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Investigating the context-dependent effects of plant mutualists on plant and herbivore performance in an agricultural legume

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ABSTRACT

INVESTIGATING THE CONTEXT-DEPENDENT EFFECTS OF PLANT MUTUALISTS ON PLANT AND HERBIVORE PERFORMANCE IN AN AGRICULTURAL LEGUME

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Nutritional symbioses between plants and plant-root mutualists are not only important players in nutrient acquisition by plants but contribute to nutrient cycling, biological community composition and even plant defense elicitation. Furthermore, several studies have purported that anthropogenic nutrient loading may disrupt these symbioses by effectively removing plant dependence on symbionts for nutrient acquisition, while some have evidenced that the presence of another symbiont may ameliorate the negative effects of nutrient loading on said first symbiont. In addition, less is known on how nutrient loading effects may influence higher trophic levels, such as insect herbivores. This study investigated how arbuscular mycorrhizal fungi (AMF) and rhizobacteria interact under nitrogen or phosphorus amendment to influence each other's abundance, plant growth and defense traits, and herbivore performance using a factorial greenhouse experiment and herbivory assay. Results illustrated no support for the negative influence of nutrient loading on symbiont abundance and fitness, nor, as a corollary of that, evidence of ameliorating effects of one symbiont on the other in dual inoculation treatments. In addition, there was evidence of nodulation in the absence of rhizobial inoculation, which calls into question the use of nodule counting as a metric of rhizobial abundance and

fitness. In addition, the insignificant response of alfalfa across nutrient and symbiont treatments for several response variables may illustrate that the domestication of alfalfa has reduced its dependence on belowground mutualists for nutrient acquisition, such as the fact that plant phosphorus and protein content were found not to be dependent upon symbiont treatment. There was also no influence of nutrient and symbiont treatments on herbivore performance, despite significant differences in foliar defensive chemistry across symbiont and/or nutrient treatments. These results do not support nutrient loading hypotheses, and thus highlight the need to consider species identities as an influence in interaction outcomes.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

INTRODUCTION

Research on how biotic and abiotic environmental factors interact and affect the strength of species interactions, and to what degree these interactions impact the distribution and abundance of a given species, has been highlighted as a major gap in community ecology research (Agrawal et al. 2007). Ecological mutualisms involve reciprocation between symbionts to the access of limiting resources, such as nutrients or physical protection, and not only shape the evolutionary and organismal biology of the respective symbionts but can also influence community and ecosystem dynamics (Artursson et al. 2006, Klabi et al. 2014, Rillig 2004, van der Heijden 2010). Terrestrial nutritional symbioses are usually partnerships between a phototroph and heterotroph (Shantz et al. 2016) and are often in the form of an association between plant roots and soil microbes. Two examples of these symbioses are arbuscular mycorrhizal fungi (AMF), present in 70-90% of the world's vascular plant species (Denison and Kiers 2011), and nitrogen-fixing bacteria associated with plants such as members of Fabaceae (legumes). Both of these symbioses can vary with abiotic factors like soil nutrients to affect host plant growth, fitness, and interactions with other community members. AMF are obligate symbionts belonging to the fungal phylum Glomeromycota (Schuβler et al. 2001, Smith and Read 2008). The fungi associate with plant roots in the soil, forming nutrient exchange sites known as "arbuscules", where AMF exchange nutrients that are tightly bound to soil particles, mainly phosphorus but also micronutrients as well, including calcium, zinc, and copper, in exchange for sugars produced photosynthetically from the plant (Barea et al. 2005, Denison and

Kiers 2011, Smith and Read 2008). AMF are efficient scourers for immobile nutrients because of their narrow hyphae with high surface area in contact with soil. While plant roots can deplete rhizosphere zones of phosphorus at a much faster rate than the concentration around the root can be replaced via diffusion, the narrow diameter and extended reach of mycorrhizal hyphae obtain phosphorous without this rapid depletion (Smith and Read 2008, Smith and Smith 2012). As a result, association with AMF can increase plant growth rate and fitness (Dension and Kiers 2011, Smith and Read 2008, Vanette and Hunter 2011). Because of this extraordinary capability to confer a limiting nutrient to plants, up to 20% of photosynthates may be allocated to the AMF symbiont (Dension and Kiers 2011).

