2015

Factors Affecting Plant Responses to Arbuscular Mycorrhizal Fungi and Soil Fungal Communities

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FACTORS AFFECTING PLANT RESPONSES TO ARBUSCULAR MYCORRHIZAL FUNGI AND SOIL FUNGAL COMMUNITIES

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Dissertation

presented in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy
in Organismal Biology and Ecology, Division of Biological Sciences

The University of Montana
Missoula, MT

September 2015

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Factors affecting plant responses to arbuscular mycorrhizal fungi and soil fungal communities

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Arbuscular mycorrhizal (AM) fungi are ancient mutualists that associate with the majority of plants. However, the factors that influence how much a plant benefits from AM fungi, or the factors that influence other root-associated fungi are unclear. I examined how plant traits related to nutrient availability can explain variation in AM-responsiveness and whether native species differ from exotics in these relationships. Leaf mass per unit of area (LMA) correlated positively with mycorrhizal growth responsiveness (MGR) and root colonization (RC) among native species. This indicates that native species with more conservative traits more strongly benefit, and benefit from, AM fungi. Furthermore, exotic species did not share this relationship, suggesting that ecological filtering can influence associations between plants and MGR.

I also investigated whether populations of the exotic plant, *Centaurea solstitialis*, collected from native versus non-native ranges, differed in AM-responsiveness. Grown alone, *C. solstitialis* from both ranges considered together derived a weak benefit from AM fungi, but in competition with the North American native *S. pulchra*, AM fungi

suppressed the biomass of *C. solstitialis*. The magnitude of this suppressive effect was greater on native versus non-native populations, suggesting that rapid evolutionary changes in how exotic plants respond to interacting AM fungal partners can affect their competitive tolerance in recipient communities.

Additions of N and of N and P can have strong effects on soil fungal community composition. However, it is unclear how individual guilds of fungi change along these gradients. I performed high-throughput sequencing on soils from the rhizosphere of *Andropogon gerardii*, the dominant C_4 grass in the Konza Tallgrass Prairie Reserve, to investigate how long-term fertilization with N and N and P affect soil fungi. Fertilization increased pathogen abundance and diversity, but AM fungal abundance and diversity was only decreased when high amounts of P were added. Further, although most AM fungal species decreased along the fertility gradient, the dominant AM fungal species increased, suggesting potential shifts in the functional attributes of those communities. These results suggest that additions of N and P can increase rhizosphere pathogen loads and increases in P can shift the composition and abundance of AM fungi.

ACKNOWLEDGEMENTS

I have so much gratitude for the countless people who helped me reach this point. I want to thank my advisor, Dr. John Maron for putting his faith in me and encouraging me to take the leap into graduate school. I am so thankful for all of the time he spent working with me to get me to this point. Thank you so much, John, for always expecting my best work. I thank the rest of my committee. In particular, I thank Ylva Lekberg, for always being there to help in every way and for encouraging me to apply modern approaches to classic ecological questions. I thank Ray Callaway for always giving me a chance, and for always knowing how to solve every problem, big or small. I thank John Klironomos, whose work originally inspired me to dive into the realm of soil ecology many years ago. Thanks for the inspiration, John! Last but certainly not least, I thank Anna Sala, for giving me my first chance at doing my own research as an undergraduate, and for her continued support through graduate school. I thank you all for your mentorship and your friendship.

I am so grateful for my family!! I could never have done any of this had it not been for my husband, Mark Waller, who has more sense than anyone I know. Lukas, my sweet little boy, who was born on the first day of school…I thank you for being such a trooper through this crazy ride. I'll never forget our soil and seed collecting misfit adventures, usually in the dark, halfway lost, and always covered in dirt. Mark and Luke, you have been such great sports from the beginning and have brought me so much perspective through it all. Thank you! I thank Mom and Dad, my ultimate yin and yang. I thank my Mom, who served proudly for many summers as our nature lady at camp. I'm so grateful for those woodland and wetland memories, collecting reptiles and peeping at

the private parts of flowers. Thank you for asking so many thoughtful questions about this dissertation over the years. I thank my Dad, for always asking me why what I'm doing is important and for teaching me the value of hard work. I know I can always count on him to have my back. Mom and Dad, your constant love and support has fueled me more than you can ever know. You shaped who I am and what I love.

I have the best friends in the world…Regan Rowley, Stephanie Lauer, Alli Staub, Darcy Chenoweth, Elizabeth Fricke, Anna Tuttle, Katie Horner, Jill Wood and the other Garmischers. Missoulians and New England-ahs too numerous to name. I thank the grad students who were on this wild ride with me, in particular Juan Oteyza, Katie Baer, Sharon Hood, Mandy Slate, Nicole Hupp, Loralee Larios, Marko Spasojevic, Riccardo Ton, Jen Palladini, Sarah Pinto, Ryan Hegstad, Karolina Fierro-Calderon, Joe Lamanna, Brett Addis, Alexis Billings, Tom Brekke, Keaton Wilson, Teal Potter and so many more…

I thank Yvette Ortega and Dean Pearson, who gave me my first shot at being a tech and got me completely hooked on Ecology. I thank co-authors, Hafiz Maherali and Gail Wilson, and field techs and volunteers, David Hooper, Whitney Wilson, Sage Stowell, Tyler Albrethsen. I thank Alexii Cornell, for helping me squeeze the guts out of all those molecules. I am thankful and inspired by the OBE faculty at the UM, who work so hard to make and maintain an outstanding department!

PREFACE

Arbuscular mycorrhizal (AM) fungi are ancient and ubiquitous plant mutualists that are responsible for significant exchanges of carbon and phosphorus between the atmosphere, plants and soil (Smith & Read 2010). AM fungi can offer multiple benefits to plants, including increased access to soil phosphorus (Smith and Read 2010), pathogen protection (Newsham et al. 1995; Maherali & Klironomos 2007; Sikes et al. 2009) and drought tolerance (Ruiz-Lozano et al. 1995). Regardless of their ubiquity and the significant ecological benefits they can confer to plants, we still have only a limited understanding of the factors that influence how much a plant benefits from AM fungi, or the factors that influence other root-associated fungi that co-occur with AM fungi.

The degree of benefit a plant receives from AM fungi can vary widely among different plant species (Johnson et al. 1997; Wilson & Hartnett 1998; Klironomos 2003), yet much of this variation remains unexplained. AM fungal benefits are measured by taking the difference in biomass between a plant grown with versus without fungi, and are referred to as the plant's mycorrhizal growth response (MGR). The MGR can affect the outcome of plant-plant competition (Hodge & Fitter 2013), and scaled more broadly, can influence plant dominance in communities (Hartnett & Wilson 1999; Urcelay & Diaz 2006). Furthermore, more AM-responsive plants allocate more carbon to AM fungi (Treseder 2013). Thus, increasing our understanding of variation in plant MGR can help us to better understand the contribution by AM fungi to processes aboveground and can also help us to better estimate fungal biomass belowground across different systems.

Plant functional traits, which characterize fundamental trade-offs between the costs of tissue construction and the benefits of capturing resources necessary to fix

carbon (Wright *et al*. 2004; Shipley et al. 2006; Donovan *et al.* 2011), may help to explain variation in responsiveness to AM fungi. For example, plants possessing traits that allow them to acquire and assimilate resources relatively quickly may receive few returns on investments in fungal symbionts, since resources that go to fungi might come at the expense of fast growth. In contrast, slower, conservative species that invest more into storage and construction than into growth may incur fewer costs relative to the long-term nutritional benefits received from AM fungi. In Chapter one of my dissertation, I tested this hypothesis by using previously published mycorrhizal growth response and colonization data from two distinct systems (Klironomos 2003; Anacker et al 2014; Avolio et al. 2014) with plant functional trait data from two distinct systems to test whether plant traits related to resource use explained variation in mycorrhizal benefits and colonization. My results suggest that plants possessing traits characteristic of conservative resource use gain more benefits from and invest more into mycorrhizal fungi when compared to species with exploitative traits.

Exotic, invasive species also show wide variation in MGR, which can differ from the native species they displace (Pringle et al. 2009). The factors explaining this variation as well as the potential ecological benefits are also unclear. In Chapter one, I also examined whether native species differ from exotics in their relationship between resource use strategies and responsiveness to AM fungi. I found that exotics did not share the same relationship as natives, and the difference appeared to be driven by different factors in each system. Specifically, exotics from the Guelph system had a more narrow range of LMA values than the natives. On the other hand, Konza exotics had a more narrow range of responsiveness than the natives, but this difference may be

unrelated to LMA. Others have shown rapid evolution in exotic genotypes of more exploitative traits such as faster growth rates (Bossdorf et al. 2005), higher nutrient use efficiency (Funk & Vitousek 2007) and lower LMA (Smith & Knapp 2001; Feng & Fu 2008), in other systems as well, which may alter the way those exotics interact with AM fungi.

The observation that exotics did not share similar relationships between traits associated with resource use strategies and MGR suggest that environmental filtering can strongly influence associations between exotic plants and MGR. Although the selective factors that affect these relationships may vary across different systems, the potential ecological advantages associated with differences in MGR between natives and exotics are unclear. In the second chapter of my dissertation, I used the exotic annual thistle, *Centaurea solstitialis,* to test whether native and exotic genotypes differed in responsiveness to AM fungi and whether range-based differences in mycorrhizal responsiveness may explain the competitive success of *C. solstitialis* in the invaded range. I found that AM fungi provided weak MGRs to native and exotic plants when grown alone and AM fungi also reduced the competitive tolerance of all plants grown in competition. However, AM fungi reduced the competitive tolerance of native populations much more strongly than the exotics, suggesting that range-based differences in the way *Centaurea solstitialis* interacts with AM fungi may affect the way they compete in their new versus native range.

Recent work investigating the effects of nutrient enrichment on soil microbial communities show that addition of N (Treseder 2008; Dean et al. 2014) and of N and P (Leff et al. 2015) can have strong effects on soil fungal community composition.

However, fungal communities are made up of many different types of organisms, ranging from plant mutualists to pathogens. Few studies have investigated how nutritional inputs can affect co-occurring guilds of microbes. This may be especially important to understand, since different groups of root-associated fungi may be affected differently by nutrient inputs, which could shift the functional attributes of soil fungal communities. For example, AM fungal communities are sensitive to changes in N and P availability (Treseder 2004; Johnson 2010), such that nutritional inputs resulting in relatively high phosphorus availability can decrease AM abundance (Johnson 1993, Johnson *et al*. 1997; Eom et al. 1999; Egerton-Warburton 2000, 2007; Corkidi *et al.* 2002; Treseder 2004) and diminish the beneficial properties of AM fungi (Johnson et al. 1997 & 2003). Some studies, mainly from the agricultural literature, have shown that pathogenic fungal abundance increases with nitrogen additions (Walters & Bingham 2007; Veresoglou *et al.* 2013), but we know virtually nothing about how nutrient inputs affect pathogenic root fungi in natural systems. If nutrient additions decrease the abundance and shift the function of AM fungal communities, while concomitantly increasing pathogenic fungal communities, plant performance may suffer.

In Chapter three of my dissertation, I sampled soils from the rhizosphere of the highly mycorrhizal community dominant, *Andropogon gerardii*. Using next-generation sequencing, I characterized communities of AM and pathogenic fungi. Indeed, I observed increases in the relative abundance of pathogenic fungi where N and P were added, and decreases in abundance and community compositional shifts of AM fungi where high rates of P were added. These results suggest that additions of N and high P,

conditions which favor higher pathogen loads and decreases in AM fungi, may change the overall function of those soil fungal communities.

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Chapter 3

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CHAPTER ONE
PLANT FUNCTIONAL TRAITS PREDICT MYCORRHIZAL GROWTH RESPONSIVENESS IN TWO DIFFERENT PLANT COMMUNITIES

ABSTRACT

Plant responses to AM fungi span a continuum from positive to negative. Similarly, root colonization (RC) by AM fungi, which may reflect benefits offered by plants to fungi, also varies widely among plant hosts. However, we currently lack a clear predictive framework for understanding what drives this variation, particularly among non N-fixing forbs. I used original and published mycorrhizal growth response and colonization data with plant functional trait data to investigate whether plant traits related to resource use explained variation in mycorrhizal benefits and colonization. I also examined whether native species differ from exotics in these relationships. After accounting for phylogenetic relationships among species, leaf mass per unit of area (LMA) was positively correlated with mycorrhizal growth responsiveness (MGR) and root colonization (RC) among native species. By contrast, there was no relationship between LMA and MGR or RC among exotic species, which demonstrated less variability in traits than natives. These results indicate that species with lower growth rates and photosynthetic capacities, as indicated by high LMA more strongly benefit, and benefit from, AM fungi. Furthermore, exotic species, whose LMA was lower than that of natives, did not show such a relationship, suggesting that ecological filtering can strongly influence associations between plants and MGR.

INTRODUCTION

The nutritional mutualism between plants and arbuscular mycorrhizal (AM) fungi is ancient, likely originating early in the evolution of plants (Remy *et al.* 1994). The mutualism is also omnipresent, as the majority of extant plants make facultative associations with the fungi (Smith & Read 2010). This symbiosis represents a significant source of resource exchange between the atmosphere, plants and soil. For example, plants may allocate up to 30% of their fixed carbon to AM fungi (Drigo 2010). In return, AM fungi provide the majority of a plant's phosphorus requirement (Smith *et al.* 2003), which may result in significant increases in plant biomass (Smith & Read 2010). However, the magnitude of these benefits can vary widely and seemingly idiosyncratically among species in common environments when phosphorus is limiting. Indeed, growth responses to AM fungi (hereafter referred to as mycorrhizal growth responses, or MGR) are best viewed as a continuum between strongly negative and strongly positive responses (Johnson *et al.* 1997; Klironomos, 2003). This continuum of responses to AM fungi among plant species is surprising given that all plants require phosphorus in relatively large quantities, phosphorus is generally limiting to plant growth, and AM fungi ameliorate this limitation. In other words, given the high phosphorus demands of plants, why aren't all plants highly responsive to AM fungi in phosphorus limiting environments?

The MGR of different plant species can be highly dependent on the abiotic context (Smith & Read 2010; Hoeksema *et al.* 2010). Plants release more carbon from their roots when nutrient stressed, making them better hosts for AM fungi, since those soluble carbohydrates in the roots are available to AM fungi before they pass into the soil

(Sylvia & Neal 1990; Schwab *et al.* 1991). When nutrients are available, however, plants allocate less carbon to fungal symbionts (Olsson et al. 2010), which often results in reduced fungal abundance and consequently, reduced resource delivery (Hetrick *et al.* 1992; Hetrick *et al.* 1993; Johnson *et al.* 1997). But whether such proximal controls on AM responsiveness are related to the ultimate factors that determine responsiveness across species remains unclear.

Because the outcome of the interaction between plants and AM fungi is proximately determined by resource supply, adaptation to *variation* in resource availability, rather than the over-all availability of resources might help explain variation in MGR. For example, fast growing annual species, which typically occur in high nutrient areas, tend to benefit less from AM fungi than slower growing perennial species, which often grow in areas where nutrients are more limiting. However, since fast growing annual species also benefit less from AM fungi than slower growing perennials in common gardens at the same fertility level (Peat & Fitter 1993; Wilson & Hartnett 1998; Hoeksema *et al.* 2010), it is likely that other factors besides nutrient availability alone drive variation in responsiveness. Thus, plant strategies (i.e. traits) related to resource supply and acquisition may contribute substantially to how strongly plant species respond to AM fungi.

Plant resource-use strategies characterize fundamental trade-offs between resource conservation and resource acquisition (Ackerly *et al.* 2000; Reich et al. 2003; Shipley *et al*. 2006; Donovan *et al.* 2011), and may strongly influence the outcome of plant-fungal interactions. Plants adapted to low resource environments are more conservative in their resource use, have tissues with higher carbon content compared to

plants from high resource areas, and a greater capacity for carbon storage rather than turnover (Grime 1977 & 1979; Coley et al. 1985; Poorter *et al*. 2009; Reich 1997 & 2014). This conservative strategy is characterized by high leaf mass per unit of area (LMA) and low nitrogen per unit of leaf mass (N_{mass}), which are traits that promote "slow" tissue development and turnover rates (Wright et al 2004). AM fungi put high carbon demands on their hosts (Douds *et al.* 2000; Bago *et al.* 2000), so plants with traits that promote carbon accumulation and storage may be better hosts for AM fungi and consequently, the costs of hosting may be lower than the benefits received. Alternatively, in environments where resource supply is high, traits specialized for resource acquisition and assimilation, such as tissues with rapid turnover times and fine root production may be favored (Chapin, 1980; Craine, 2009). These traits may allow them to obtain nutrients independent of symbionts. Thus, hosting AM fungi may come at the expense of growth for resource acquisitive species, so those plants would be expected to benefit less and to invest less in AM fungi, which would be reflected in lower rates of colonization and lower MGR. The quantitative traits LMA and N_{mass} can be used to predict some plant resource-use strategies (Wright *et al.* 2004) and these traits may also help us better understand how plants interact with AM fungi.

