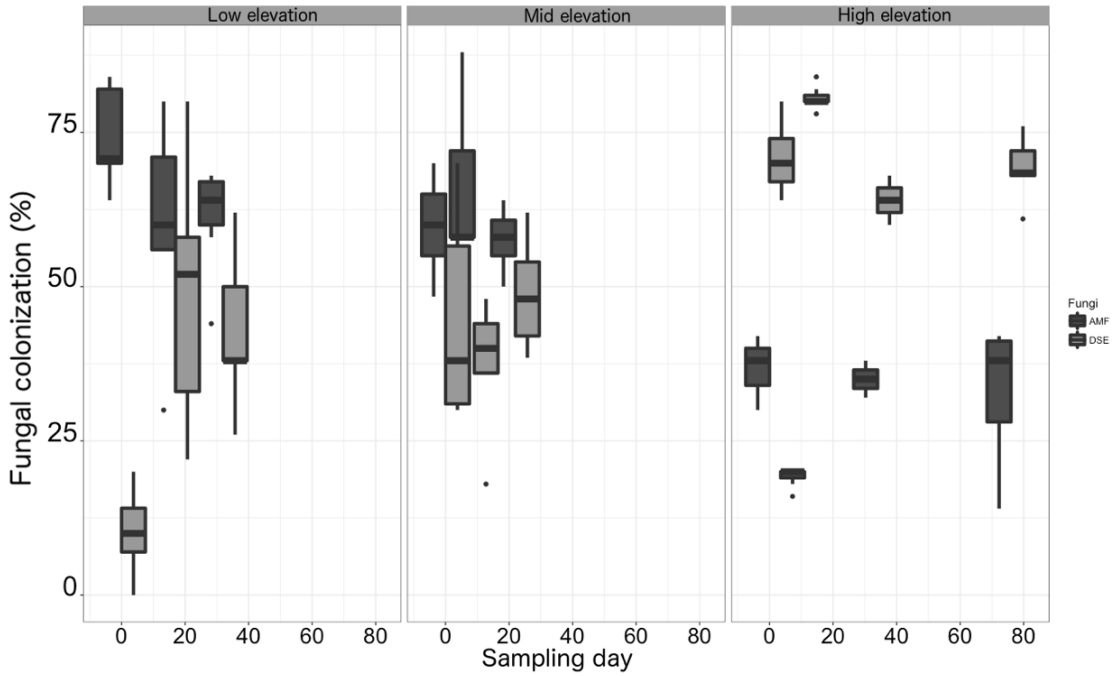

Figure 22. Seasonality in AMF and DSE colonization at three elevations in Colorado USA.