Rhizobium is the general term for soil nitrogen-fixing bacteria that become established in legume root nodules; rhizobial phylogeny is currently debated, consisting of six genera that may or may not be monophyletic (Aoki et al. 2013, van Berkum et al. 2003). Unlike AMF, rhizobia are not obligate and populations can persist in soil for up to a decade after legume hosts are removed (Denison and Kiers 2011). Rhizobia have the capacity to fix atmospheric nitrogen, N_2, into the plant-usable form ammonia via the use of the nitrogenase enzyme (Denison and Kiers 2011). Rhizobial inputs of nitrogen into the environment are estimated at almost 70 million metric tons, providing an ecosystem service worth up to US $60 billion in the production of food, fuel and fiber (Shantz et al. 2016).

Plant-symbiont relationships generally have been studied as pairwise interactions, although researchers have pointed out that studying situations with single symbionts and plants may under- or overestimate effects that are modified by a third partner or multiple other community members (Bennett et al. 2006, Larimer et al. 2010, Morris et al. 2007, Stanton 2003).

For example, evidence suggests that AMF uptake of micronutrients may play a crucial part in rhizobial fitness and nodulation, and fixed nitrogen by rhizobia can aid in chitin synthesis necessary for AMF cell wall and hyphal development (Barea et al. 2005). Synergism, referring in this thesis to the response of a plant when inoculated with two symbionts that exceeds that of their additive effects, has been observed in multiple studies. Synergism on plant biomass (Toro et al. 1998, Toro et al. 1997, Ferrari and Wall 2008, Pacovsky et al. 1986, Wang et al. 2011) and nutrient uptake and use (Toro et al. 1997, Harris et al. 1985, Jha et al. 1993, Tajini et al. 2012) has been noted. In addition, dual inoculations of AMF and rhizobia can benefit the symbionts, increasing the abundance of AMF, rhizobia, or both (Toro et al. 1998, Toro et al. 1997, Ferrari and Wall 2008, Janos 1980, Jia et al. 2004, Pacovsky et al. 1986, Tajini et al. 2012, Vazquez et al. 2001, Wang et al. 2001). Thus, it is increasingly important to consider the interactions between mutualists and their influences on their plant hosts.

Given that abiotic conditions can influence the magnitude and direction of interspecific and even intertrophic-level interactions (Agrawal et al. 2007), there is increasing interest in determining how nutrient loading in an ecosystem may disrupt these nutritional symbioses and the advantages they impart. Nutrient loading is the anthropogenic influx of nutrients into an environment that consequently "overloads" the system with these nutrients, relative to the amounts occurring from natural sources (Shantz et al. 2016). For instance, a recent meta-analysis illustrated that nutrient loading consistently decoupled symbioses in terrestrial and aquatic systems, with phototrophs benefitting at the expense of heterotrophs (Shantz et al. 2016). Similarly, soil fertilization can have negative effects on abundance and performance of both AMF and rhizobia symbioses. Colonization of plant roots can decrease with phosphorus

fertilization, as this releases plant dependence on AMF for phosphorus and thus degrades the plant response to the mutualism (Cornwell et al. 2001, Grman and Robinson 2013, Jha et al. 1993, Shantz et al. 2016, Wang et al. 2011). One study suggested that phosphorus fertilization physically deformed arbuscules and downregulated the expression of plant proteins that transport phosphorus from AMF (Breuillin et al. 2010). A similar effect on rhizobia occurs with application of nitrogenous fertilizers, reducing root nodulation, N_2 fixing activity and plant uptake of fixed compounds such as ureide (Fujikake et al. 2002, Herridge et al. 1984, Jia et al. 2004, Larimer et al. 2014, Shantz et al. 2016, Sodek and Silva 1996, Vazquez et al. 2001, Voisin et al. 2003,.