Although plant traits associated with resource use may help identify causes of variation in MGR, past work focused on root traits has not found strong associations. For example, some interspecific comparisons indicate that coarse rooted species should respond more positively to AM fungi than fine rooted species (Baylis, 1970, 1975; Fitter 2004). C_3 grasses with fibrous root systems, have been found to depended weakly on AM fungi, whereas C_4 grasses with coarser root systems tended to be highly dependent

(Hetrick *et al.* 1991; Wilson & Hartnett 1998). However, it is not clear these root traits cannot be separated from aboveground traits, and no consistent relationship was observed between root architecture and AM dependence of prairie forbs (Hetrick *et al.* 1991). Furthermore, a recent meta-analysis suggests that functional variation in root traits is a poor predictor of responsiveness to AM fungi on a broad scale (Maherali 2014). Even though roots are where the symbiosis occurs, leaves are the primary plant organs responsible for acquiring carbon and carbon is the currency paid by plants to AM fungi. Thus, aboveground traits, such as LMA and N_{mass}, which are leaf traits that are highly correlated with the resource environment where plants occur (Wright *et al.* 2004), may be more suitable predictors of variation in MGR.

To test whether leaf traits that are indicative of plant resource strategies are associated with mycorrhizal responsiveness, I used original and published MGR data for 124 native and exotic species that occur in two distinct ecological communities. I combined these data with LMA and N_{mass} obtained from trait databases and tested whether these traits were correlated with MGR and AM colonization. I analyzed native and exotic species separately because previous studies have shown that successful exotic species behave in fundamentally different ways than natives, often have faster growth rates (Bossdorf *et al.* 2005) and possess suites of traits that enable rapid resource acquisition, such as lower LMA (Smith & Knapp 2001; Feng & Fu 2008) and higher nutrient use efficiency (Funk & Vitousek 2007) than the natives they displace. Thus, I predicted that exotic species would have a narrower distribution of trait values, and would be less likely to make the same trade-offs related to resource capture as native species. I predicted that native plant species possessing leaf traits characteristic of

conservative resource use should benefit more from AM fungi and have greater colonization than native species possessing leaf traits characteristic of exploitative resource use.

METHODS

I used plant growth response to mycorrhizae and leaf trait data from two different systems (the Konza Prairie Long-Term Ecological Research Site, hereafter Konza, and the Guelph Long-Term Mycorrhizal Research Site, hereafter Guelph). The Konza data came from a published study investigating mycorrhizal responsiveness among 95 tallgrass prairie species (Wilson & Hartnett 1998). Plant species in the Wilson and Hartnett (1998) study were grown from seeds purchased from commercial sources in field-collected, steam-pasteurized soil. For each species, seven seedlings were either inoculated with 200 spores each of *Glomus etunicatum* and *Gl. mosseae* or used as controls without adding spores. Plants were fertilized biweekly with a 20:0:25 N-P-K fertilizer for 16 weeks and harvested, dried and weighed.

To quantify the MGR of old field plants in southern Ontario, 115 plants were grown from seeds collected in the University of Guelph Arboretum and other old fields in the Guelph region. Plants were grown in 20 cm diameter, 3 L nursery pots containing a 4:1 ratio of sand to promix BX, a peat-based growth medium (Premier Tech, Rivière-du-Loup, QC). The potting soil was sterilized prior to planting by exposing it to a 32 kGy (±10%) dose of gamma-irradiation from a Cobalt-60 source over a period of 7 days (McMaster University Nuclear Reactor, Hamilton, ON). For each species, 10 pots were each inoculated with 200 spores of commercially produced *Glomus intraradices* inoculum (Premier Tech, Rivière-du-Loup, QC), and an additional 10 pots were grown as

controls without spores. Stratified seeds were placed in pots after fungal spores were added. During the two-week seedling germination and establishment period, pots were watered every day, and then watered to saturation 2-3 times per week for the remainder of the experiment. All plants were fertilized with 300 ml of a ¼ strength Hoagland's solution every two weeks. Aboveground plant parts were harvested after 348-372 days growth, dried, and weighed. The experiments were carried out over 3 years (subsets of species were grown in year long trials that began in May 2003 and finished in April 2006).

The mycorrhizal response ratio was calculated as the log response ratio, MGR = $\ln[X_i/X_n]$, where X_i is inoculated biomass and X_n is non-inoculated biomass (Hoeksema et al. 2010). I obtained leaf trait data for 43 of the 95 species from Konza and 80 of the 115 species from Guelph from the TRY (Kattge *et al.* 2011, www.try-db.org), LEDA (www.leda-traitbase. org) and the GLOPNET (Wright *et al.* 2004) databases. I supplemented the data from these databases with published data from the literature, found by searching Google Scholar using the keywords "mycorrhiza*", "SLA" and individual species' names. The original references for the data from TRY, LEDA, GLOPNET and the additional studies are in the Supporting Information.

I constructed a phylogeny for the Konza and Guelph datasets using Phylomatic (Webb *et al.* 2008), which establishes taxonomic relationships based primarily on the third report of the Angiosperm Phylogeny Group (APG III 2009). With the Guelph dataset, I used the 'bladi' function in Phylocom (Webb *et al.* 2008) to calculate branch lengths from fossil-calibrated node ages (Wikstrom *et al.* 2001) and to create an ultrametric tree for analysis. With the Konza dataset, branch length calibration was

conducted in BEAST using a tree reconstructed with maximum likelihood using RAxML-HPC2 v 7.2.6 (Bell *et al.* 2010).

To account for the effects of shared ancestry among the species in the dataset, I used phylogenetic generalized least squares regression (PGLS) with the package Ape in R (R Development Core Team 2010, http://cran.r-project.org) to investigate the relationship between the mycorrhizal growth response ratio (MGR) and root colonization (RC) with LMA and N_{mass}. RC was calculated using standard protocols (McGonigle 1990). I did not have colonization data for the Guelph dataset, so I limited the colonization analysis to the Konza dataset.

Because absolute growth responses to AM fungi likely depend on the environmental conditions in which the interaction plays out (e.g. soil and greenhouse conditions, pot size, etc.; Hoeksema et al. 2010), I assessed relative differences in responses among plants from Konza and Guelph separately.

I performed a Bartlett test to determine whether I met the assumptions of an ANOVA to test for differences in mean LMA and MGR between native and exotic species. Because the assumption of unequal variance was not met for some of these variables, I performed t-tests to compare the mean MGR and LMA between native and exotic species in each system, since a t-test is more robust to the violation of unequal variance.

I used a natural log transformation of variables to correct heteroscedasticity, as determined by a Breuch Pagan test. However, the relationships between MGR and LMA in the Konza native and exotic datasets were still heteroscedastic after transformation, so I explored the data for potential outliers and found three clear outliers in the native

dataset. A search of the PLANTS database revealed two of these outliers were species commonly found in wetland areas. Since AM fungi are not typically found in these environments, I removed those species from this study. The other outlier was the native C3 grass *Koeleria pyramidata* in the Konza dataset, which was also removed. Removal of these outliers corrected the heteroscedasticity. Since I was unable to meet the assumptions of the linear regression model in the Konza exotic dataset, I used the non-parametric Spearman's test to examine relationships between traits and MGR.

RESULTS

Mycorrhizal growth responsiveness (MGR) correlated positively with dry leaf mass per unit of area (LMA) for native species in both the Guelph old-field (Fig. 1a, r^2=0.24; $F_{1,27}$=9.73, p=0.0043, b(SE)=0.17(0.05), λ=0.66) and the Konza tallgrass prairie system (Fig. 1b., r^2=0.37; $F_{1,23}$=15.04, p=0.0008, b(SE)=2.33(0.60), λ=0). When the outlier *Koeleria macrantha* is included, the relationship is somewhat weaker, but still significant (r^2=0.29; $F_{1,24}$=11.37, p=0.0025, b=2.16(0.64), λ=0). MGR scaled positively with N_{mass} in the Guelph dataset (Fig. 2, r^2=0.47; $F_{1,12}$=12.42, p=0.0042, b(SE)=-0.78(0.22), λ=0.50). Root colonization (RC) scaled positively with LMA among the native species in the Konza dataset (Fig. 3, r^2=0.24; $F_{1,23}$=9.9, p=0.0073, b=0.47(0.16), λ=0.42).

For exotic species only, mycorrhizal responsiveness was independent of LMA (Fig. 4a, Guelph: r^2=0.03; $F_{1,49}$=2.40, p=0.1278, b=0.13(0.09), λ=0.59; Fig. 4b, Konza: ρ=-0.005, p=0.5075, n=17) and N_{mass} (Guelph: r^2=-0.09, p=0.8743, $F_{1,15}$=0.03, λ=0.20). Root colonization was similarly independent of LMA (Konza: ρ=0.04, p=0.4405, n=17).

There was no evidence for unequal variance in ln LMA among natives and exotic species in the Konza dataset, or in MGR among natives and exotics in the Guelph dataset. However, the Bartlett test did show unequal variance between native and exotic species for ln LMA in the Guelph dataset (Bartlett's K^2=14.84, p<0.0001) and for MGR in the Konza dataset (Bartlett's K^2=4.36, p<0.0368). Specifically, the ln LMA among Guelph natives ranged from 2.85 to 4.81 but the exotics only ranged from 3.27 to 4.40. The ln MGR among the Konza natives ranged from -0.14 to 5.25, whereas the exotics only ranged from -0.29 to 3.71. The mean ln MGR among Konza Prairie natives (2.28 +/- 0.36) was significantly higher than that of the exotics (0.55 +/- 0.26, t_{40}=-3.88, p=0.0004), and the ln LMA of the natives' (4.03 +/-0.10) was marginally higher than the exotics (3.79 +/-0.08, t_{40}=-1.88, p=0.0674). Provenance had no significant effect on the mean ln MGR (natives: 0.18 +/-0.03, exotics: 0.16 +/-0.03) or the mean ln LMA (natives: 3.84 +/-0.09, exotics: 3.86 +/-0.04) in the Guelph dataset (ln MGR: t_{62}=-0.33, p=0.7428; ln LMA: t_{37}=-0.25, p=0.8063).

DISCUSSION

These findings indicate that leaf traits associated with plant resource use strategies (LMA and N_{mass}) are good predictors of plant responsiveness to and colonization by AM fungi. Leaf traits such as LMA and N_{mass} can be related to a plant's investment in longevity, and variation in these traits has consequences for interactions with other organisms, such as herbivores (Coley et al. 1985), and as I show here, fungal symbionts. This work, along with a spate of other recent research (Orwin *et al.* 2010; Laughlin 2011; de Vries *et al.* 2012; Legay *et al*. 2014; Ke *et al.* 2015) suggests that leaf traits can offer insights into identifying the causes of variation in plant responses to soil biota. However,

all results to date are strictly correlative, and as such, they still require a mechanistic explanation. One hypothesis is that this relationship is driven by differences in the quality and quantity of carbon compounds made available to AM fungi among plant species. These differences may be complex, but using plant traits associated with resource-use strategies should make these species-level differences clearer in the context of the plant-AM fungal symbiosis.

If a higher capacity to reward AM fungi increases a plant species' benefit from the fungi (Lekberg et al. 2010; Kiers *et al*. 2011), one hypothesis to explain the variation in mycorrhizal benefits across the resource-use spectrum is that conservative species have more carbon available to reward AM fungi. Species possessing traits specialized for resource conservation store large pools of carbohydrates in roots relative to species possessing traits specialized for resource acquisition (Chapin, 1980; Chapin *et al.* 1993; Craine, 2009). Although storage may come at the expense of growth for slower growers, if this carbon is available to reward symbionts, it may give plants a long-term performance advantage that have higher pay-offs than allocation to growth. Although differences in carbon allocation patterns among plants that vary in their resource-use strategies is fairly well-understood (Hobbie, 1992; Aerts & Chapin 2000; Westoby *et al.* 2002; Craine, 2009), the hypothesis that greater carbon storage increases plant benefit from the mycorrhizal symbiosis has not been explicitly tested.

A second hypothesis for the association between plant resource-use strategy and MGR is that the quantity of carbon exudates differs among conservative and exploitative plant species. Plants growing in low fertility conditions, specifically nitrogen-limited environments may increase nutrient availability by diffusing stored carbon into the soil

through their roots (Sylvia & Neal 1990; Schwab *et al.* 1991). This soil priming may stimulate decomposition by otherwise carbon-limited saprophytic fungi, which may increase nitrogen availability for plants through breakdown of organic material (Craine, 2009; Orwin *et al.* 2012). Prior to leaving plant roots, this carbon may also stimulate AM fungi (Sylvia & Neal 1990; Schwab *et al.* 1991). However, although plants have higher exudation rates when nutrient stressed, interspecific variation in the amount of C that plants allocate in the form of exudates and the degree of plasticity in this trait is unknown. It is reasonable to expect that plants adapted to low fertility environments allocate more carbon below-ground in the form of exudates, since they have more stored carbohydrates in their roots (Chapin, 1980), but the only studies to my knowledge that have investigated exudation variation have tested only a few species from managed systems (Jones *et al.* 2009).

These results also reveal a positive relationship between LMA and root colonization. We might expect such a relationship, since plant growth responses often increase with increasing colonization, potentially as a result of increased P delivery (Lekberg & Koide 2005; Treseder, 2013). However, this result may also lend support to the hypothesis that plants with higher LMA offer more carbon to AM fungi. If plants with more conservative strategies offer more resources to AM fungi, and AM fungi reciprocate with increased phosphorus delivery, this would support for the model of reciprocal trade in mycorrhizal symbioses (Lekberg et al. 2010; Kiers *et al*. 2011).

The plant species investigated here co-occur across relatively subtle resource gradients. Hence, these species likely possess adaptations closer to the middle of the resource availability spectrum compared to species that occur in geographically separated

and distinct resource environments. Thus, there might be even stronger patterns if intraspecific variation in resource use across large resource gradients were explored (see Schultz *et al.* 2001) or species at the far ends of the resource use spectrum were compared. For example, plants adapted to extreme resource limitations associate with different types of fungi (Cornelissen *et al.* 2001) or utilize strategies for resource uptake that are independent of AM fungi, such as the exudation of organic acids or phytosiderophores (Dakora & Philips 2002; Jones *et al*. 2004). Alternative resource use strategies such as these may also explain outliers among the species investigated here, such as some of the C_3 grasses from the tallgrass prairie, particularly *Koeleria pyramidata*.

The relationship between leaf traits and mycorrhizal responsiveness was remarkably consistent across both grassland and old-field plants, even though mycorrhizal responses varied by orders of magnitude between Konza and Guelph species. The species from Konza Prairie performed very poorly in the absence of AM fungi, but extremely well in their presence. Alternatively, species from Guelph grew well without, but slightly better with AM fungi (Konza species' average proportional response to AM fungi was 66% and the Guelph species' was 22%). These differences in responsiveness may be explained by intrinsic differences in resource availability between the two systems. Konza Prairie is an N and P-limited system (Eom*, et al.* 1999; Johnson *et al.* 2003 & 2015), particularly where historical fire regimes are maintained (Seastedt *et al.* 1991). The experimental plants from the Konza dataset were fertilized with N, but not P (Wilson & Hartnett 1998). The alleviation of N-limitations, but not P may have increased the magnitude of the benefits those plants received from AM fungi, which has

been observed in other studies as well (Eom *et al*. 1999; Edgerton-Warburton, et al. 2007). The fertilizer used in the Guelph experiment, however, did include some P, which might explain their weaker responses. Interestingly, species from both sites shared the same general relationship between their leaf traits and mycorrhizal responsiveness, regardless of these differences in growth response.

Another intriguing pattern that emerged from this analysis is that in contrast to natives, exotics lacked a relationship between the leaf traits investigated and mycorrhizal responsiveness. There was a considerable range of variation in both LMA and MGR among native species from both systems, but comparably little variation in LMA and MGR for the exotics from Guelph and Konza, respectively. Had I seen the same variation in these traits among exotics as I did among natives for these variables, it is possible that I may have observed a similar relationship as the natives. Instead, it appears that the exotics from each system converged along a single trait axis. Exotic species from Konza, as a group, derived significantly fewer mycorrhizal benefits than natives, while exotics from Guelph had significantly lower LMA as a group. This may be reflective of newly evolved traits in exotic species in their new range. Some evidence supports this idea, as many exotic species possess traits that allow them to exploit resources better than co-occurring native neighbors (Levine *et al.* 2003; Liao *et al.* 2008). If exotic species are able to escape ecological costs that natives cannot, it may help to explain the competitive success of some exotics versus natives in their new range.

Previous studies have focused on classifications that have left much of the variation in mycorrhizal responsiveness unexplained. Plant functional groups do a very good job of explaining the variation between some large groups of plants. For example,

C_4 grasses are a highly mycorrhizal group compared to cooler season C_3 grasses (Wilson & Hartnett 1998; Hoeksema *at al.* 2010). Functional group is a discrete description and strongly linked to phylogeny. Although mycorrhizal responsiveness shows a phylogenetic signal (Anacker et al. 2014; Reinhart *et al.* 2012), phylogeny does a poor job of predicting responsiveness on a broad scale (Reinhart *et al.* 2012). By correcting for phylogenetic relatedness among the species investigated here, I show that variation in mycorrhizal responsiveness and percent AM colonization are driven by factors other than evolutionary history alone. Further, this analysis includes species from a range of functional groups, such as non N-fixing forbs, whose variation in mycorrhizal responsiveness has been previously unexplained. Future research could explore the hypotheses raised here to explain the mechanism behind the relationship between functional traits and mycorrhizal responsiveness.