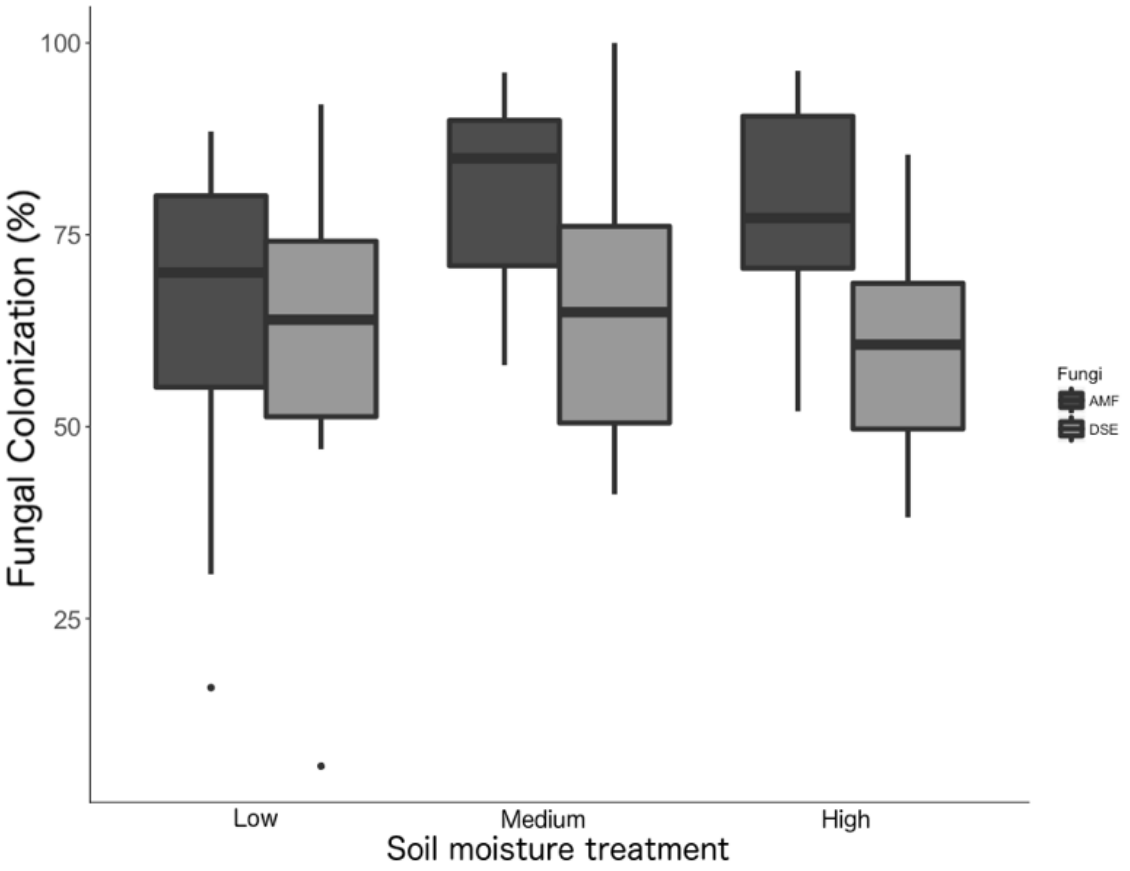

Figure 23. AMF and DSE colonization in response to changes in soil moisture.

high moisture contrast, mean = 0.963, 95% confidence interval: 0.215, 1.713; p = 0.007) relative to low soil moisture treatments. Once we accounted for differences in initial plant height, we found that soil moisture had no influence on aboveground biomass ($F_{(2,20)}$ = 0.85, p = 0.44), fine root biomass ($F_{(2,20)}$ = 1.56, p = 0.23), total root biomass ($F_{(2,20)}$ = 1.63, p = 0.21), or total biomass production ($F_{(2,20)}$ = 1.63, p = 0.21). Thus, we found that although soil moisture increased plant height and leaf number, there were no changes in total biomass, suggesting changes in plant phenotype.

Soil nutrient manipulation

Overall, we found changes in nutrient availability had no effect on AMF ($F_{(4,30)}$ = 0.69, p = 0.61) or DSE ($F_{(4,30)}$ = 1.01, p = 0.42) colonization in either *Poa alpina* or *Juncus drummondii*. Additionally, we observed no differences in aboveground biomass ($F_{(4,305)}$ = 1.59, p = 0.18), or root:shoot biomass ($F_{(4,150)}$=1.24, p = 0.29) in *Poa* or *Juncus* in response to nutrient addition. Thus, changes in nutrient availability had limited ability to shape the colonization patterns of AMF and DSE.