While plant hosts may be released from the energetic constraint of symbiosis via nutrient loading, plants and plant communities as a whole may risk losing benefits of microbial symbionts beyond nutrient acquisition. For instance, AMF have been shown to promote plant tolerance to heavy-metal-contaminated and high-salinity soils (Amir et al. 2013, Soliman et al. 2012). In addition, the high surface area of absorptive hyphae can aid in plant drought tolerance (Koide 1991, Ortiz et al. 2015). At community and ecosystem levels, AMF have been shown to influence microbial community makeup in the rhizosphere (Artursson et al. 2006), increase plant diversity and productivity (Bauer et al. 2012, Collins and Foster 2009, Hartley and Gange 2009, Klabi et al. 2014), promote carbon and phosphorus cycling in ecosystems (Rillig 2004, van der Heijden 2010), as well as reduce erosion because of the high surface area of AMF mycelium and the creation of stable soil aggregates via secretion of exudates (Barea et al. 2005, Koide 1991, van der Heijden 2010). AMF have also been investigated for use as a bioinoculant to replace synthetic fertilizer use, as AMF not only can aid in nutrient uptake by their plant hosts, but AMF

have been shown to drastically reduce nutrient loss and runoff (Owen et al. 2014, van der Heijden 2010). The benefits of rhizobia include the fixation of more than 60% of plant nitrogen from a biological source and the role of legumes in maintaining nitrogen pools in plant communities (Barea et al. 2005, Spehn et al. 2002).

Perhaps even more compelling is the evidence that AMF and rhizobia may influence plant defenses against plant enemies. Several studies have shown that AMF can modulate floral volatile organic compounds that attract parasitoid predators of insect herbivores (Bennett and Bever 2007, Hartley and Gange 2009, Schausberger et al. 2012). Other studies have demonstrated that AMF and rhizobia can increase plant tolerance of herbivory and pathogenic attack via supplementation of nutrients (Kula et al. 2005, Morris et al. 2007). AMF and rhizobia also compete with root pathogens for root space, thereby competitively excluding pathogens that otherwise might infect their plant hosts (Denison and Kiers 2011). Finally, both types of symbionts may provide protection from herbivores through increased expression of defense traits (Johnson and Bentley 1991, Koricheva et al. 2009, Vanette and Hunter 2011). AMF and rhizobia may prime inducible defenses (Dean et al. 2014, Pietrese et al. 2008, Pozo et al. 2008, Ruiz-Lozano et al. 1999, Verhagen et al. 2004, Yan et al. 2002). Priming causes accelerated expression of jasmonic-acid-regulated defense chemicals, including protease inhibitors (PI) and peroxidase (POD) (Pozo et al. 2008). Therefore, consequences of anthropogenic pollution and nutrient loading can lead to the decoupling of these mutualisms that are important for a variety of processes at organismal, community, and ecosystem levels.

In light of the negative effects of nutrient application on maintenance of mutualisms, there is evidence that amelioration of nutrient loading on symbionts can occur in dual-inoculated

systems, i.e. plants colonized by both AMF and rhizobia (Larimer et al. 2014), although more research is needed to confirm this possible interaction and its mechanism. In particular, single- and dual-symbiont effects on plant defenses in different nutrient contexts are largely unknown (Heil 2011). Here, I investigate how AMF and rhizobia interact in different nutrient contexts to influence each other, plant growth, and defense against herbivores in an agriculturally significant legume. My research sought to answer three questions: 1) Does soil amendment in single-inoculation treatments with nitrogen and phosphorus decrease rhizobial and fungal abundance, respectively, relative to controls? 2) Do dual inoculation treatments result in reduced magnitude of the inhibitory effect of nutrient amendment on symbiont performance, due to the increased tolerance provided by a second mutualist? 3) Are plant defensive chemistry and herbivore performance (measured as larval mass) modulated by nutrient and symbiont treatments?