ACKNOWLEDGEMENTS

This study was supported by the TRY initiative on plant traits (Kattge *et al.* 2011, http://www.try-db.org), the LEDA traitbase and the GLOPNET database, archived in the online supplementary materials in (Wright *et al.* 2005) and available at www.nature.com/nature/journal/v428/n6985/suppinfo/nature02403.html. We thank Drs. Loralee Larios and Marko Spasojevic for providing helpful comments on the manuscript.

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FIGURE LEGENDS

Figure 1. Relationship between leaf dry mass per unit area (LMA) and mycorrhizal responsiveness for the native species from the **a)** Guelph Long-Term Mycorrhizal Research Site (r^2=0.24, p=0.0043, n=29) and **b)** the Konza Prairie Long-Term Ecological Research Site (r^2=0.37, p=0.0008, n=25). All values are displayed on a natural logarithmic scale.

Figure 2. Relationship describing nitrogen content per unit of mass (N_{mass}) to mycorrhizal responsiveness of the native species from the Guelph Long-Term Mycorrhizal Research Site (r^2=0.47, p=0.0042, n=17). All values are displayed on a natural logarithmic scale.

Figure 3. Relationship describing leaf dry mass per unit area (LMA) to root colonization of the native species from the Konza Prairie Long-Term Ecological Research Site (r^2=0.24, p=0.0073, n=25). All values are displayed on a natural logarithmic scale.

Figure 4. a) Relationship describing leaf dry mass per unit area (LMA) with mycorrhizal responsiveness of the exotic species from the Guelph dataset (r^2=0.03, p=0.1278, n=51). All values are displayed on a natural logarithmic scale. **b)** Correlation between leaf dry mass per unit area (LMA) with root colonization of the exotic species from the Konza Prairie dataset. (ρ=-0.005, p=0.5075, n=17). All values are displayed on a natural logarithmic scale.

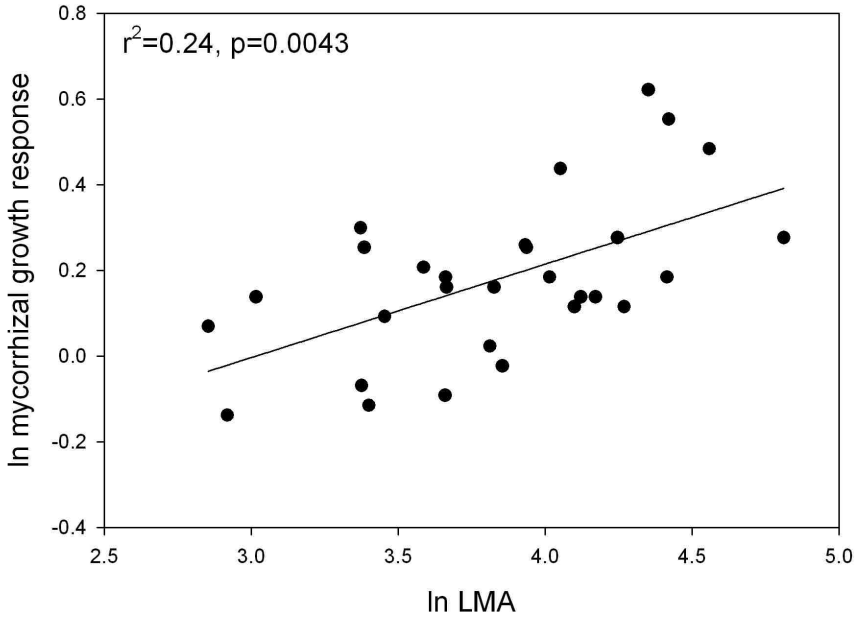
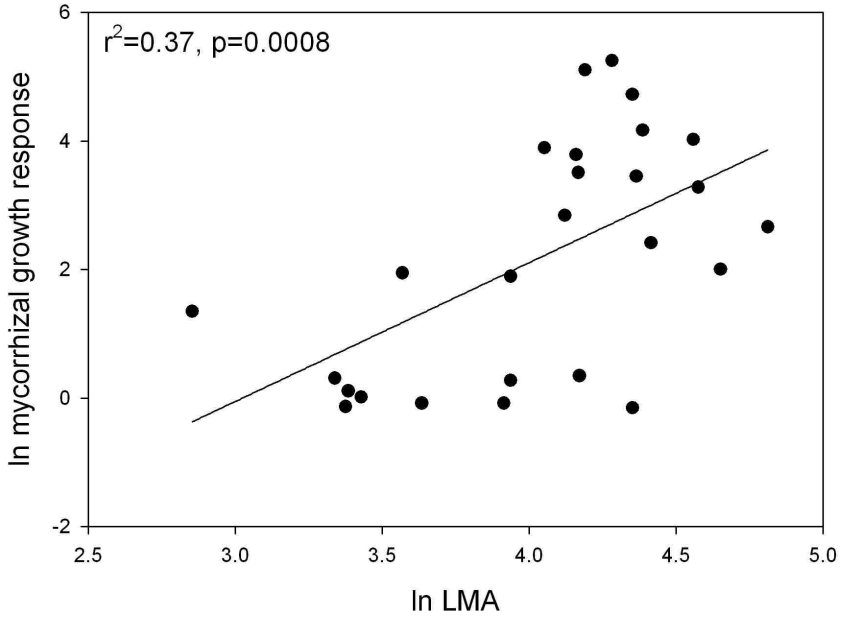


Figure 1 a)



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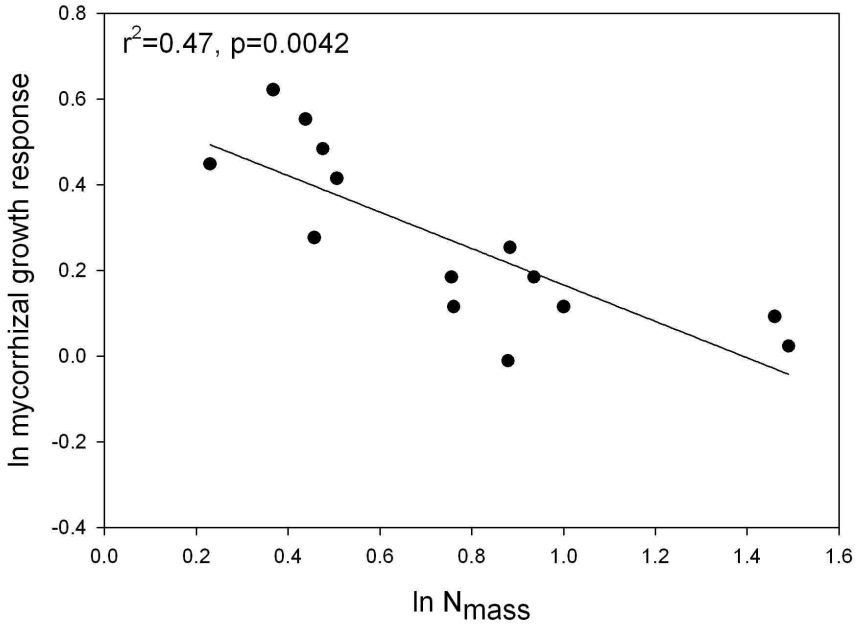

Figure 2

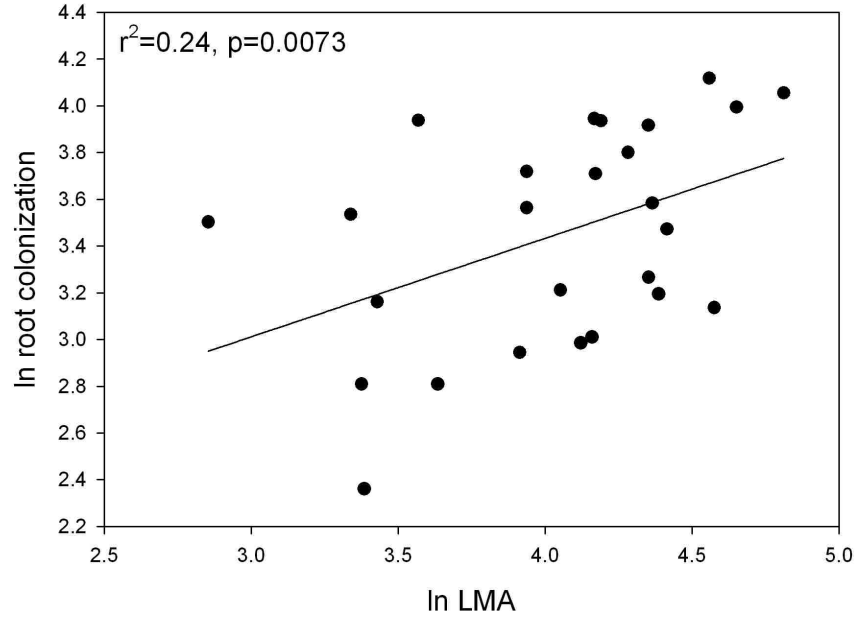

Figure 3

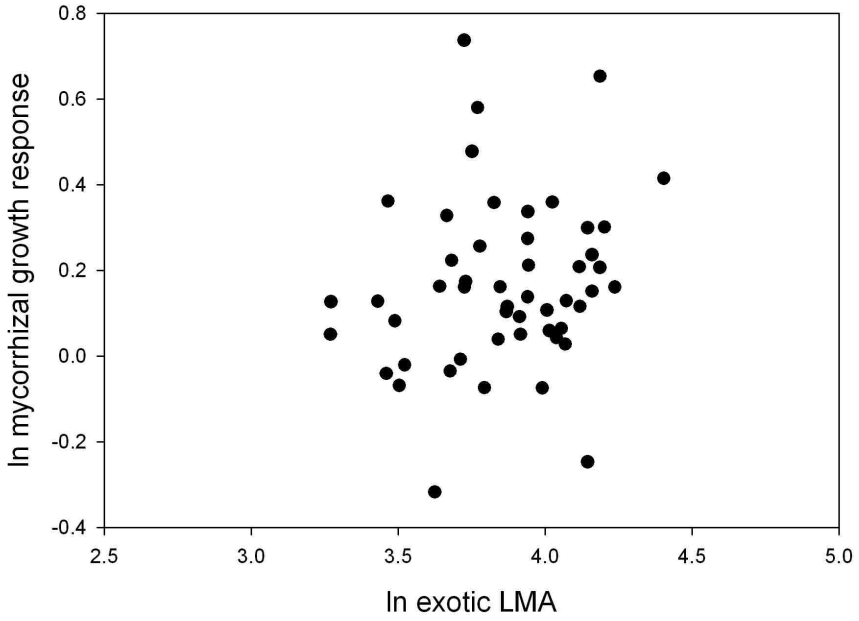

Figure 4 a)

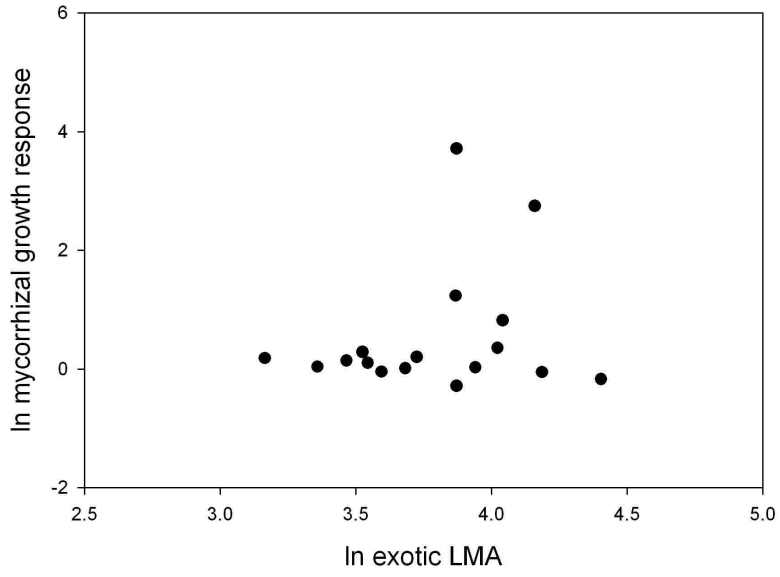

Figure 4 b)

CHAPTER TWO

REDUCED MYCORRHIZAL RESPONSIVENESS AND INCREASED COMPETITIVE TOLERANCE IN AN INVASIVE EXOTIC PLANT

ABSTRACT

Some invasive species undergo rapid evolution in their new range. Novel species' traits can affect the way they interact with soil biota in their new range. Soil microbes such as arbuscular mycorrhizal (AM) fungi have a powerful influence on the outcome of plant-plant competition. Hence, when a species is relocated to a new range, novel selection pressures there may lead to evolution of traits that affect the way plants interact with AM fungi, and consequently, with their new neighbors. I explored whether populations of the annual exotic star thistle (*Centaurea solstitialis*), collected from native versus non-native ranges, differed in responsiveness to arbuscular mycorrhizal (AM) fungi and whether range-based differences in mycorrhizal responsiveness correspond with how strongly *C. solstitialis* tolerates competition with the North American native bunchgrass, *Stipa pulchra*. Grown alone, *C. solstitialis* from both ranges considered together derived a weak benefit from AM fungi. However, AM fungi suppressed the biomass of *C. solstitialis* grown in competition with *S. pulchra*, and the magnitude of this suppressive effect was greater on native versus non-native populations. These results suggest that rapid evolutionary changes in how exotic plants respond to interacting AM fungal partners can affect their competitive tolerance in recipient communities.

INTRODUCTION

When plant species are relocated to new ranges they are often faced with novel selection pressures that may favor the evolution of traits that maximize fitness in their new range. Newly evolved traits may change the way they interact with species in the new range that may result in ecological advantages for exotic plants. Most studies that have explored rapid evolutionary change in exotic plants have focused on those traits that might be influenced by escape from specialist herbivores (Wolfe et al. 2002 & 2004; Zangerl & Berenbaum 2005; Agrawal *et al.* 2012; Uesugi & Kessler 2013) or pathogens (Maron *et al*. 2004b). However, rapid evolution of traits may be unrelated to biotic escape, and may also occur in response to abiotic conditions (i.e. latitude, Maron *et al.* 2004a), or hybridation events (Campbell et al. 2006). Regardless of the selective driver, newly evolved traits might affect subsequent interactions with species in the new range, which could in turn affect their success in the new range.

Few studies have explored whether rapid evolution of novel traits in exotic genotypes affects the way plants respond to mutualist communities in their introduced range (but see Seifert *et al.* 2009; Porter *et al*. 2011). If selection favors traits that reduce dependence on particular mutualists in the new range, this may lessen the costs of maintaining those associations, potentially resulting in reduced allocation to symbionts and more to growth. For example, Siefert *et al.* (2009) showed that non-native genotypes of the invasive plant, *Hypericum perforatum*, had reduced dependency on arbuscular mycorrhizal (AM) fungi compared to native European genotypes. They found that non-native versus native genotypes received a reduced benefit from AM fungi and had finer root architecture, a morphology consistent with reduced mycorrhizal dependence.

However, I know of no other studies that report whether exotic genotypes differ from native genotypes in terms of AM fungal dependency, and no studies testing whether this loss affects competitive interactions between exotic invaders and native species in the new ranges of invaders. Thus, it is unclear how widespread this phenomenon is, or whether reduced mycorrhizal responsiveness provides any ecological advantages to exotic invaders. However, other correlative results support the idea that low mycorrhizal dependency contributes to the success of some invaders (Pendleton & Smith 1983; Richardson *et al.* 2000; Pringle *et al*. 2009).

Associations between plants and AM fungi are typically assumed to be mutualistic, but growth responses of native species to AM fungi can range from strongly negative to strongly positive (Johnson *et al.* 1997; van der Heijden *et al.* 1998), as can the growth responses of invasives (Klironomos 2002 & 2003; Vogelsang *et al*. 2009). Where a plant species might lie on this spectrum of responses to AM fungi depends on its characteristics (Janos 1980; Koziol & Bever 2015), the identity of AM fungal colonists (Vogelsang *et al.* 2006), and the ecological context in which the association plays out (Johnson *et al.* 1997; Klironomos 2003). Given that mycorrhizal responsiveness is host-dependent and heritable trait (Hetrick *et al*. 1996), evolutionary escape from mycorrhizal associations may be favored in the new range if interactions with novel mutualist communities provide reduced benefits that don't outweigh the costs of maintaining these associations.