Discussion

Understanding the combination of biotic and abiotic factors that structure belowground communities across environmental gradients and the identification of ubiquitous patterns of belowground biogeography is one of the final frontiers in modern ecology. To circumvent philosophical, methodological, and logistical limitations that have plagued belowground biogeography for decades, we first conducted a global synthesis, combining biotic and abiotic factors structuring plant-associated fungal colonization patterns along elevational gradients to generate testable hypotheses. Overall, our global synthesis suggested that climatic factors such as mean annual temperature as well as variation in temperature, variation of precipitation and total precipitation, plant host, and soil properties determined fungal colonization rates. However, we found that AMF and DSE differed in the combination of factors that correlated with colonization patterns. For instance, obligate plant-associated AMF correlated strongly with plant host functional group and seasonal temperature variation, compared to facultative-associated DSE which was structured strongly by soil pH and mean annual temperature, although both functional groups may be performing semi-analogous functions. As a follow-up, we tested several of these hypotheses with observational and manipulative experiments at a single, representative gradient site in Colorado, USA. Overall, we found little agreement between the hypotheses that were generated by our global synthesis and the observational and mechanistic experiments to test them. For instance, our global synthesis predicted that AMF colonization would be higher in hot, dry, sites and DSE colonization would be higher in cool, wet sites, however, we found no link between temperature and soil moisture and fungal colonization patterns in an

observational field experiment across growing season or a manipulative soil moisture experiment (and hopefully nutrient experiment). This suggest that either 1) other factors that co-vary with temperature and precipitation are more important in shaping fungal colonization patterns, 2) fungal responses to temperature and precipitation are mediated through the plant host, or 3) there are differences in short-term factors driving contemporary fungal colonization patterns and long-term fungal distribution patterns along environmental gradients.

Covariance of biotic and abiotic factors along elevational gradients

Across global elevational gradients, AMF abundance is generally higher at low elevation and DSE abundance is generally higher at high elevation sites, although we found exceptions to this generalization. Of course, AMF and DSE are not responding to 'elevation'; instead, their diversity patterns are driven by some factors that covary with elevation, like temperature, precipitation, plant hosts, nutrient availability, soil pH, etc (Cantrell *et al.* 2013, Coince *et al.* 2014, Siles & Margesin 2016). Globally, the combination of biotic and abiotic factors driving species abundance and distribution differ site to site because of differences historical disturbances, geological age, relative area, topography, species composition, and climate regimes, which may cloud or prevent ubiquitous patterns in species distributions (Mayor *et al.* 2017; Sanders & Rahbek 2012). Despite this, the reduction of diversity of aboveground organisms with increasing elevation is one of the best-documented patterns observed in nature (Sundqvist *et al.* 2013; Rahbek 1995). Thus, for aboveground organisms, temperature emerges as a dominant driver of aboveground communities, despite site to site differences (Körner 1998, cite). However, in belowground communities, the link of diversity and abundance and temperature has not been as clear (Hendershot *et al.* submitted; Fierer & Jackson 2006). In fact, a wealth of papers have demonstrated that ecological legacies and evolutionary history (Treseder *et al.* 2014; Andam *et al.* 2016, Henning *et al.* submitted), disturbance history ( ), chance (Fukami 2015), soil properties (Fierer & Jackson 2006), and dispersal limitation (Peay *et al.* 2010) can all be crucial factors that shape the distribution of belowground communities. Thus, the ability of researchers to pinpoint biotic and abiotic mechanisms driving belowground distributions may be linked to the factors they choose to measure, possibly not the true factors driving communities. In the case of temperature, correlation between AMF and DSE colonization may simply co-vary with a suite of actual drivers shaping belowground communities.

Temperature and precipitation link to belowground community is mediated indirectly through the host plant

Undoubtedly, the colonization patterns of plant-associated fungal groups are intimately linked to their host plant (Smith and Read 2009; Ranelli *et al.* 2015). Our global synthesis suggested that relationships of AMF and DSE

colonization to temperature and precipitation differed by plant functional group. This suggests that either constrains of host range, plant manipulation of fungal associations to maximize services received (Bever *et al.* 2009, Kiers *et al.* 2011), shifts in plant carbon allocation patterns belowground (Bragazza *et al.*. 2014), or other in-direct mechanisms may control fungal colonization patterns more tightly relative to direct responses of fungi to changes in temperature and moisture. For instance, in a 20-year warming experiment at RMBL, increased drought stress led to increased AMF colonization in two species of graminoid; however, AMF colonization was unaffected by warming in three other graminoid species (Rudgers *et al.* 2014). Additionally, 20 years of warming seemed to benefit DSE colonization as well as DSE-associated *Carex* spp. (Rudgers *et al.* 2014), directly counter to the predictions made by our synthesis. The mutual benefit of DSE and DSE-associated plant species in response to global change, may suggest that plant-soil feedbacks may control the response of plant communities and fungal communities by proxy to ongoing global change.