METHODS

Mesocosm Setup and Experimental Treatments

I manipulated AMF (presence/absence), rhizobia (presence/absence), and soil nutrients (control, added P, or added N) in a factorial design for 12 treatment combinations (n = 26 plants/treatment, 312 total). The host plant was *Medicago sativa* (alfalfa), a cool-season legume in the family Fabaceae. Alfalfa is widely cultivated around the world and is an important source of fodder for livestock (Small 1996). Seeds were surface sterilized in a 1:15 bleach dilution for 15 minutes and then rinsed. Seeds were coated in rhizobial inoculant, consisting of *Rhizobum leguminosarum (biovar trifolii)* and *Sinorhizobium meliloti* (INTX Micorbials, LLC, Kentland, IN, USA) or sterilized rhizobial inoculant for control plants. Plastic horticultural pots were filled with 450 mL of sterilized soil (Fafard 3B Growing Mix, Sun Gro Horticulture Canada Ltd., Agawam, MA, USA), and 15 seeds were added to the top of the soil layer in each of their corresponding treatment pots. Next, 2 mL of either viable or sterilized inoculum for the AMF species *Rhizophagus irregularis* on a perlite carrier was added to corresponding treatment pots (MYKE®, Premier Tech Biotechnologies, Quebec, Canada). Seeds and inoculant treatments in pots were then covered with an additional 150 mL of soil, watered with deionized (DI) water and covered with sterilized rice hulls to reduce invertebrate pests and retain moisture. Pots were arranged in randomized blocks with one pot per treatment combination per block, and blocks were rotated on greenhouse benches weekly. Plants were grown for 20 weeks, with application

of nutrients every other week (25 mL of either NH_4NO_3 solution at 1.24 mmol or KH_2PO_4 solution at 0.73 mmol or 25 mL of DI water; Larimer et al. 2014). The concentrations utilized for nutrient application have been previously observed in literature to elicit significant legume and symbiont responses (Larimer et al. 2014). In addition, the fertilizer concentrations are greater than reported needs of nitrogen and phosphorus for alfalfa (Koenig et al. 1999) and therefore are sufficient treatments to simulate nutrient loading in pot mesocosms. Plants were also thinned out every other week in order to achieve one viable plant per pot, starting at 2 weeks after sowing and continuing on until week 11.

Eight complete blocks (96 plants) were harvested to measure biomass. On the 141st and 142nd days after seeding, the plants were clipped at just above the root crown for aboveground biomass, whereas the roots were carefully rinsed to remove soil. Aboveground and belowground biomass portions were dried for one week at 60˚ C and weighed separately. For leaf tissue analysis, a different set of eight blocks was used. Three 0.1 g leaf samples were taken from each plant and stored at -80˚ C. Remaining aboveground biomass of these plants was dried and ground for P analysis. Roots were washed, and the number of nodules were counted to quantify rhizobial abundance. Last, a sample of fine roots were clipped from around the entire root mass and stored at -80˚ C until staining with trypan blue (Koske and Gemma 1989) to measure AMF colonization using the magnified intersect method (McGonigle et al. 1990).

Digestible protein content of foliar tissues was measured as a proxy for nitrogen content using the Bradford assay (Bradford 1976; Bio-Rad, Hercules, CA, USA). For alfalfa leaves, the sample extracts were diluted for this particular protocol to enhance spectrophotometric measurements to better differentiate between different concentrations of nitrogen. Phosphorus

content of stem and leaf tissues was measured using the Murphy-Riley procedure (Murphy and Riley 1962). For general plant defensive chemicals, activities of POD and PI were measured using established protocols (Moore et al. 2003, Thaler et al. 1996). All of these colorimetric analyses were conducted using a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Herbivory Assay

I assayed treatment effects on herbivores using larvae of *Spodoptera frugiperda*, a Noctuid generalist chewing insect known to a feed on legumes (Batista-Pereira et al. 2002, Schmelz et al. 2007). Several hundred *S. frugiperda* eggs (Benzon Research Inc., Carlisle, PA, USA) were incubated at 28˚C for six days with nonexperimental alfalfa feeder leaves in a growth chamber to facilitate hatching and allow larvae to grow before the assay. Leaves were clipped from plants in the remaining 10 blocks and placed in individual petri dishes with moist filter paper. A single third-instar larva was placed on the leaf clippings in each petri dish. Although larvae were all the same age, individuals were chosen to maintain consistent size within each block. The petri dishes were then sealed with Parafilm to maintain moisture. Larvae were allowed to feed for 101 hours and checked every 10 hours to ensure that they were still alive and that adequate leaf material was still available for them to feed. When leaf reserves were exhausted, several more leaves were clipped and placed in the petri dish. Larvae were then starved for 6 hours to void their guts before being weighed.