Relative to other studies investigating how biotic interactions can affect interspecific competition between plants, there have been few studies investigating the role of AM fungi in plant-plant competition. Those addressing this topic typically grow

plant species that vary in their mycorrhizal responsiveness and demonstrate that the competitive advantage favors the plant that can derive the greatest benefit from the symbiosis (Fitter 1977; Grime *et al*. 1978; Allen 1990; West 1996; Hartnett & Wilson 1999; Smith *et al.* 1999; Scheublin *et al*. 2007). However, I know of no studies to date that have focused directly on the costs of hosting AM fungi in a competitive environment. However, correlative results support the idea that low mycorrhizal dependency contributes to the success of some invaders (Pendleton & Smith 1983; Richardson *et al.* 2000; Pringle *et al*. 2009).

To explore these ideas, I conducted a common garden experiment where I grew multiple populations of *Centaurea solstitialis* (yellow starthistle) from the native (European) and from the heavily invaded non-native (North American) ranges. I manipulated competition and the presence of AM fungi in order to determine whether plants grown from seed collected in the non-native range have evolved novel traits when compared to plants originating from the native range and whether range-based differences cause them to interact differently with AM fungi when grown alone or when grown with the competitor *Stipa pulchra*. *S. pulchra* is a bunchgrass native to North America and is the dominant competitor occurring in grasslands invaded by *C. solstitialis*. Thus, my goal was to quantify whether the relative advantages or disadvantages of associating with AM fungi for native versus exotic *C. solstitialis* plants change when they are grown alone compared to when they are forced to compete with a heterospecific.

METHODS

In April of 2009, I collected seeds of *C. solstitialis* from a minimum of 10 maternal plants in each of 12 populations in North America and Europe, respectively (Appendix A). Soil was collected during the summer of 2008 from ten native grassland sites across the invaded range in western North America (Appendix B). I chose sites where neither *C. solstitialis* nor other strong invaders (as defined in Ortega & Pearson 2005) were present, but in typical habitat for *C. solstitialis*. This reduced the probability of collecting soils containing specialist pathogens of *C. solstitialis*, while still making it likely that collected soils contained generalist AM fungal species that these plants likely encounter. I grew plants in soil from the introduced range only because I was interested in making inferences about the impacts of North American AM fungi on *C. solstitialis* populations. At each site, I took a minimum of 15 soil cores (10 x 6 cm^2) at randomly-chosen locations covering approximately 0.75 hectare, and then pooled these samples to provide 80 liters of inoculum. I shipped soil to Guelph University where I extracted the AM fungal spores using fine mesh filters (45 μm). These spores were mixed with autoclaved field soil to provide 10 liters of inoculum, which was then shipped to the University of Montana campus in Missoula, MT and stored at 2.5^oC for one week.

I increased the AM fungal spore abundance from soil collected at each site by cultivating these AMF with two plant species known to be good hosts to AM fungi, *Sorghum bicolor* and *Allium porrum*. To accomplish this I mixed together soils collected from the ten populations containing AM fungal inoculum and added this inoculum to 15 liters of Turface®, Buffalo Grove, IL, U.S.A. (a calcite clay medium) and 15 liters of silica sand in a large, shallow tub (66 x 40 x 17.5 cm^3) to produce 40 liters of mixed soil-

AM fungal inoculum. I surface-sterilized *S. bicolor* and *A. porrum* seeds in a 1% sodium hypchlorite solution and sowed the seeds across the surface of the soil and fertilized every three weeks with a half strength 20-2-20 (N-P-K) fertilizer. After six weeks of growth I harvested a small sample of plants and stained a subsample of roots (Brundrett *et al*. 1984) to confirm mycorrhizal colonization using standard procedures (McGonigle *et al.* 1990). After twelve weeks of growth I stopped watering and allowed plants to die back naturally and then cut the *S. bicolor* and *A. porrum* roots into small pieces and mixed them back into the sand-Turface® mix to use as the inoculum.

To determine whether there were differences in responsiveness to AM fungi between native (European) and non-native (North American) populations of *C. solstitialis* I compared the growth responses of these populations in soils with and without AM fungi, in the presence and absence of competition. To accomplish this, I mixed and triple autoclaved a potting medium of 20 parts sand, 40 parts Turface® and 40 parts soil gathered locally in Missoula, MT. I added 425 ml of this sterile potting mix to each of 480 (500 ml) pots. To half of these pots I added an additional 75 ml of the AM fungal inocula. To the other half I added 75 ml of the sand-Turface®-soil mix without AM fungal spores. To each pot with and without the inoculum, I added two seeds from a single maternal *C. solstitialis* and then thinned to one individual per pot. This provided me with ten replicates (each representing a single maternal plant) from each population in each AM fungi treatment. Daytime temperatures ranged between 18-26^{o}C and 12-18^{o}C at night. A constant photoperiod was maintained throughout the experiment with supplemental light that ensured 14 hours of light and 10 hours of dark each day.

To determine how variation in responsiveness might interact with competitive tolerance I grew native and non-native populations of *C. solstitialis* in competition with the C_3 native North American bunchgrass, *Stipa pulchra*. I sowed 4-5 *S. pulchra* seeds (Larner Seeds, Bolinas, CA) into each of 500 pots, half containing sterile soil and the other half containing the AM fungal inoculum. Pots were watered daily and thinned to one individual per pot. After letting *S. pulchra* grow alone for three weeks, I added *C. solstitialis* seeds to each of 480 pots, using the same methods and amount of replication as above. To measure the mycorrhizal responsiveness of *S. pulchra* I prepared 20 pots (ten with the fungal inoculum and ten in sterile soil) with only a single *S. pulchra* individual.

Plants grew for a total of 18 weeks, after which I harvested all plants, separating shoots from roots. Harvested plants were dried at 65 ^{o}C for three days, and roots and shoots were then weighed. I retained a subset of roots from each population, kept separate by treatment, to measure AM fungal colonization using the standard gridline intersect method (McGonigle *et al*. 1990).

Analyses

I used the GLIMMIX module within SAS 9.0 (Cary, N.C., U.S.A.) to analyze the data. To determine whether non-native *C. solstitialis* populations from North America have rapidly evolved reduced responsiveness to AM fungi compared to native populations from Europe, I used a generalized linear mixed model with a lognormal distribution to test for the effects of range, AM fungi, competition and their interactions on total biomass, root to shoot ratios and relative growth rates. For *S. pulchra,* I used a generalized linear mixed model with a normal distribution to test for the effects of range,

AM fungi, competition and their interactions on total biomass. I treated population and individual (i.e., each maternal plant) as random factors in these models. To test for differences in mean fungal colonization rates, measured as the proportion of roots colonized with hyphae, arbuscules and vesicles per population, I used a generalized linear model with a beta distribution to determine the effects of range, competition and their interactions on plants grown with AM fungi. I also used this model to compare the percentage of vesicles in roots.

To test for proportional differences in mycorrhizal responses among ranges, I used a modified Relative Interaction Intensity (RII) Index (after Armas *et al.* 2004) to determine how native and non-native *C. solstitialis* populations responded to AM fungi. I defined RII with AM fungi as (Biomass+AMF – Biomass-AMF) / (Biomass+AMF + Biomass-AMF). This index was chosen because 1) it is bounded between +1 and -1, so it controls for extreme values and 2) it is symmetrical around zero, so values on either side of zero provide a comparable effect of AM fungi, reflecting either a positive or negative interaction depending on the sign. For each maternal plant from each population, I calculated the mean RII with AM fungi by comparing the biomass of one maternal offspring grown with AM fungi with the biomass of its sibling when grown in sterile soil. I calculated the mycorrhizal response for each competition treatment separately. I performed a generalized linear mixed model with a normal distribution to test for the effect of range, competition and their interactions on the responsiveness of *C. solstitialis* to AM fungi. I treated population and individual as random factors in this model.

RESULTS

Mycorrhizal colonization

Inoculation with AM fungi resulted in colonization of *C. solstitialis* roots, whereas uninoculated plants were not colonized ($F_{1,46}$=381.00, P<0.01). There was no significant difference in root colonization between native (European) and non-native (North American) populations ($F_{1,47}$=0.22, P>0.05). However, *C. solstitialis* roots were over twice as colonized by AM fungi when grown in competition than when grown alone ($F_{1,51}$=59.61, P<0.01). Roots in the AM fungi treatment were 37±3% colonized when grown alone, versus 81±3% when grown in competition (AM fungi x competition: $F_{1,51}$=50.62, P<0.01). Furthermore, colonized *C. solstitialis* plants had a far higher percentage of vesicles in their roots when grown in competition (58±7%) than when grown alone (7±3%).

Plant growth

AM fungi ($F_{1,66}$=6.36, P<0.01) and competition by the North American bunchgrass *S. pulchra* ($F_{1,66}$=1019.77, P<0.01) each significantly reduced the total biomass of *C. solstitialis* overall (Table 1). There was a significant interactive effect of AM fungi and competition (AM fungi x competition; $F_{1,67}$=16.35, P<0.01). AM fungi had a positive effect on plants grown alone, but competition shifted the plant response to AM fungi from positive to negative. Also, even though the total biomass of *C. solstitialis* plants did not differ between populations from the native and non-native ranges (when averaged across AM fungi and competition treatments, $F_{1,22}$=1.93, P>0.05) the negative effect of AM fungi on plant biomass in competition was only apparent in native

populations (range x AM fungi x competition; $F_{1,67}$=4.03, P<0.05). Hence, while mycorrhizal fungi increased the negative effect of competition by *S. pulchra* on plants from native *C. solstitialis* populations, plants from non-native populations showed no difference in competitive tolerance to *S. pulchra* whether provided with AM fungi or not.

Neither range ($F_{2,35}$=0.81, P>0.05), nor AM fungi ($F_{1,33}$=0.01, P>0.05), nor their interaction ($F_{2,35}$=0.35, P>0.05) significantly affected *S. pulchra* biomass. This suggests that the growth depression observed in *C. solstitialis* plants inoculated with AM fungi was independent of any effect of the fungi on the competitor and the difference in competitive tolerance of plants from native versus non-native populations was likely an effect of AM fungi rather than any direct competitive effect of *S. pulchra* per se.

Relative interaction intensity with AM fungi

The interactive effects of AM fungi, competition and range on growth of *C. solstitialis* plants were also evident when I examined the proportional response of biomass to AM fungi. Although there was no difference between native and non-native populations in their proportional response to AM fungi when grown without a competitor ($F_{1,39}$=1.50, P>0.05), competition switched the proportional effect of AM fungi from weakly positive when grown alone to strongly negative when grown in competition ($F_{1,39}$=18.41, P<0.01, Fig. 1). Furthermore, the effect of competition on the AM fungal response differed between plants from the native and non-native ranges (range x competition; $F_{1,39}$=4.32, P<0.05, Fig. 1). When grown alone, there was no significant difference in the proportional effect of AM fungi on plants from either range (contrast, t=-0.61, P>0.05). However, when grown in competition, plants from native populations

were significantly more suppressed by AM fungi than plants from non-native populations (contrast, $t=2.32$, $P<0.05$).

Root to shoot ratios

Overall, *C. solstitialis* plants allocated more biomass to roots when grown with mycorrhizae ($F_{1,64}=12.15$, $P<0.01$), but more to shoots when grown in competition ($F_{1,64}=245.16$, $P<0.01$). However, the AM fungi effect varied depending on whether *C. solstitialis* was grown in competition or not (AM fungi x competition; $F_{1,64}=4.13$, $P<0.05$). When grown alone, AM fungi had no significant effect on the root to shoot ratio of *C. solstitialis*. However, when grown in competition, mycorrhizal *C. solstitialis* allocated significantly more biomass to shoots than non-mycorrhizal plants (contrast, $t=3.86$, $P<0.01$).

There was no difference in root to shoot ratios between native and non-native populations of *C. solstitialis* overall ($F_{1,22}=1.47$, $P>0.05$). The effect of range was only apparent when plants were grown in competition (range x competition; $F_{1,64}=4.11$, $P<0.05$, Figs. 2 and 3). Plants from non-native populations tended to allocate more to roots than native populations when grown alone, but there was no difference between ranges when grown in competition. Range also had a marginally significant interaction with AM fungi (range x AM fungi; $F_{1,64}=3.76$, $P=0.06$), as the positive shift in allocation to roots in response to AM fungi tended to be greater for native compared to non-native populations of *C. solstitialis*. This pattern did not vary significantly with competition (range x AM fungi x competition; $F_{1,64}=0.02$, $P>0.05$), but the AM fungal-induced shift

in root allocation was most apparent for native populations of *C. solstitialis* in the presence of competition.

DISCUSSION

This experiment comparing the effects of AM fungi on the growth of native (European) and non-native (North American) genotypes of *C. solstitialis* provide novel insights into the potential for rapid evolution to occur in an invasive exotic plant and the ecological implications of such a shift. When grown alone, non-native genotypes did not show reduced mycorrhizal responsiveness compared with native genotypes in terms of effects on absolute biomass. But when grown with the strong competitor, *S. pulchra*, native *C. solstitialis* genotypes showed a much greater difference in absolute biomass between mycorrhizal and non-mycorrhizal treatments compared with non-native genotypes. Specifically, in competition, *C. solstitialis* from the native range were suppressed by AM fungi, whereas *C. solstitialis* plants from the non-native range were unaffected by the presence of AM fungi. These results suggest that non-native genotypes have evolved reduced responsiveness to AM fungi, but this was only apparent when plants are grown in competition.

Most experiments that have demonstrated a competitive advantage mediated by AM fungi have shown that the competitive advantage goes in favor of the species with the highest responsiveness to AM fungi (Grime *et al.* 1987; Hartnett & Wilson 1999). However, in this experiment, although *S. pulchra* was the dominant competitor, it showed no growth response to AM fungi under any conditions. Thus, it is unlikely that AM fungi directly increased the competitive ability of *S. pulchra*. Instead, differences in responsiveness by native versus non-native genotypes of *C. solstitialis* explain the

differences I observed when those plants were grown in competition versus alone. By culturing soils that never contained either of the focal species, the chance of soils containing specialist pathogens was minimized, suggesting that the effects of soil biota in this experiment were due primarily to AM fungal colonization.

What might explain the higher growth depression of mycorrhizal native genotypes relative to non-natives, evident specifically in competition? Plant species that are more responsive to AM fungi may have less control over exploitation by AM fungi than plant species that are less responsive to AM fungi (Grman 2012). Thus, when conditions do not favor mycorrhizal mutualisms, species that are more responsive are more likely to experience costs of hosting AM fungi. In this experiment, overgrowth and shading by *S. pulchra* plants likely reduced photosynthetic rates of *C. solstitialis*, creating carbon limitation and thereby causing growth depressions when grown with AM fungi. The differences in root to shoot ratios across competition treatments support this interpretation. All *C. solstitialis* genotypes allocated significantly more biomass above-ground when grown in competition compared to when grown alone. This is a common plant response when above-ground resources become limiting (Poorter & Nagel 2000).

Although I observed no differences in absolute biomass between mycorrhizal and non-mycorrhizal plants from both ranges when grown alone, I did observe differences in biomass allocation that suggest a reduction in mycorrhizal dependency in genotypes from the non-native range. When grown alone, non-native genotypes allocated more biomass to roots than native genotypes, but without a decrease in total biomass. Furthermore, in the absence of AM fungi and competition, there was a trend towards greater allocation to roots by non-native genotypes compared to native genotypes. This suggests that non-

native genotypes may be able to exploit a greater soil volume for nutrient acquisition without depending on the nutritional services delivered by AM fungi. This might benefit non-native genotypes in a realistic setting where nutrients are limiting. Because I supplemented plants with a weak fertilizer through the course of the greenhouse experiment, I may not have captured the true magnitude of the difference between mycorrhizal and non-mycorrhizal plants when grown alone.

There is a relatively low probability that founder effects can explain the shift in responsiveness observed in non-native genotypes in this experiment. Molecular analysis on the neutral genetic variation in 22 *C. solstitialis* populations sampled from the southernmost to the northernmost extent of the invaded range revealed high genetic diversity within populations, counter to what one would expect if only a few genotypes were introduced into North America (Sun 1997). However, genetic information about both founder *and* source populations would be optimal in order to conclude that there has been an adaptive shift by non-native genotypes (Bossdorf *et al.* 2005).

Most research on how AM fungi affect plant-plant competition has focused on how AM fungi can directly increase the competitive ability of plants that have inherently high mycorrhizal responsiveness. Since mycorrhizae can elicit substantial growth responses in plants that depend on AM fungi, these growth responses should certainly lead to improved competitive outcomes (Smith & Read 2008). However, since AM fungi decreased the competitive tolerance of native genotypes, but not non-native genotypes, this study highlights the potential *disadvantage* of being more responsive to AM fungi. Specifically, selection can act to reduce responsiveness when AM fungal benefits do not outweigh the costs, resulting in more competitive genotypes in some environments.

Future studies should focus on the costs involved with mycorrhizal responsiveness in different ecological contexts so that we may better understand how selection pressures in a changing global environment may influence the interactions between plants and mycorrhizal fungi.

ACKNOWLEDGEMENTS

This work was funded by an NSF grant DEB 0614406 to Drs. John L. Maron and Ragan M. Callaway. I thank Jose Hierro and Dani Montesinos for help collecting seeds and Giles Thelen and Jill Gebhart Wood for help collecting soil. Tyler Albrethsen, Whitney Warren and David Hooper provided support in the greenhouse.