Differences in short-term seasonality patterns and long-term responses to climate regimes

The match between short-term changes in fungal-colonization seasonality patterns with longer-term responses of fungal distributions along environmental gradients rests on the assumption that short-term responses of fungi will match long-term responses of fungi. However this assumption has not been tested. In plant response to global change, short-term responses of plants to warming experiments greatly under-predict the response of plants to long-term warming (Wolkovich *et al.* 2012). Matching short-term changes in fungal colonization patterns to long-term changes in fungal distributions may mask multiple processes such as dispersal differences, barriers to dispersal, species assemblages, or historical legacies at these sites (Peay *et al.* 2007). Further, although we measured fungal colonization in response to changes of warmer, drier environments, we did not measure changes in nutrient availability, plant nutrient need, changes in pH, which have all been shown to vary throughout a growing season (Ryel *et al.* 1996; Lingfei *et al.* 2005).

Linking global patterns to mechanism

The use of global-scale, publically available datasets has greatly increased our ability to perform global or mutli-site analyses (Hendershot *et al.* submitted). Since emerging on the scene in 2005, studies incorporating WorldClim climate layers have increased steadily from year to year (Hijmans *et al.* 2005). Incorporation of these available data layers makes it easy to identify correlations between species distributions and temperature and precipitation. However, our study demonstrates that caution must be given when interpreting these correlations as causation. Across an elevational gradient, temperature is undoubtedly one of the main drivers that predictably changes from low to high elevation, however it is not the only factor (Bahram *et al.* 2011). Our global

analysis predicted strong relationships between precipitation and temperature, however empirical tests of temperature and precipitation effects revealed very little influence on fungal colonization patterns. Together, the results from our study suggest that although the use of publicly-available, global-scale data have increased our ability to perform global syntheses, caution must be given when inferring causation from correlation.

CONCLUSION

Mutualisms are globally ubiquitous species interactions that are crucial for the maintenance of global biodiversity and mediating species responses to global change. Historically, mutualisms have been treated as +/+ two-species interactions. However, mutualisms are now treated as complex networks of interacting species that fall on a continuum from beneficial (+), to neutral (0), to pathogenic (-) (Janzen 1980; 1985; Bronstein 1994; 2015). The outcome of mutualistic interactions is determined by the partners involved as well as the climatic contexts they reside within (Bever *et al.* 2009; Palmer *et al.* 2010; Hoeksema *et al.* 2010; Kiers *et al.* 2011). Because global change is altering both climate contexts of ecosystems and the composition and relative abundances of the partners involved, mutualistic networks may determine the response of ecosystems to global change. As an example, plant-bacterial and fungal endophytes are responsible for maintaining plant diversity and productivity as well as carbon fluxes to the atmosphere. However, we are only beginning to understand the relationships among climate, edaphic factors, biogeography, diversity, and function in plant-endophyte communities. The four chapters of my dissertation in combination with additional collaborative research projects have allowed me to ask three inter-related questions critical to understanding and predicting how plant endophytic communities will mediate ecosystem response to ongoing climate change: (1) What are the drivers of soil biodiversity at local and global scales? (2) Do fine-scale changes in plant symbionts scale to influence community interactions and ecosystem function across the landscape? (3) How will global change re-shape interactions among microbial communities, plant hosts, and ecosystem function? To pursue these questions, my research merges tools from microbial ecology, biogeography, community ecology, and ecosystem ecology and combines fine-scale mesocosm studies, manipulative field experiments, and large-scale observational experiments. Below, I will highlight some of my current work that addresses these research questions and my future research plans.

Current Research

What are the drivers of soil biodiversity at local and global scales?