Statistical Analysis

Biomass (root, shoot, root:shoot and total), larval mass, P and protein content, AMF colonization and PI and POD activity were analyzed with linear mixed-effect models, specifying AMF, rhizobia, nutrient treatments, and their interactions as fixed independent variables and block as a random factor, using the nlme package (Pinheiro et al. 2010) in R version 3.2.2 (R Development Core Team 2016). The response variables of larval mass, protein content, phosphorus content, PI and POD activity were log transformed to normalize residuals. Nodule number was analyzed using a generalized linear mixed model with Poisson error distribution and an individual-level random factor to account for overdispersion (Agresti 2002) in the lme4 package (Bates et al. 2012). Fixed factors were evaluated using likelihood ratio tests.

RESULTS

Plant Growth and Performance

Inoculation with rhizobia significantly increased aboveground biomass (Table 1) by nearly 19% (Figure 1A). There was also an interaction between AMF and nutrient treatments on aboveground biomass. While unfertilized plants did not differ in aboveground biomass with and without AMF, AMF increased aboveground biomass with N addition and decreased it with P addition (Figure 1B). Relative allocation of resources to above- and belowground growth, as measured by root:shoot ratio, was affected by all three treatments (Table 1). In non-fertilized plants, single-symbiont treatments increased allocation of resources belowground but allocated more biomass aboveground when plants were inoculated with both (Figure 2). Nitrogen-fertilized plants allocated more resources belowground only in the absence of both symbionts, while phosphorus-fertilized plants' allocation scheme was unaffected by symbiont treatments (Figure 2). Shoot P content was only affected by nutrient treatment, being 16% greater with P addition relative to control (Table 1, Figure 3). Treatments did not affect leaf protein content.

Table 1: Likelihood Ratio Test Analysis for Plant Performance Response Variables.

	Aboveground Biomass		Belowground Biomass		Total Biomass		Root:Shoot Biomass	
	LRT	*p*	*LRT*	*p*	*LRT*	*p*	*LRT*	*p*
AMF	0.22	0.640	0.09	0.769	1.62	0.204	96.37	**<0.0001**
Rhizobia	8.20	**0.005**	0.71	0.401	4.49	**0.037**	3.17	0.079
Nutrient	1.50	0.230	1.94	0.150	0.18	0.669	0.40	0.529
AMF x Rhizobia	2.19	0.143	3.94	0.051	1.00	0.372	0.47	0.627
AMF x Nutrient	4.03	**0.022**	0.77	0.468	2.66	0.076	0.74	0.483
Rhizobia x Nutrient	0.65	0.524	1.05	0.355	3.59	0.062	0.87	0.354
AMF x Rhizobia x Nutrient	1.84	0.166	0.92	0.401	0.52	0.594	5.42	**0.006**

Bold print indicates $p < 0.05$.