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TABLES

Table 1. Plant biomass means and standard errors

Region	AM fungal treatment	Competition treatment	Mean biomass (g)	Standard error
Europe	AM fungi	No competition	1.89	0.7
Europe	No AM fungi	No competition	1.53	0.20
North America	AM fungi	No competition	2.03	0.27
North America	No AM fungi	No competition	1.91	0.25
Europe	AM fungi	Competition	0.06	0.01
Europe	No AM fungi	Competition	0.15	0.02
North America	AM fungi	Competition	0.10	0.01
North America	No AM fungi	Competition	0.14	0.02

FIGURE LEGENDS

Figure 1. Proportional response of *C. solstitialis* to AM fungi, measured by the relative interaction intensity (RII) index (+/- SE) of non-native (North American, black bars) or native (European, grey bars) populations of *C. solstitialis* and AM fungi from North America, with and without competition from the North American native bunchgrass, *Stipa pulchra.* Bars represent the mean of population means (n=12 North American populations and 12 European populations). Positive values represent a net benefit from AM fungi. Negative values represent a net negative interaction with AM fungi.

Figure 2. Root to shoot biomass ratio (+/- SE) of non-native North American (black circles) or native European (white circles) populations of *C. solstitialis*, grown alone with and without AM fungi.

Figure 3. Root to shoot biomass ratio (+/- SE) of non-native North American (black circles) or native European (white circles) populations of *C. solstitialis*, grown in competition with *S. pulchra* with and without AM fungi.

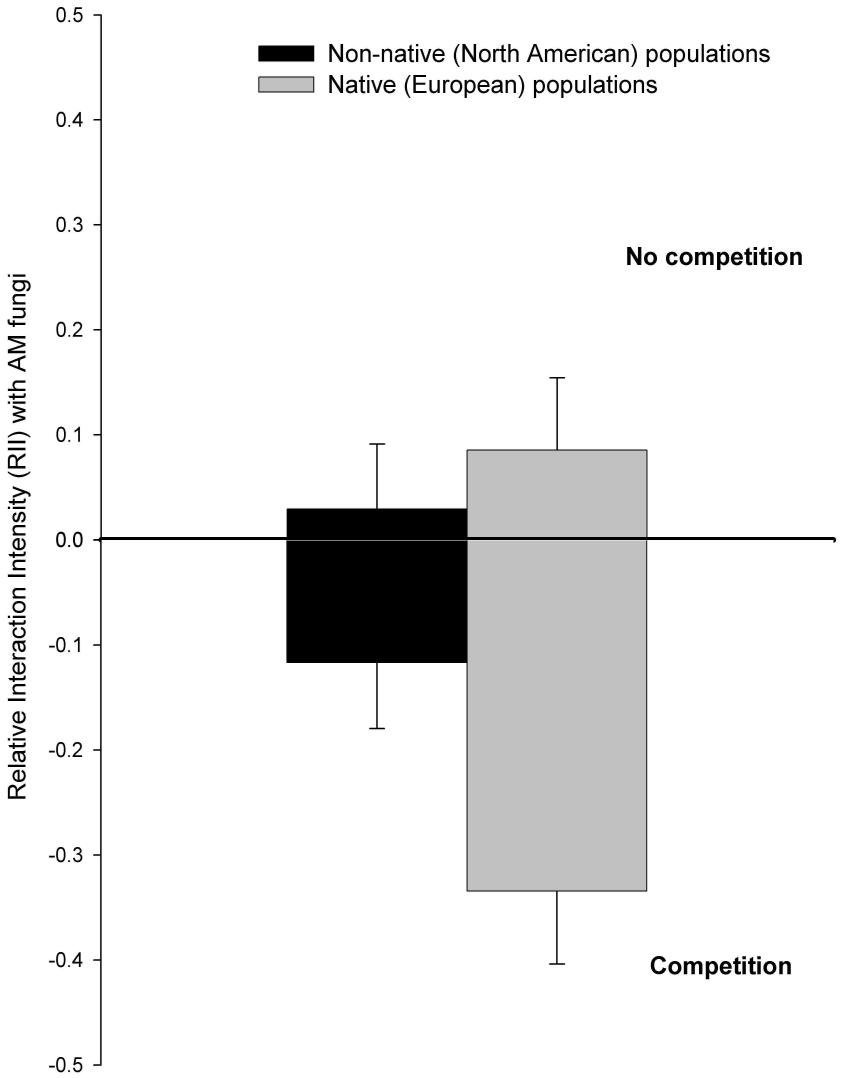
Non-native (North American) populations
Native (European) populations
No competition
Competition
Relative Interaction Intensity (RII) with AM fungi
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
-0.5

Figure 1

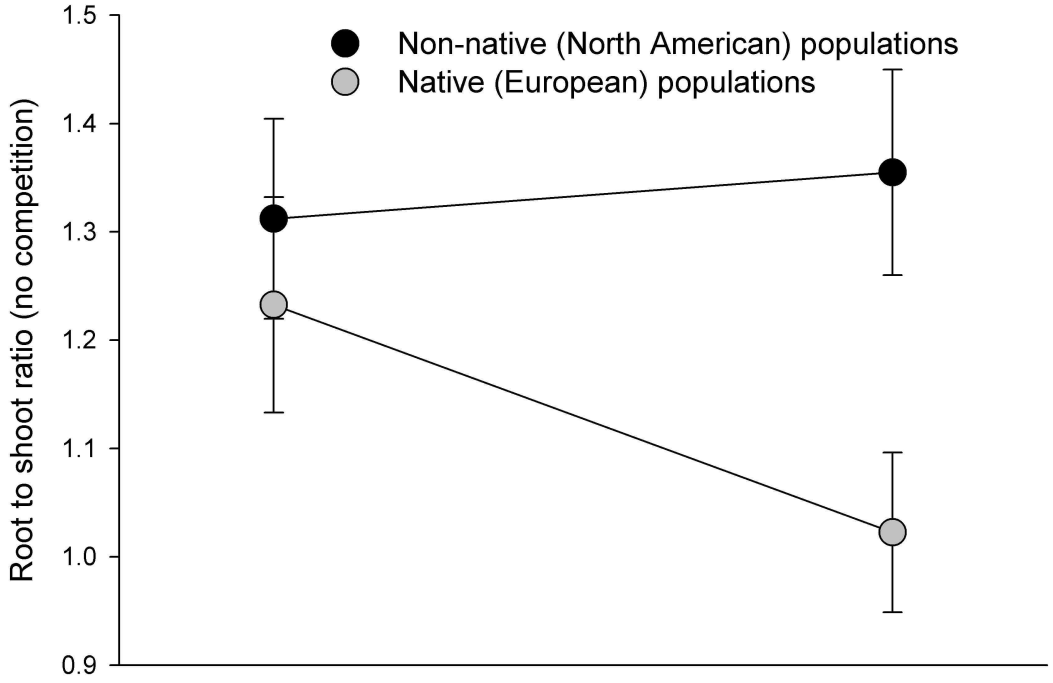
Non-native (North American) populations
Native (European) populations
Root to shoot ratio (no competition)
1.5
1.4
1.3
1.2
1.1
1.0
0.9

Figure 2

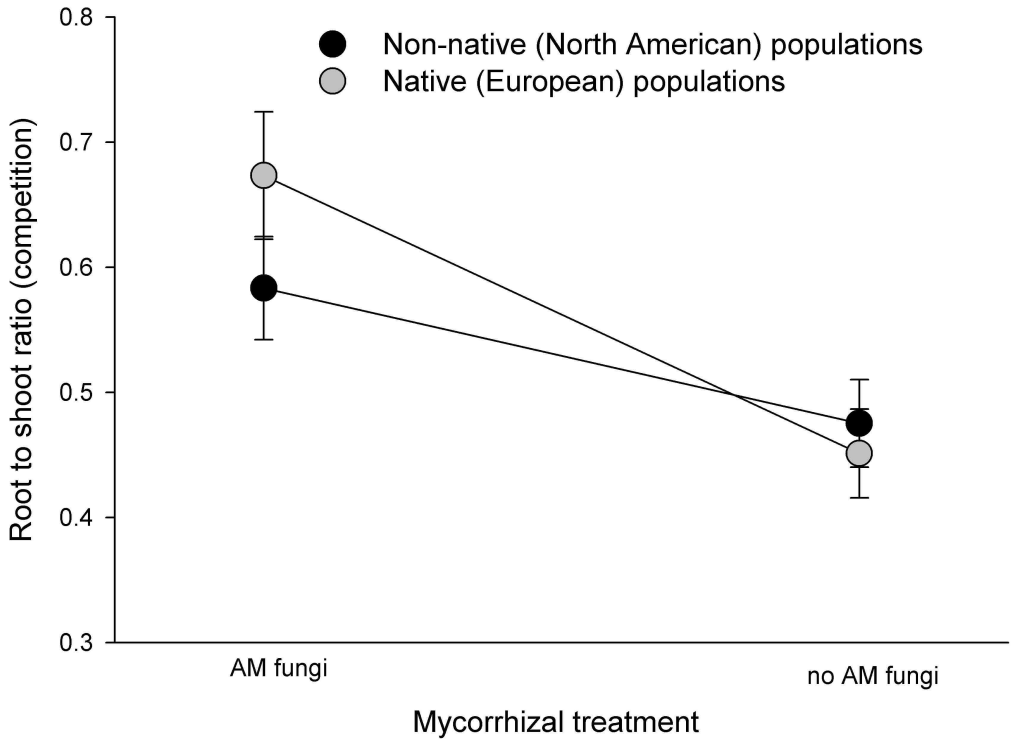
Non-native (North American) populations
Native (European) populations
Root to shoot ratio (competition)
0.8
0.7
0.6
0.5
0.4
0.3
AM fungi
no AM fungi
Mycorrhizal treatment

Figure 3

CHAPTER 3
ADDITIONS OF NITROGEN AND PHOSPHORUS AFFECT THE ABUNDANCE AND COMMUNITY COMPOSITION OF ROOT-ASSOCIATED MUTUALISTIC AND PATHOGENIC FUNGI

ABSTRACT

Anthropogenic rates of nitrogen deposition are increasing worldwide into systems that vary widely in phosphorus availability. Nutrient enrichment clearly can impact soil microbial communities, yet we do not fully understand how enrichment affects individual microbial guilds, particularly how N additions may affect different guilds in systems that vary in P availability. Plant-associated fungi, such as arbuscular mycorrhizal (AM) and pathogenic fungi, may show different responses to enrichment with N and different levels of P, which could feedback to affect plant hosts differently. I performed high-throughput sequencing on soils from the rhizosphere of the highly mycorrhizal tallgrass prairie dominant, *Andropogon gerardii*, to investigate how long term fertilization with N and N and P affect the community structure and abundance of AM and pathogenic fungi. Fertilization with N and N and P increased non-AM fungal abundance and diversity, but AM fungal abundance and diversity was only decreased when N was added with high amounts of P. The non-mycorrhizal group that experienced the greatest increase in fertilized soils were identified taxonomically as plant pathogens. Further, I observed shifts in abundance of the dominant AM fungal OTUs across the fertilization gradient, suggesting potential shifts in the functional attributes of those communities. These results suggest that additions of N and P can increase pathogenic fungi in the rhizosphere of the dominant C_4 grass in that system, and increases in P can shift the composition and abundance of AM fungi.

INTRODUCTION

The soil harbors an enormous diversity of plant-associated microbial taxa that range from mutualistic to parasitic, yet the ecology of these organisms is often

investigated one guild at a time. For example, arbuscular mycorrhizal (AM) fungal community composition and abundance often changes along nutritional gradients in natural systems (Johnson 1993, Johnson et al. 1997; Edgarton-Warburton 2000, 2007; Corkidi et al. 2002; reviewed in Hoeksema et al. 2010). However, relatively less is known about how nutritional gradients affect other groups of root-associated fungi, such as pathogens, or whether shifts in soil nutrient availability affect co-occurring groups of microbes differently. Soil microbial communities may interact more than previously thought, and these interactions may have strong implications for plant performance (Kennedy & Bruns 2005; Boddy 2000; Peay *et al.* 2008; Van Elsas *et al.* 2012). Here, I investigate the effects of long-term nutrient enrichment on different functional groups of root-associated fungi, with a particular focus on arbuscular mycorrhizal (AM) and pathogenic fungi.

Perhaps the most common way nutrient enrichment can affect soil microbial communities is via their direct effects on plant hosts. For example, nitrogen inputs often result in increases in productivity and competitive dominance by some plants (Grime 1977; Tilman 1982, 1987; Tilman & Wedin 1991; Foster & Gross 1998; Gough *et al.* 2000; Shaver *et al.* 2007). This can increase the abundance of soil fungal pathogens in the rhizosphere of those plants (Wardle *et al*. 2004), which are known to attack plant roots in a frequency-dependent fashion (Anderson & May 1981; Burdon & Chilvers 1982; Holt & Pickering 1985; Begon *et al.* 1992; Begon & Powers 1994). Crops that have received high nitrogen inputs often suffer from pathogen attack (Tompkins *et al.,* 1992; Jensen & Munk, 1997; Mascagni *et al*. 1997; Hoffland *et al.,* 2000; Veresoglou *et al.* 2013), likely due to the increased plant productivity following nitrogen fertilization.

In contrast, phosphorus amendments that enhance plant vigor typically decrease plant susceptibility to pathogens, and consequently, their abundance in soil (Walters & Bingham 2007; Veresoglou *et al.* 2013), although there are exceptions (Perennoud 1990). There are too few studies investigating the effects of co-enrichment of N and P on plant susceptibility to fungal pathogens to make any meaningful conclusions (Veresoglou et al. 2013). Thus, nitrogen additions that increase plant density might increase the abundance of species-specific pathogens, and phosphorus additions may increase plant vigor and reduce pathogens, but it is unclear how additions of N and P might affect pathogens.

Nutrient enrichment can also change host attributes in ways that are independent of density that can affect soil fungal communities. For instance, plants often reduce carbon allocation belowground when phosphorus is in ample supply (Marschner *et al.* 1996), which can translate to fewer resources available for AM fungi (Johnson *et al.* 2003). AM fungi are ubiquitous mutualists that can provide significant quantities of phosphorus in exchange for host carbon (Smith & Read 2008). Thus, phosphorus additions that render mycorrhizal nutritional services redundant can result in decreases in AM fungal abundance and can shift the composition of these communities towards taxa that are less specialized for resource acquisition (Johnson 1993). Indeed, since phosphorus limitations promote optimum conditions for symbiotic benefit exchange between plants and AM fungi, AM fungal abundance is often relatively high under low soil P availability, but low when P availability is high (Johnson 1993, Johnson *et al*. 1997; Egerton-Warburton 2000, 2007; Corkidi *et al.* 2002; Treseder 2004). Nitrogen additions can also decrease AM fungal abundance (Porras-Alfaro *et al.* 2007) or have no effect (Treseder 2008). However, nitrogen additions often increase AM fungal

abundance when inputs result in increased P limitations (Eom *et al*. 1999; Treseder & Allen 2002; Egerton-Warburton *et al.* 2007; Garcia *et al.* 2008; Johnson 2010; Grman & Robinson 2013). Because plants and AM fungi exchange carbon for phosphorus, nitrogen additions that stimulate carbon synthesis can balance C for P trade between symbionts (Johnson 2010).

AM and pathogenic root-associated fungi can interact with each other in ways that can affect plant performance (Maherali & Klironomos 2007; Sikes *et al.* 2009), yet no study to my knowledge has investigated the effects of fertilization with N and P on both of these guilds simultaneously. Much is known about how nutrient additions affect AM fungal communities in natural systems (Johnson *et al.* 1997; Egerton-Warburton *et al.* 2000 & 2007; Smith & Read 2008; Johnson *et al*. 2003; Treseder 2004), relative to pathogen communities and we know virtually nothing about how nutrient additions can affect patterns of co-occurrence between these groups. AM fungi can provide a substantial amount of pathogen protection to plants (Newsham *et al.* 1995a & b; Maherali & Klironomos 2007; Sikes *et al.* 2009), so understanding how these groups are simultaneously affected by nutrient additions may be of particular importance. For example, nutrient inputs that increase nitrogen availability relative to phosphorus can increase AM and pathogenic fungal abundance, so these soils may possess bioprotective properties against the harmful effects of soil pathogens. In contrast, additions of N with P may increase pathogen loads but decrease AM fungal abundance, which may diminish bioprotective services in these soils.

My objectives are to investigate how N and P additions affect the abundance of co-occurring fungal guilds and species turnover within guilds. To accomplish this, I

sampled soils from the rhizosphere of *Andropogon gerardii* in plots from a decade-long fertilization experiment at the Konza Prairie Long-Term Ecological Research Area. *A. gerardii* is a highly mycorrhizal community dominant (Eom et al. 1999) whose mycorrhizal associations can be sensitive to nutrient additions (Grman 2012). I used high-throughput sequencing to characterize communities of AM and pathogenic fungi in these soils to assess changes in fungal community composition and relative abundance across experimental nutrient treatments.