Since the time of Wallace and Darwin, global biodiversity patterns have fascinated scientists; however, the most diverse area in the world, the soil, remains a mystery. To understand the biodiversity and abundance patterns of belowground communities, as well as the factors that drive them, I conducted a meta-analysis synthesizing 325 globally distributed microbial communities with Quentin Read and undergraduate collaborator Nick Hendershot (Hendershot *et al.* 2017). Overall, we found that unlike aboveground organisms, the diversity and abundance of soil microbial communities show no ubiquitous trend with elevation or latitude. Further, we found no pattern in abundance or diversity with precipitation, soil pH, land-use history, taxonomic group, or biome type. We

concluded that for several reasons, belowground communities may not display ubiquitous, global patterns like aboveground organisms. One methodological problem we identified was that many studies cannot distinguishable between functional attributes of the organisms, studies often lump free-living and plant-associated belowground groups. We hypothesized that selection pressures and possibly distribution ranges would differ between free-living and symbiotic organisms.

As a follow-up, I focused strictly on plant-associated fungal communities to explore and predict colonization patterns at local and global scales (Chapters III and IV). For instance, in Chapter IV, I Combined fungal colonization data from multiple plant hosts in the Colorado Rocky Mountains, Chinese Tibetan Plateau, northern Sweden, and the Swiss Alps with previously published fungal colonization data and publically available climate data to explore the drivers of global colonization patterns. Additionally, in Chapter III and IV, I conducted several manipulative and observational experiments to explore the factors that drive local fungal colonization patterns at a single gradient site in the Colorado Rocky Mountains. Overall, I found mismatches between the factors that structured fungal colonization patterns at global scales and the factors the structured fungal colonization at local scales. This suggests that the composition of both aboveground and belowground communities, biotic and abiotic legacies, other edaphic and climatic properties may be important factors shaping plant-endophyte interactions along climatic gradients.

Do fine-scale changes in plant symbionts scale to influence community interactions and ecosystem function across the landscape?

Changes in the composition of symbiotic communities can alter plant host phenotype and performance (Chapter I, II, III), which scales to shape plant community dynamics and ecosystem function. Using mesocosm approaches, I demonstrated that changes in endophytic community composition regulate host morphology and physiology (Chapter I & II). Endophytic bacterial strains influence host gene expression and metabolite expression (Timm *et al.* 2016), which influence host morphology and physiology (Chapter I). In mixed endophytic communities, changes among closely-related, low-abundance bacterial strains mediate plant resource allocation and acquisition (Chapter II). However, changes in gene expression or phenotype are not easily predicted using predictive genomics or plant responses to single-strain inoculations, suggesting that microbe-microbe interactions may drive the outcome of endophyte-host function interactions. Changes in endophyte community composition cascade to shape plant community dynamics and ecosystem function. For example, changes in fungal symbiont communities can feedback to shape plant seedling survival (Chapter III), and at larger spatial scales, plant diversity and productivity (Henning *et al.* in review).

How will global change re-shape interactions among microbial communities, hosts, and ecosystem function?

Global change alters plant productivity, carbon allocation to symbionts, and ecosystem inputs, which feedback to alter the structure and function of belowground symbiotic communities. Global change factors such as warming, nutrient deposition (Chapter III, Henning *et al.* in press, Read *et al.* 2017), plant composition shifts (Chaper III), invasion (Kuebbing *et al.* 2015), and changes in aboveground herbivore populations (Shelton *et al.* 2014) directly and indirectly alter belowground community composition and function. Changes in belowground communities then feedback to influence plant community composition and ecosystem function (Henning *et al.* in review). Thus, interactions between plants and belowground symbionts may shift ecosystems to new stable states following global change or will allow ecosystems to be resistant and resilient to ongoing global change (Read *et al.* 2017, Chapter III). For instance, after 4 years of nitrogen addition and dominant plant removal in a montane meadow community, we observed no changes in fungal colonization patterns (Chapter III), as colonization of dark-septate endophyte colonization levels remained consistent following removal of the dark-septate endophyte-associated plant species *Festuca thurberi*. Similarly, after four years of Festuca removal, we found plant communities converged to a composition whose root and leaf traits resembled *Festuca thurberi* (Read *et al.* 2017*)*. Further, the plant species that compensated for the loss of *Festuca*, were all dark-septate endophyte-associated (Henning unpublished), suggesting that root endophytes may allow ecosystems some resiliency and resistance to global change. However, this hypothesis has not been directly tested and is likely contingent on the climate contexts and regional species pools.