METHODS

Site description and sampling

Konza Prairie Long-term Ecological Research Site (LTER) is located in the Flint Hills near Manhattan Kansas, USA. The plant community is typical of the tallgrass prairie ecosystem, dominated by C4 grasses, such as *Andropogon gerardii* Vitm. (big bluestem), *Schizachyrium scoparium* (Michx.) Nash (little bluestem), *Sorghastrum nutans* (L.) Nash (Indiangrass) and *Panicum virgatum* L. (switchgrass). Subdominant plants include C3 grasses and a diverse assemblage of perennial forbs. Despite having very low amounts of inorganic P (Eom et al. 1999), the soils in the experimental plots are primarily N-limited, which is exasperated by biannual burning (Seastedt 1991).

Avolio and colleagues (2014) initiated a fertilization experiment in 2002 in an area that had been burned biannually since 1973. Beginning in 2003 and ending in 2013, they established 30 (5 x 5 m) plots (n=6 per treatment) that either received N only (at 10 g m^{-2}), N + low P (at 2.5 g m^{-2}), N + medium P (at 5 g $m^{-2)}$, and N + high P (at 10 g m^{-2}) or no nutrient amendments. Nutrients were applied in early June each year. N was added to plots as ammonium nitrate (10 g m^{-2}) and P was added as super-phosphate.

Fertilization with N and P led to initial increases in productivity by *A. gerardii* and other C_4 grasses in those plots, which returned to ambient levels halfway into the decade-long experiment (see Avolio et al 2014). I used their data to assess how nutrients, plant community composition and mycorrhizal colonization in the roots of *A. gerardii* plants correlate with soil fungal community changes in fertilized plots (See Avolio *et al.* 2014 for additional methodological details and results).

In July of 2013, after a decade of fertilization, I collected four distinct soil samples from the rhizosphere of *A. gerardii* from each plot. Samples from each plot were pooled, sieved through a sterile sieve, and transported on ice to the University of Montana laboratory where they were stored at -18^{o} C until processed.

DNA extraction, amplification and sequencing

There is currently no one DNA region and primer set that captures the entire fungal kingdom properly, so I amplified two regions to get the best coverage of both AM and non-AM fungi. Amplification of the internal transcribed spacer 2 (hereafter ITS) can capture a very broad range of fungal taxa to the species level, but its ability to capture Glomeromycotan taxa is more limited (Schoch *et al.* 2012). In contrast, the partial small subunit of the nuclear ribosomal RNA (hereafter SSU), combined with the Glomeromycotan-specific primers, can be used to sequence Glomeromycotan taxa to the species level, but other fungal groups are poorly resolved. Because of the wide coverage of fungal taxa in the ITS dataset, I used that region when comparing AM versus non-AM fungal abundance across treatments. However, when analyses were focused on AM fungal community composition and species turnover, I used the SSU data.

In January of 2015, DNA was extracted from 250 mg of soil per plot using the Powersoil™ DNA extraction isolation kit (MoBio Laboratories, Inc. Solana Beach, CA), following the manufacturer's instructions. I amplified the SSU gene and the ITS individually for each sample. PCR was carried out in 12.5 µL reaction volumes containing 1 µL of DNA extract as template, 20 pmol of each primer in 1X GoTaq Green Master Mix [(Green GoTaq Reaction Buffer, 200 µM dATP, 200 µM dGTP, 200 µM dCTP, 200 µM dTTP and 1.5 mM $MgCl_2$), Promega, USA]. Each reaction was performed in duplicate. I amplified the ITS region and all fungi using a mixture of the fungal specific primers fITS7 and ITS7o (Ihrmark *et al.* 2012; Kohout *et al*. 2014) and the general eukaryotic primer ITS4 (White *et al.* 1990) under the following conditions: initial denaturing at 95 ^{o}C for 2 minutes, followed by 35 cycles at 95 ^{o}C for 1 min., 57 ^{o}C for 1 min., 72 ^{o}C for 1 min. with a final elongation for 10 mins. at 72 ^{o}C. I amplified the partial SSU region with the AM fungal specific forward primer WANDA (Dumbrell *et al.* 2010) and the reverse primer AML2 (Lee *et al*. 2008) under the following conditions: initial denaturing at 95 ^{o}C for 2 minutes, followed by 35 cycles at 95 ^{o}C for 1 min., 54 ^{o}C for 1 min., 72 ^{o}C for 1 min. with a final elongation for 10 mins. at 72 ^{o}C. To confirm the presence of the target amplicon, all reactions were analyzed on 1.5% agarose gel electrophoresis using a 100 bp ladder (O'GeneRuler DNA Ladder, Thermo Scientific, USA) as a size standard. PCR controls and extraction blanks showed no amplification. PCR products were purified using magnetic beads (Agencourt AMPure P, Beckman Coulter Genomics, Danvers, MA, USA) and pooled in equimolar concentration prior to sequencing. Paired-end sequencing (2 x 300 bps) was conducted on an Illumina MiSeq

(San Diego, CA, USA) sequencer at the iBEST sequencing facility at the University of Idaho in Moscow, ID.

Bioinformatics

I performed the bioinformatics analysis using QIIME (Quantitative Insights Into Microbial Ecology, version 1.9.0, Caporaso *et al.* 2010a), an open-source software pipeline. I checked the quality of the forward and reverse reads using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). As expected, I obtained much greater sequencing depth of Glomeromycotan taxa from the SSU region than the ITS region, since the SSU region is a better region for capturing that phylum (Stockinger *et al*. 2010). I obtained a total of 317,258 raw ITS reads from the MiSeq Illumina sequencer, which was reduced to 45,993 after quality filtering. I obtained 3,395,425 raw sequence reads from the SSU region, which was reduced to 1,585,084 after quality filtering. The median length of the ITS paired-end reads was 351 bps. Because of poor quality at the end of the single reads, I was unable to align the forward and reverse reads and therefore used the SSU forward reads only, which had a median length of 261 bps. This length is acceptable because it covers most of the variable region required for taxa identification (Hart *et al.* 2015). I assigned operational taxonomic units (OTUs) based on a 97% sequence similarity using UCLUST (Edgar *et al.* 2010). I used the most abundant sequences as seeds for the clustering. I detected chimeras using ChimeraSlayer (Haas *et al*. 2011). Due to the great sequence dissimilarity of fungi within the ITS, I did not align the ITS sequences, but I did align the SSU sequences using PyNAST (Caporaso *et al.* 2010b).

I obtained a total of 1708 operational taxonomic units (OTUs) from the ITS region and 29,105 from SSU after clustering to 97% similarity and removing doubletons and chimeras. I used a closed-reference taxa assignment using the Basic Local Alignment Search Tool (BLAST, Altschul *et al.* 1990) against the SSU Maarj*AM* reference database (Opik *et al*. 2010) and the ITS Tedersoo reference database (from Tedersoo *et al.* 2014). The Tedersoo database has been curated to include functional as well as taxonomic assignments, which allowed us to assign functional attributes (i.e. plant pathogen) to the sequences. I used this database to identify and focus on plant pathogens and AM fungi, but I also identified saprophytic fungi, which I include in a minor part of the analysis. I did not resample the ITS data because a) there was low variation in the sequencing depth among the treatments and b) Glomeromycotan OTUs had lower representation than the other fungi in the ITS region. Because of these factors, rarefaction may have reduced the sensitivity of the ITS analysis (Hart *et al*. 2015).

QIIME could not assign taxonomy to 213 of the 29,105 SSU OTUs. I compared these sequences directly against sequences in the Basic Local Alignment Search Tool (BLAST: http://blast.ncbi.nlm.nih.gov) using the BLAST algorithm (Altschul *et al.* 1990). This search assigned a Glomeromycotan identity to only 89 OTUs, so I retained these but removed the remaining no blast hits from the sample-OTU matrix. I rarefied the 26,581 SSU sequences to an even depth of 75% of the total number of sequences in the sample with the lowest sequence abundance. Collapsing the sample-species matrix resulted in a total of 164 AM fungal virtual taxa. Approximately-maximum-likelihood phylogenetic trees for the aligned SSU sequences were built using FastTree 2.1.3 (Price *et al.* 2010).

Statistical analysis

I used R (R Development Core Team 2010, http://cran.r-project.org) for all of the statistical analyses unless mentioned otherwise and transformed the data to meet the assumptions of the linear models. To assess how relative AM, pathogenic and saprophytic fungal abundance from the ITS region were affected by the fertilization treatment, I performed one-way ANOVAs on total sequence abundance using fertilization treatment as a fixed effect and plot as a random effect. Tukey's Honestly Significant Difference (HSD) tests were used to determine differences between sample units. To assess how well total N and total P explained the variation in AM and pathogen community abundance, I performed simple linear regressions using the total sequence abundance in the OTU tables from the ITS region and the soil N and P availability.

I used QIIME to calculate alpha diversity among ITS sequences identified as pathogens and AM fungal virtual taxa from the SSU region. I assessed whether these metrics of alpha diversity were affected by fertilization using a one-way ANOVA with fertilization treatment as a fixed effect and plot as a random effect. To assess changes in particular species of AM fungi, I performed one-way ANOVAs to assess the effects of treatment on the abundance of each of the nine AM fungal families represented in the data, as well as the 35 most abundant OTUs (those with more than 3000 total sequences) from the SSU region. I assessed the effect of treatment on the community composition (as species turnover, Anderson 2011) of AM fungi (using SSU) and pathogen communities (using ITS) by analyzing the average Gower dissimilarity matrices in permutational multivariate analyses of variance (PERMANOVA, Anderson 2001). I used QIIME to calculate Gower (Gower 1971) dissimilarity matrices among samples and used the *adonis* function in the R vegan package (Oksanen *et al.* 2015) with 999

permutations to asses the effect of treatments (N, P, N:P) on community composition. I also used vegan to make PCoA plots to visualize beta-diversity (Lozupone & Knight 2005).

RESULTS

Fertilization effects on AM and pathogenic fungal sequence abundance

The fertilization treatments did not affect the total abundance of fungi (measured as the number of sequences from the ITS region, Table 1, $F_{4,25}$=1.14, P>0.05), but it did affect the relative proportions of distinct guilds of fungi (Table 1 and Fig. 1). Fertilization reduced the total number of AM fungal sequences (Fig 2a., $F_{4,25}$=4.32, P<0.01) in rhizosphere soils that received the highest fertilization application (N high P) relative to the others (Tukey HSD, P<0.05). Sixty of the OTUs from the ITS region were assigned a pathogenic function according to the database from Tedersoo (2015). Their abundance increased in all rhizosphere soils that were fertilized relative to controls (Fig. 2b & Table 1, $F_{4,25}$=7.43, P<0.0004). There was one clear outlier among the pathogenic OTUs in the ITS dataset. Removal of this outlier did not change the statistical significance of the differences in abundance among the samples, so we left it in. There was no effect of the fertilization treatment on saprophytic fungi ($F_{4,25}$=0.33, P>0.05, Fig. 1).

I used the data from the ITS region to compare changes in abundance among the 15 most abundant taxa known to be potentially pathogenic. *Fusarium* species were the most common plant pathogens overall, making up 53% of the total pathogenic sequences. The three most common pathogens overall were an uncultured *Fusarium sp., F. solanii*

and *F. tricinctum*. Neither the uncultured *Fusarium* nor *F. tricinctum* differed significantly in abundance among the fertilization treatments, but fertilization increased the abundance of *Fusarium solanii* ($F_{4,25}$=10.02, P<0.0001) and *F. fujikuroi* ($F_{4,25}$=4.48, P<0.01). *F. solanii* abundance was highest when both N and P were added, *F. fujikuroi* abundance was higher in all fertilization treatments relative to controls (Tukey HSD, P<0.05). Of the 35 most abundant AM fungal OTUs (Appendix C), I found that fertilization had a significant effect on 22 different taxa (Table 2). Of these, 18 were reduced with fertilization, whereas only 5 responded positively to fertilization.

Nitrogen availability explained much of the variation in pathogen abundance (Fig. 3a, r^2=0.65, P<0.0001), whereas phosphorus explained much less (r^2=0.30, P<0.001). In contrast, phosphorus availability explained the majority of the variation in AM fungal abundance (Fig. 3b., r^2=0.65, P<0.05), whereas nitrogen was not a significant predictor of any of that variation (P>0.05).

Alpha and beta diversity of AM and pathogenic fungi

Fertilization increased the taxonomic diversity of pathogens ($F_{4,25}$=9.05, P=0.0001), but not Simpson's diversity ($F_{4,25}$=1.99, P>0.05). Fertilization had a significant effect on the taxonomic (Fig. 4a, $F_{4,25}$=4.07, P=0.002), phylogenetic (Fig. 4b, $F_{4,25}$=4.67, P<0.01) and Simpson's ($F_{4,25}$=4.27, P<0.01) diversity of AM fungi. Among the fertilized treatments, AM fungal phylogenetic diversity was significantly lower in the highest fertilization treatment (N high P), but not different from controls (Tukey HSD, P<0.05). Taxonomic diversity only differed between N only and N high P, where the N high P soils had fewer OTUs represented (Tukey HSD, P<0.05). The family-level

difference in diversity appeared be driven by a decrease in OTUs from the family Gigasporaceae, which was the only family that differed in sequence abundance among treatments (Table 1, $F_{4,25}$=5.62, P=0.002). OTUs from that family were significantly lower in control and N high P soils than they were in N only soils. Sequence abundance from the family Gigasporaceae increased linearly with N:P (Fig. 5, r^2=0.28, P=0.001). The species turnover of AM fungal communities differed significantly among treatments (Fig. 6a, P<0.05) but pathogen communities did not (Fig 6b, P>0.05).

DISCUSSION

Fertilization shifted the composition and abundance of two important groups of soil fungi in this study. Pathogenic fungi in the rhizosphere of *A. gerardii* increased in all fertilized plots relative to controls, whereas total AM fungal abundance was reduced with fertilization, but only when N was added with high P. A significant proportion of the most abundant AM fungal taxa decreased with fertilization, particularly in rhizosphere soils where N and high P was added. In contrast, no single pathogen species decreased in abundance along the fertilization gradient, but several common pathogens did increase, again, particularly in soils with N and high P added. Thus, heavy fertilization with N and high P acts to decrease AM fungal abundance and alter community composition while pathogen loads increase with N regardless of P availability.

Individual families of AM fungi responded similarly to fertilization. Specifically, of the 22 taxa affected by fertilization, the 18 that decreased with fertilization were from the family Glomeraceae. In contrast, Gigasporaceae tended to increase with fertility. The only non-Glomeraceae taxa, *Gigaspora decipiens* (VTX00039), a *Scutelospora* (VTX0318) and an *Acaulospora* (VTX00425) all increased in abundance with

fertilization. *Gigaspora* and *Scutellospora* responded particularly strongly to nutrient inputs that increased P-limitations in soil, whereas the *Acaulospora* increased somewhat linearly along the gradient of N and P.

These observations support previous work demonstrating trait conservatism within families of AM fungi (Hart & Reader 2002) and resource stoichiometry driving AM fungal communities (Johnson 2010). AM fungi from the family Gigasporaceae are known to benefit plants most at low levels of P, primarily because they are able to extend a much greater proportion of their hyphal biomass into the soil than into roots. This morphology allows them to acquire and deliver more phosphorus than taxa from Glomeraceae or (to a lesser extent) Acaulosporaceae, which put proportionately less hyphae into soil and more into the root (Hart & Reader 2002; Maherali & Klironomos 2007). Gigasporaceae taxa were much more abundant in rhizosphere communities where N additions increased P-limitations. Furthermore, AM fungal diversity peaked in samples where all three families were well represented, reinforcing previous work that niche processes may direct local assembly (Lekberg *et al.* 2007) and increase fungal richness (Maherali & Klironomos 2007; Powell et al. 2009).

In contrast, most Glomeraceae taxa decreased with fertilization, but there were two notable exceptions. The most abundant AM fungal OTU, VTX00219 and another highly abundant OTU, VTX00411, both increased by an order of magnitude in all soils that were fertilized compared to controls. It is unclear what exactly drives this increase for these particular *Glomus* taxa, or how much influence they have on plant performance, but they are clearly more competitive than other Glomus taxa in fertilized soils.

Leff et al. (2015) recently showed that fertilization with N and P increased dominance of bacterial species with more ruderal life history traits. The proliferation of VTX00219 and VTX00411 in fertilized plots is suggestive of a ruderal life history strategy. It is unclear how these particular taxa affect their hosts, since I did not have biomass or demographic data for *A. gerardii* in these plots. However, *A. gerardii* cover did not differ among treatments at the time of sampling (Avolio et al. 2014) suggesting that these taxa were not necessarily more harmful to their hosts in this context. On the contrary, these OTUs may be good candidates to screen for their pathogen protective ability, since they were present at high abundance in soils with high pathogen loads, but there were no noticeable differences (in terms of cover) in plant susceptibility to pathogens across treatments.