In addition to the work outlined in my dissertation, I have explored the climate contexts shaping ecosystem response to warming and shifts in plant composition within a 4-year warming × plant removal experiment at a low and high elevation site in Colorado, USA in collaboration with Quentin Read. This experiment is part of a larger multi-site experiment that is currently distributed to 12 mountain sites globally as part of the Warming and Removal in Mountains (WaRM) network established by Aimée Classen and Nate Sanders. This experiment tests (1) how global change factors individually and interactively influence aboveground and belowground communities and (2) how changes in belowground communities will regulate plant community composition and soil carbon storage and fluxes. Early results have demonstrated that ecosystem responses to warming and removal are contingent on traits of the dominant plant species and the traits of the subordinate plant species. For instance, at low elevation, removal of a dominant, perennial sunflower, *Wyethia amplexicaulis* increases soil respiration rates and decreases net ecosystem exchange. At low elevation, warming reduces soil respiration, but only in removal plots and warming increases net ecosystem exchange, but only in *Wyethia* intact plots. At high elevation removal of dominant sedge, *Juncus drummondii* and warming does not change soil respiration rates or net ecosystem exchange rates. After four years of experimentation, we have not started measuring changes in

belowground communities in response to our treatments, but it's clear that aboveground communities and ecosystem are responding to experimental treatments.

Future directions

Ongoing global change threatens global biodiversity; however, it is unknown how the direct and indirect influences of global change will re-shape the function of future ecosystems. For instance, my research found that nitrogen addition can have multiple and simultaneous effects on plant and fungal community composition and ecosystem function, however the responses of ecosystems to nitrogen addition are contingent on edaphic, climatic, contemporary regional species pools, as well as past species pools. Thus, as my research program continues to develop, I will continue pursuing questions on how climate and edaphic contexts shape the distribution of interacting organisms, i.e. limits on the potential species pools, how climate and edaphic contexts directly shape the outcome of species interactions, and how biotic and abiotic legacies mediate the response of ecosystems to multiple and simultaneous global change drivers. To answer these questions, I will continue to combine large-scale observational research, manipulative field experiments, and mechanistic greenhouse pot and mesocosm experiments.

Additionally, a long-term goal is to expand these questions beyond plant-fungal and plant-bacterial endophyte communities to incorporate multiple aboveground and belowground communities. By incorporating belowground endophytic, plant, and aboveground seed-dispersal, protection, pollination, or pathogen networks, I will get a more holistic view of the drivers regulating community and ecosystem dynamics. The incorporation of multi-trophic plant interactions will allow me to explore questions pertaining to plant resource allocation to multiple symbiotic communities, factors that shape resource allocation strategies, and fitness tradeoffs for plants maintaining multiple symbionts. For instance, aboveground leaf pathogens regulate plant photosynthetic potential and carbon allocation toward root endophytic fungal communities. Additionally, plants that are forced to allocate carbon to multiple aboveground and belowground mutualistic symbionts may face fitness tradeoffs as they allocate carbon to receive the best return on investment, and how the legacies of past disturbance events or mutualistic partners shape the resource allocation strategies of contemporary plant hosts. Since resource allocation strategies have important implications for how and where individuals are investing carbon and nutrient resources, at larger spatial scales, multi-trophic interactions may regulate the amount of carbon that ecosystems are able to store.