Fertilization also affected the fungal OTUs identified as plant pathogens, but their abundances only increased. Over half of the total sequences identified as pathogens were from the species complex *Fusarium*. *Fusarium* taxa are generally considered to be "hemibiotrophic" because infection begins in live tissue, but eventually kills and feeds on those tissues (Ma *et al.* 2013). Because these pathogens have to kill their host to gain their full carbon benefits, *Fusarium* and other biotrophic pathogens' success can depend on good plant nutrition (Walter & Bingham 2007). Hence, phosphorus additions (Veresoglou *et al.* 2013) as well as AM fungal colonization (reviewed in Borowicz 2001) can decrease plant susceptibility to pathogenic fungi.

It is notable that the *A. gerardii* cover did not differ between control and fertilized plots at the time of sampling (Avolio *et al.* 2014), regardless of the relatively high pathogen abundance in their rhizospheres. One explanation for this result is because I

sampled soils and not roots, so sequenced communities may not accurately reflect the realized communities affecting plant roots. I think this is unlikely, due to the long duration of this experiment, so that the roots in those soils likely had high exposure to the pathogen communities in those soils. Another explanation is that *A. gerardii* had tolerance to the pathogens in these soils than other C_4 grasses did not have. The cover of all of the C_4 grasses except *A. gerardii* decreased in fertilized plots (Avolio *et al.* 2014), suggesting that those grasses may have succumbed to the higher pathogen pressure in those plots.

This analysis suggests that inputs of N and P can have different effects on co-occurring guilds of soil fungi. Plant pathogens, for instance, exhibited a significant increase in abundance in fertilized soils relative to controls. As well, *A. gerardii* and other C_4 grasses showed considerable increases in productivity soon after being treated with N and N+P, relative to controls (Avolio et al. 2014). Thus, pathogen increases in the rhizosphere of *A. gerardii* may have been a result of density-dependent accumulation following fertilizer-driven increases in host production (Anderson & May 1981; Burdon & Chilvers 1982; Holt & Pickering 1985; Begon *et al.* 1992; Begon & Bowers 1994).

In contrast, AM fungi decreased in abundance when high P was added with N. This result is not surprising, as decreases in AM fungal abundance following P additions are common (Johnson 1993), largely because plants tend to allocate fewer resources belowground when phosphorus supplies are high (Marchner 1996). However, since root-associated pathogens conceivably utilize the same resources, it is unclear why pathogens did not also suffer at high P. On the contrary, pathogen abundance was as high in

rhizosphere soils with high P as they were where P-limitations would be expected to increase belowground resources.

One explanation is that fungal pathogens are more competitive for host carbon than AM fungi when P is in high supply. High phosphorus could negatively affect C acquisition by AM more than pathogenic fungi, since AM fungi depend on carbon rewards that are related to phosphorus supply (Bever et al. 2009). For example, cheating by AM fungi is most likely when phosphorus is in high supply, since AM fungi can acquire carbon without reciprocating with phosphorus under those conditions (Johnson & Oelmuller 2009). To reduce the success of cheating partners, plants can actively reward beneficial fungi with carbon (Lekberg et al. 2010; Kiers et al. 2011), since rewards to beneficial partners can increase their fitness over cheaters. Fungal pathogens, however, are not dependent on carbon rewards by plants. Rather, (hemi)biotrophic pathogens such as *Fusarium* infect hosts by overcoming their immune defenses (Glazebrook 2005). Because of this difference in the way AM versus pathogenic fungi acquire host carbon, pathogens may be able to invade roots that AM fungi cannot when plants actively reduce allocation to AM fungi. This might suggest a hidden cost to preferential rewards in the AM symbiosis.

It is important to note the likelihood that some of the OTUs identified as plant pathogens in the taxonomic database (Tedersoo *et al.* 2015) were not causing damage to their host at the time of sampling. The term pathogen can be a complicated term. For example, many well-known fungal species that can cause significant damage to hosts (e.g. *Fusarium* species) can also grow asymptomatically in host tissues for some time (Leslie *et al.* 1990; Ma *et al.* 2013). Likewise, AM fungi, which are best known for their

beneficial properties, can also be somewhat pathogenic to their hosts in some contexts (Johnson 1993; Johnson *et al.* 1997; Klironomos 2002). Here, our use of the term plant pathogen is used to describe species outside of the phylum Glomeromycota whose identity is associated with the potential for significant damage to their hosts at some point in their ontogeny, but may also have a latent phase.

It is well acknowledged that AM fungi can structure plant communities by increasing the competitive ability of highly mycorrhizal species, such as *A. gerardii*, by increasing their access to nutrients (Hartnett & Wilson 1999; Urcelay & Diaz 2003; Smith & Read 2008; Wagg et al. 2011). However, the role of other soil microbes that may interact with AM fungi has been previously overlooked. Interactions between these two groups in particular may be an important focus, since AM fungi can offer substantial protection from pathogens (Newsham *et al.* 1995; Maherali & Klironomos 2007; Sikes *et al.* 2009). Using next-generation sequencing, I found that additions of N and of N+P increased plant pathogen loads and high levels of phosphorus decreased AM fungal abundance in the rhizosphere of a dominant, highly mycorrhizal plant.

ACKNOWLEDGEMENTS

This work was funded by an NSF EPSCoR grant to Ragan Callaway. I thank the Konza Prairie Long-term Ecological Research Site and Eva Horne and Jeff Taylor for help in the field. Thanks to Scott Collins and Melinda Smith for designing the experimental plots I used for sampling, Sally Koerner, Kevin Wilcox, Kimberly La Pierre and Meghan Avolio for soil nutrient and plant species data and Gail Wilson for mycorrhizal colonization data. Alexii Cornell assisted with sequencing and bioinformatics and Tom Brekke provided bioinformatics support.

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TABLES

Table 1. The proportion of sequences (+/- S.E.) of a) plant pathogenic, AM, and saprophytic fungi from the ITS dataset and b) each Glomeromycotan family in the 18 S dataset from each fertilization treatment.

	Fertilization treatment				
a. ITS	control	+N	+N low P	+N med. P	+N high P
Pathogens	0.08 (.01)[b]	0.19 (0.02)[a]	0.17 (0.02)[a]	0.20 (0.02)[a]	0.24 (0.01) [a]
AM	0.02(0.002)[a]	0.03 (0.01)[a]	0.03 (0.01)[a]	0.03 (0.01)[a]	0.01 (0.002)[b]
Saprobe	0.50 (0.03)[a]	0.50 (0.04)[a]	0.48 (0.01)[a]	0.46 (0.04)[a]	0.47 (0.01)[a]
b.18S					
Glomeraceae	0.66 (0.04)[a]	0.65 (0.04)[a]	0.60 (0.08)[a]	0.63 (0.11)[a]	0.40 (0.10)[a]
Acaulosporaceae	0.08 (0.05)[a]	0.14 (0.06)[a]	0.18 (0.09)[a]	0.10 (0.07)[a]	0.19 (0.07)[a]
Archaeosporacea	0.15 (0.03)[a]	0.08 (0.03)[a]	0.05 (0.02)[a]	0.10 (0.04)[a]	0.25 (0.1)[a]
Gigasporaceae	0.01(0.003)[a]	0.06 (0.01)[a]	0.03 (0.02)[a]	0.02(0.002)[a]	0.01 (0.005)[a]
Paraglomeraceae	0.01(0.001)[a]	0.01(0.001)[a]	0.04 (0.03)[a]	0.06 (0.05)[a]	0.04 (0.01)[a]
Clairoideoglom.	0.08 (0.01)[a]	0.06 (0.02)[a]	0.07 (0.02)[a]	0.08 (0.02)[a]	0.09 (0.05)[a]

Table 2. The total number of sequences (+/- S.E.) of the 22 most abundant Glomeromycotan OTUs (SSU) that were

	Fertilization treatment			
Taxon name	control	+N	+N low P	+N med.
Glomus MO-G5, VTX00219****	107 (42)[b]	3900 (1174)[a]	1925 (869)[a]	2708 (81
Glomus MO-G8, VTX00130**	1661 (281)[a]	262 (116)[b]	651 (183)[a]	1133 (19
Glomus viscosum, VTX00063**	585 (92)[ab]	295 (105)[b]	890 (181)[a]	1030 (26
Glomus MO-G23, VTX00222*	830 (131)[a]	576 (208)[ab]	435 (87)[ab]	462 (151
Claroideoglomus lamellosum, VTX00193*	746 (137)[a]	342 (222)[ab]	240 (81)[ab]	430 (324
Gigaspora decipiens, VTX00039*	99 (58)[b]	800 (168)[a]	431 (230)[ab]	238 (39)[a]
Acaulospora Deepika15 Acaulo P2, VTX00425****	76(12)[c]	243 (52)[b]	176 (54)[c]	419 (98)[b]
Glomus perpusillum, VTX00287***	233 (46)[a]	277 (85)[a]	395(112)[a]	488 (212
Glomus sp., VTX00093**	210 (41)[a]	473 (82)[a]	217 (48)[a]	176 (79)[b]
Glomus sp., VTX00315****	417 (62)[a]	271 (87)[a]	130 (21)[a]	32 (18)[b]
Glomus MO G15, VTX00135***	583 (103)[a]	64 (13)[ab]	157 (46)[a]	27 (14)[c]
Glomus Alguacil 14b Glo10, VTX00423*	352 (88)[a]	94 (31)[ab]	224 (78)[a]	108 (26)[a]
Glomus Wirsel OTU13, VTX00140*	286 (49)[a]	121 (26)[a]	112 (47)[ab]	109 (59)[a]
Scutellospora LER04, VTX00318*	45 (13)[ab]	315 (94)[a]	154 (80)[ab]	65 (7)[ab]
Glomus Torrecillas 12b Glo G13, VTX00409*	114 (22)[a]	56 (34)[b]	161 (30)[a]	198 (49)[a]
Glomus MO G22, VTX00125**	264 (51)[a]	137 (42)[a]	80 (17)[ab]	71 (21)[b]
Glomus MO G7, VTX00199*	72 (34)[ab]	261 (22)[a]	95 (46)[ab]	136 (79)[a]
Glomus LES15, VTX00411****	4 (2)[b]	212 (51)[a]	120 (47)[a]	136 (35)[a]
Claroideoglomus Torrecillas 12b Glo G5*,* VTX00402*	177 (45)[a]	32 (68)[ab]	86 (42)[ab]	180 (144
Glomus Glo2, VTX00096**	74 (10)[ab]	209 (41)[a]	93 (26)[ab]	97 (28)[ab]
Glomus Kottke08.11, VTX00217**	143 (29)[a]	360 (59)[a]	154 (34)[a]	136 (63)[a]
Glomus mosseae, VTX00067****	182 (53)[a]	2 (1)[b]	85 (33)[a]	85 (33)[a]

* <0.05, **<0.01, **<0.001, ***<0.0005, ****<0.0001

FIGURE LEGEND

Figure 1. Bars represent the mean relative abundance of pathogens, AM and saprophytic fungi found in each soil treatment.

Figure 2. Bars represent the mean number of ITS sequences (+ SE) of a) AM ($p=0.0083$) and b) pathogenic ($p=0.004$) fungal origin found in each soil treatment. Different letters represent differences at the $p<0.05$ level.

Figure 3. Relationship between a) N and pathogenic fungal sequence abundance from the ITS region ($r^2=0.65$, $p<0.0001$, $n=30$) and b) P and AM fungal sequence abundance ($r^2=0.12$, $p=0.0402$, $n=30$). Symbols represent, closed circles: control plots, open triangles: +N only, open circles: +N low P, closed triangles: +N medium P, squares: +N high P. All values are represented on a logarithmic scale.

Figure 4. Bars represent the mean (+SE) a) taxonomic and b) phylogenetic diversity of AM fungi in each soil treatment. Different letters represent differences at the $p<0.05$ level.

Fig. 5. Relationship between N:P and Gigasporaceae sequence abundance from the ITS region ($r^2=0.28$, $p=0.0014$, $n=30$). Symbols represent, closed circles: control plots, open triangles: +N only, open circles: +N low P, closed triangles: +N medium P, squares: +N high P. All values are represented on a logarithmic scale.

Fig. 6. PCOA plots of beta diversity of the a) AM (SSU) and b) pathogenic (ITS) fungal communities in each soil treatment. Circles represent 95% confidence intervals.

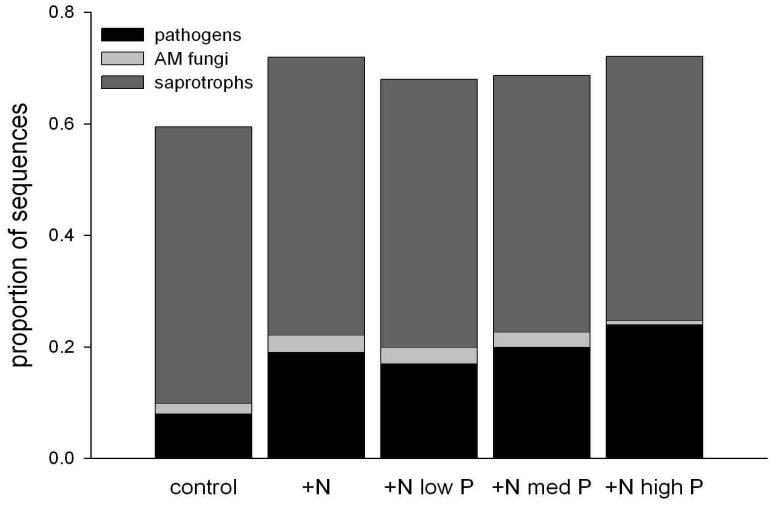
pathogens
AM fungi
saprotrophs
proportion of sequences
0.0
0.2
0.4
0.6
0.8
control
+N
+N low P
+N med P
+N high P

Fig. 1

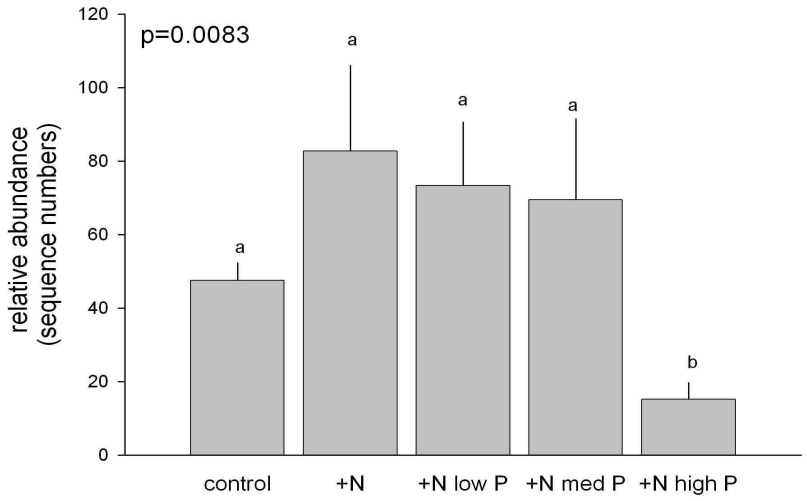

Figure 2 a)

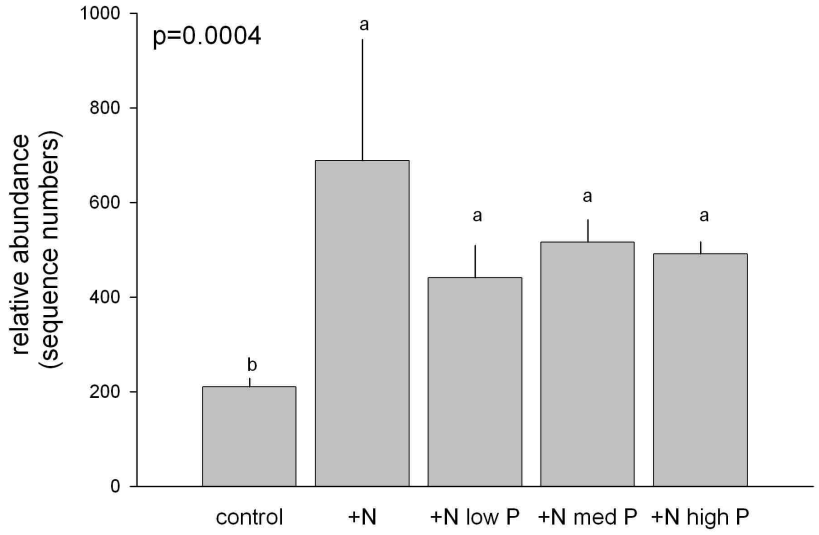

Figure 2 b)

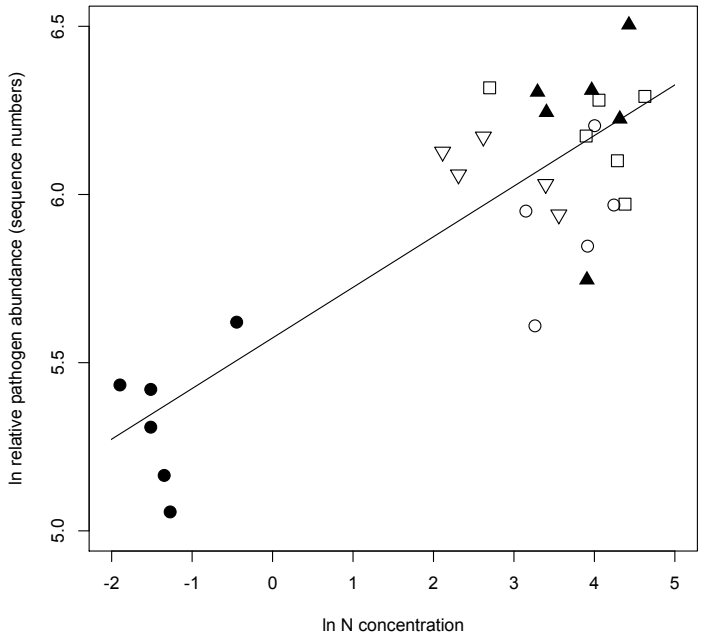
ln relative pathogen abundance (sequence numbers)
6.5
6.0
5.5
5.0
-2
-1
0
1
2
3
4
5
ln N concentration

Fig. 3 a)

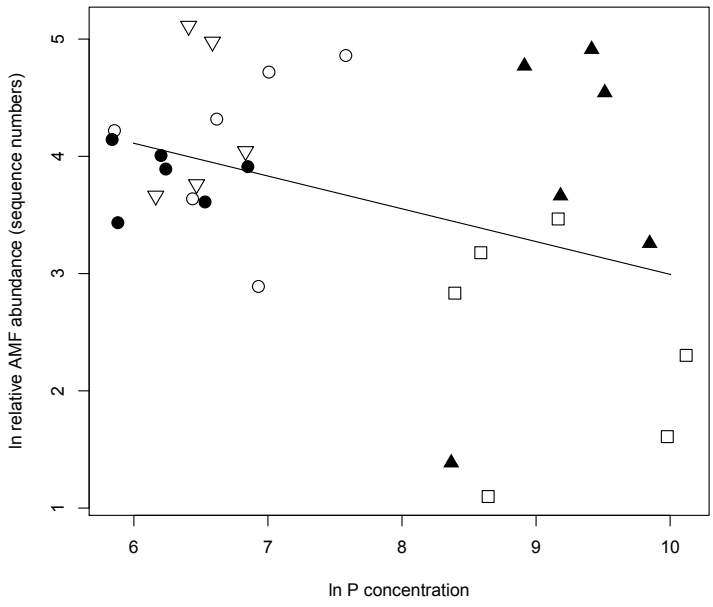
ln relative AMF abundance (sequence numbers)
5
4
3
2
1
6
7
8
9
10
ln P concentration

Fig. 3 b)

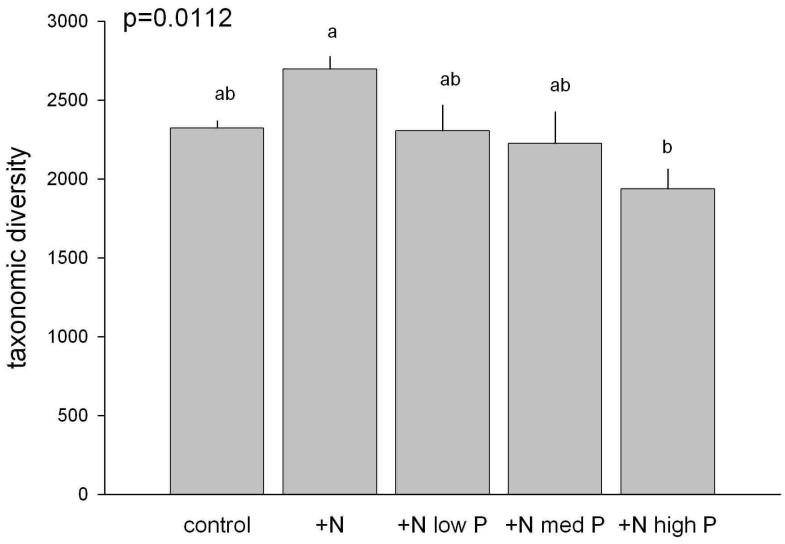
p=0.0112
taxonomic diversity
3000
2500
2000
1500
1000
500
0
ab
a
ab
ab
b
control
+N
+N low P
+N med P
+N high P

Fig. 4 a)

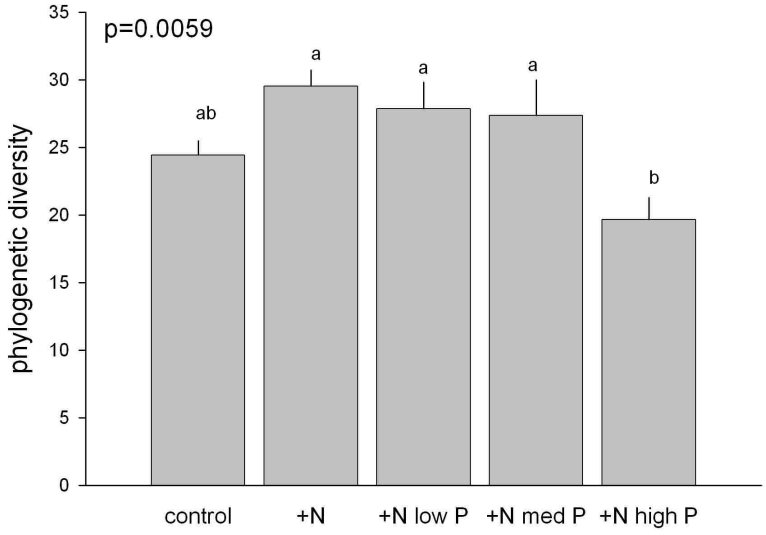
p=0.0059
phylogenetic diversity
35
30
25
20
15
10
5
0
ab
a
a
a
b
control
+N
+N low P
+N med P
+N high P

Fig. 4 b)

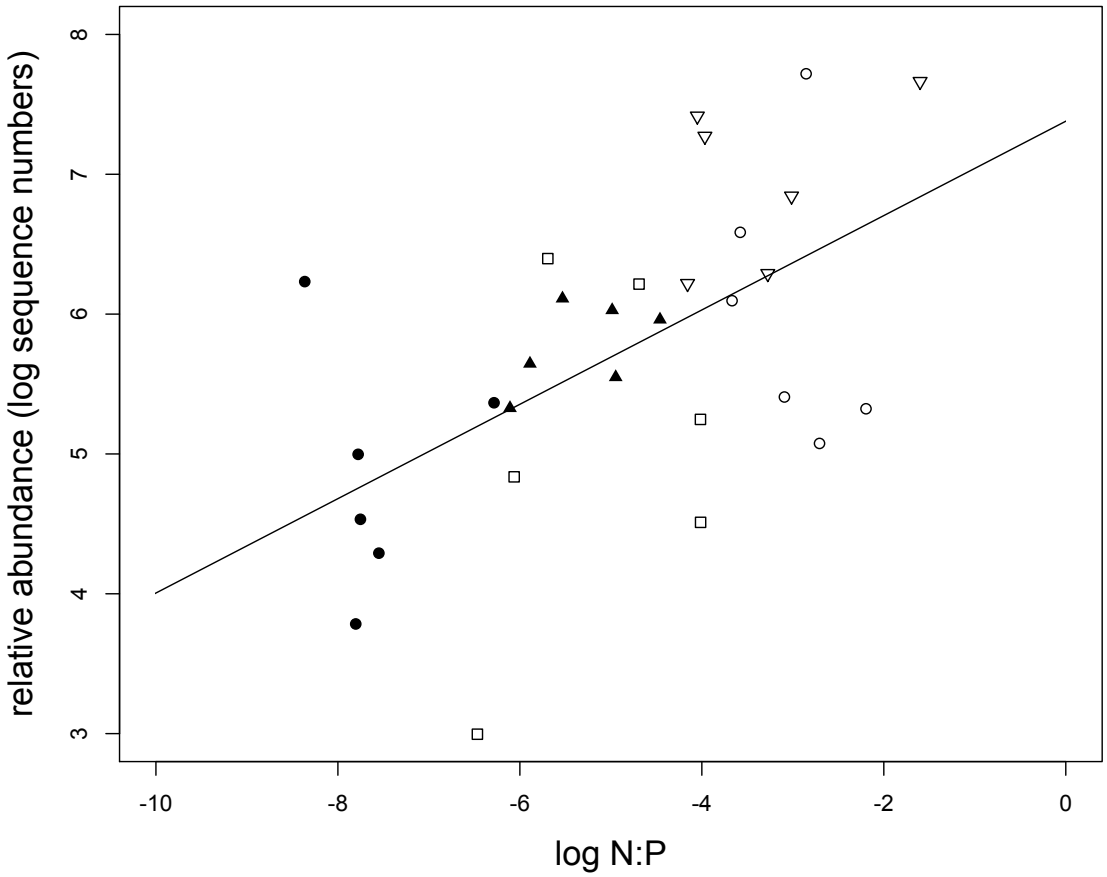
relative abundance (log sequence numbers)
3
4
5
6
7
8
-10
-8
-6
-4
-2
0
log N:P

Fig. 5

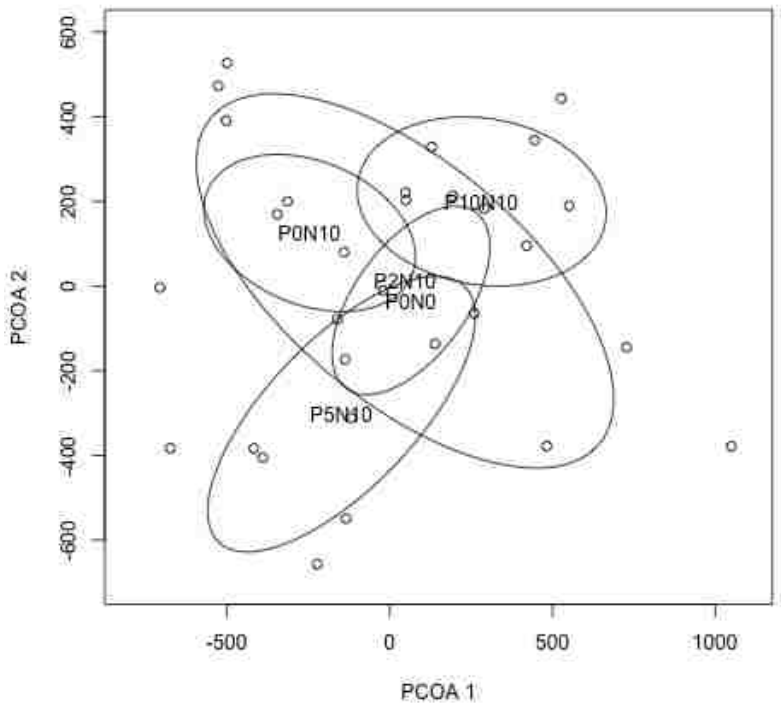
P10N10
P0N10
P2N10
P0N0
P5N10
PCOA 2
PCOA 1
600
400
200
0
-200
-400
-600
-500
0
500
1000

Fig. 6 a)

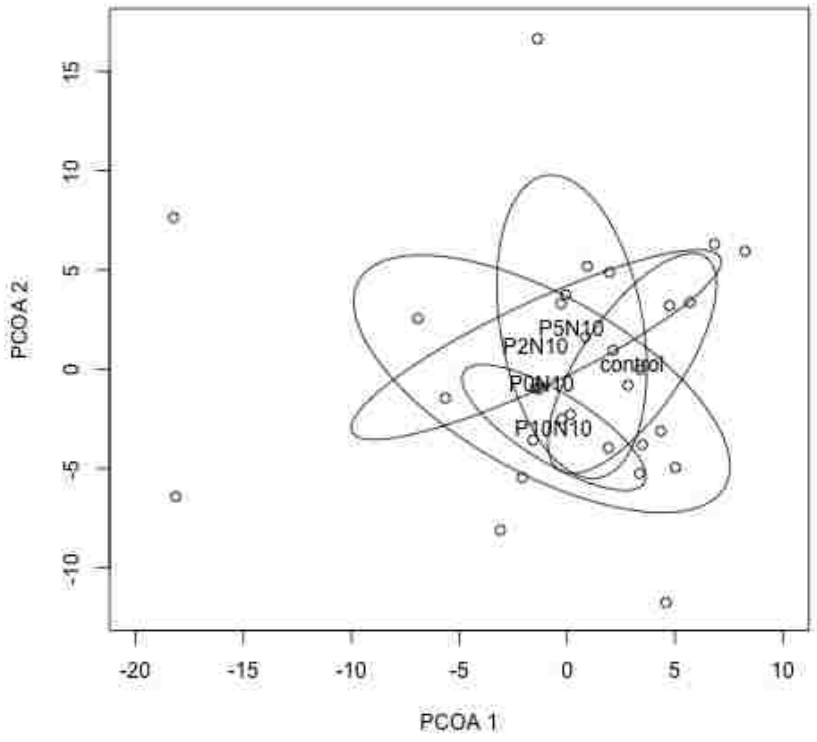
P5N10
P2N10
control
P0N10
P10N10
PCOA 2
PCOA 1
15
10
5
0
-5
-10
-20
-15
-10
-5
0
5
10

Fig. 6 b)

APENDICES
APPENDIX A
Sites in North America and Europe where seeds were collected

Pop'n	Location	Country	Elev.	Latitude	Longitude
1	Wallula, WA	USA	107	45.56	119.20
2	California	USA	967	41.59	122.36
3	California	USA	816	41.31	122.28
4	Colfax, WA	USA	601	46.52	117.20
5	California	USA	694	40.59	122.25
6	California	USA	193	40.25	122.16
7	California	USA	210	39.12	121.06
8	California	USA	9	38.31	45.64
9	California	USA	8	38.16	121.49
10	California	USA	26	38.24	122.56
11	Lewiston, ID	USA	519	46.28	116.44
12	Walla Walla, WA	USA	287	46.23	118.47
13	Georgia	Georgia	667	41.00	44.08
14	Hungary	Hungary	81	47.19	21.02
15	Hungary	Hungary	81	46.53	20.26
16	Georgia	Georgia	544	41.36	44.47
17	Georgia	Georgia	478	41.26	44.37
18	Hungary	Hungary	129	46.58	18.41
19	Romania	Romania	357	47.42	26.02
20	Turkey	Turkey	129	46.58	18.41

21	Turkey	Turkey	50	37.50	27.51
22	Turkey	Turkey	1015	37.01	30.22
23	Romania	Romania	88	47.11	27.28
24	Romania	Romania	97	46.15	27.39

APPENDIX B
Sites where soil was collected

Location	Country	State/Province	Elev.	Latitude	Longitude
The Dalles	USA	Oregon	100	45.63	-121.21
Lind	USA	Washington	417	46.97	-118.56
Rose Lake	USA	Idaho	661	47.52	-116.51
Southern Bitterroot	USA	Montana	1184	46.13	-114.04
Butte	USA	Montana	1688	46.01	-112.53
Clearwater	USA	Montana	1165	47.02	-113.36
Miles City	USA	Montana	722	46.41	-105.84
Devil's Lake	USA	North Dakota	443	48.11	-98.86
George Reserve	USA	Michigan	290	42.47	-84
Ontario-Guelph LTMRS	Canada	Guelph	334	43.54	-80.25

APPENDIX C

The Genus, virtual taxa number and total number of sequences for the 35 most abundant Glomeromycotan OTUs (SSU).

Glomus NES27, VTX00331	104063	*Claroideoglomus Douhan*9, VTX00056	
Archaeospora sp., VTX00009	75580	*Glomus sp*., VTX00315	
Glomus MO-G5, VTX00219	62820	*Glomus* MO-G15, VTX00135	
Acaulospora Acau16, VTX00102	59151	*Glomus* Kottke08-11, VTX00217	
Glomus Douhan3, VTX00212	32966	*Glomus Alguacil1*4b Glo10, VTX00423	
Claroideoglomus Glo G8, VTX00340	24807	*Paraglomus brasilianum*, VTX00239	
Glomus MO-G8, VTX00130	24705	*Glomus Yamato*09 D4, VTX00075	
Glomus viscosum, VTX00063	18798	*Glomus Wirsel* OTU13, VTX00140	
Glomus MO-G23, VTX00222	15066	*Scutellospora* LER04, VTX00318	
Kuklospora PT6, VTX00249	13165	*Glomus Torrecillas*12b Glo G13, VTX00409	
Claroideoglomus lamellosum, VTX00193	10888	*Glomus* MO-G22, VTX00125	
Gigaspora decipiens, VTX00039	10387	*Glomus* MO-G7, VTX00199	
Acaulospora Deepika15 Acaulo P2, VTX00425	10116	*Glomus* LES15, VTX00411	
Glomus MO-G3, VTX00113	9316	*Paraglomus laccatum*, VTX00281	
Glomus perpusillum, VTX00287	8540	*Claroideoglomus Torrecillas*12b Glo G5, VTX00402	
*Paraglomus Alguacil*12b PARA1, VTX00350	8316	*Glomus* Glo2, VTX00096	
Glomus MO-G18, VTX00064	7056	*Glomus mosseae,* VTX00067	
Glomus sp., VTX00093	6836		