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VITA

Jeremiah A Henning was born in Fond du Lac, Wisconsin in 1984, to William J. and Traci Henning and graduated from Oakfield High School in May 2002. Jeremiah's academic career began at the University of Wisconsin Oshkosh in Fall of 2002, he was the first member of his family to enroll in college. At the University of Wisconsin Oshkosh, Jeremiah struggled to find his place and underperformed in all his classes. However, after a challenging couple of years, Jeremiah became engaged in his education with mentorship from Dr. Roberta Maguire, Dr. Robert Wise, Dr. Greg Adler, and Dr. Stephen Bentivenga, who were instrumental in helping Jeremiah see his potential. During this time, Jeremiah was exposed to the work of Drs. Dan Simberloff and E.O. Wilson on the Theory of Island Biogeography (1969) as part of a Community Ecology course taught by Dr. Greg Adler. This work and course inspired Jeremiah to become a community ecologist. Shortly, after this course, Jeremiah took an introductory mycology course taught by Dr. Stephen Bentivenga which exposed him to the weird world of fungi and he was hooked on ecology and fungi. Jeremiah was fascinated by how fungi seemed to break every biological rule of how organisms are supposed to live, feed, and reproduce. He started conducting independent research in the lab of Dr. Stephen Bentivenga on fungi and land management shortly afterward.

Jeremiah graduated with a B.S. in Biology in 2007 and Jeremiah enrolled in the M.S. program at University Wisconsin Oshkosh continuing his undergraduate research project with Dr. Stephen Bentivenga. His masters work explored successional trajectories of arbuscular mycorrhizal fungal communities in a reconstructed grassland (Henning *et al. in press*). Jeremiah graduated with a Masters of Biology in December of 2009 from the University of Wisconsin Oshkosh. He was offered a prestigious technical position at the University of Indiana, Bloomington in 2011 to work with Dr. Jim Bever and Dr. Peggy Schultz to work on a diversity of fungal projects. During his time at Indiana, Jeremiah developed new skills, formulated his research interests, and focused his research questions on understanding the linkages between belowground community composition and ecosystem function and how belowground diversity maintains aboveground diversity. He was able to collaborate in a wide-variety of projects from understanding how heavy deer herbivory-shaped above- and belowground community structure and function (Shelton *et al.* 2014), understanding how belowground community composition shaped the success of grassland restorations (Middleton *et al.* 2015), to understanding how permafrost melt shaped fungal communities (Schüette *et al.* in prep). Answering these research questions brought Jeremiah to a place where he never thought he would be, living in the great state of Tennessee.

In 2012, Jeremiah was recruited to the University of Tennessee on a prestigious Chancellor's Funds fellowship. He joined the lab of Aimée Classen, in the department of Ecology & Evolutionary Biology. During this time, Jeremiah

honed his skills as a frequent traveler, orphaned graduate student, constant collaborator, and tried his hardest to think like an ecosystem ecologist. Jeremiah worked on a diverse set of scientific endeavors from invasion biology (Kuebbing *et al.* 2015), macroecology (Hendershot *et al.* 2017, Henning *et al.* in prep), molecular plant-microbe interactions (Henning *et al.* 2016, Timm *et al.* 2016, Henning *et al.* in prep), fungal community ecology (Lugo *et al.* in review), plant community ecology (Read *et al.* 2017, Read *et al.* in press), and global change biology (Classen *et al.* 2015). Jeremiah was well funded as a graduate student in Tennessee, receiving grant funding from the National Science Foundation, Oak Ridge National Laboratory, Rocky Mountain Biological Laboratory, Department of Energy, the British Mycological Society, to conduct research and to travel the world. In addition to the work conducted at the University of Tennessee, Jeremiah held appointments at the Rocky Mountain Biological Laboratory, Oak Ridge National Laboratory, as well as Peking University. However, one of Jeremiah's greatest accomplishments was his mentorship of many students at the University of Tennessee and Rocky Mountain Biological Laboratory, many of whom have gone to receive prestigious national and international grants and enroll in PhD and professional school programs. Jeremiah completed his PhD in May of 2017 from the University of Tennessee.