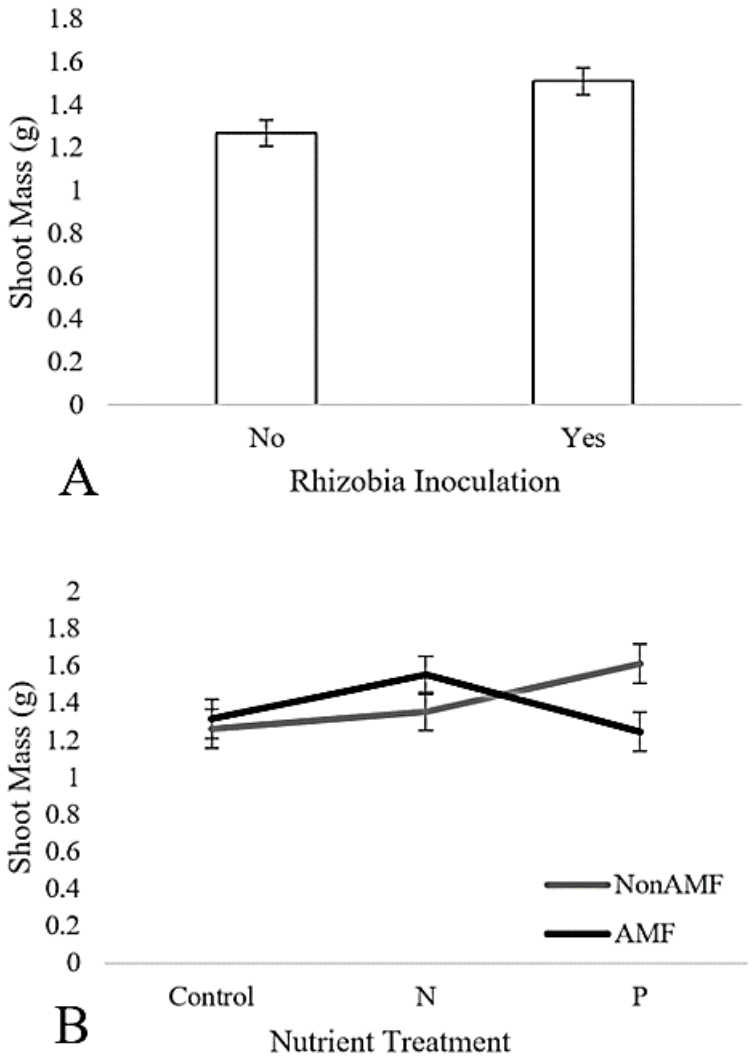

Figure 1: Mean aboveground biomass for a) rhizobia inoculation treatments and b) nutrient x AMF treatments. Con = control, non-nutrient amended plants, N= nitrogen amended plants, and P= phosphorus amended plants. Error bars represent 1 ± SE.

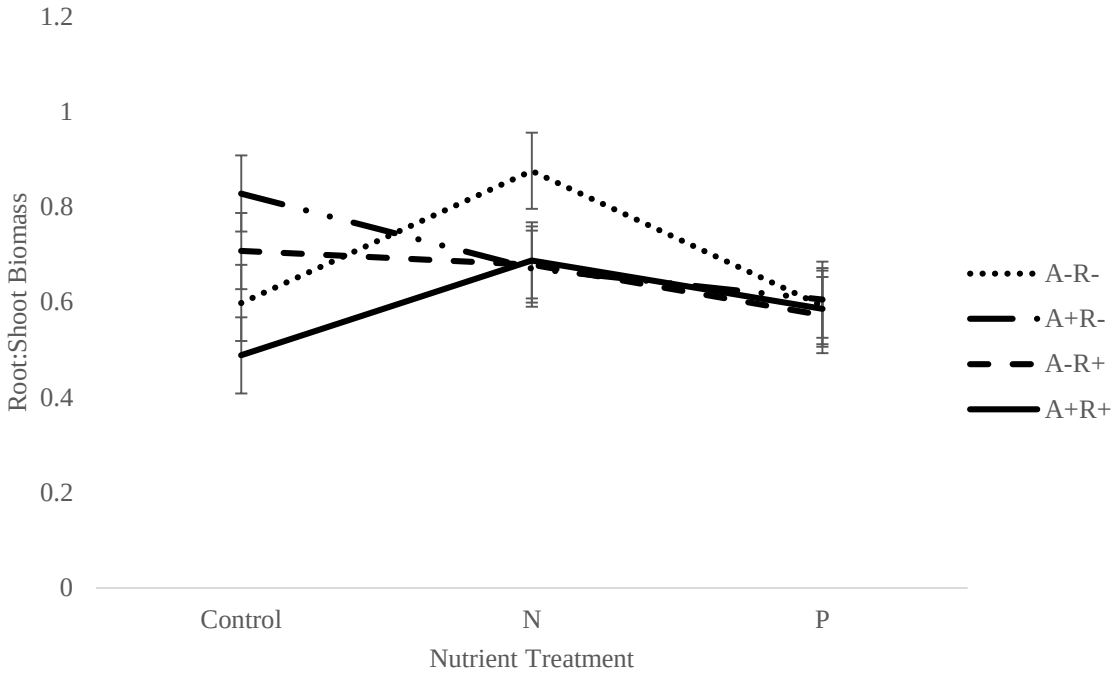

Figure 2: Mean root:shoot ratio for AMF x Rhizobia x Nutrient treatments. Treatment abbreviations are as follows: Control = control, non-nutrient amended plants, N= nitrogen amended plants, and P= phosphorus amended plants. A- = non-AMF inoculated, A+ = AMF inoculated, R- = non-rhizobia inoculated, and R+ =rhizobia inoculated treatments. Error bars represent 1 ± SE.

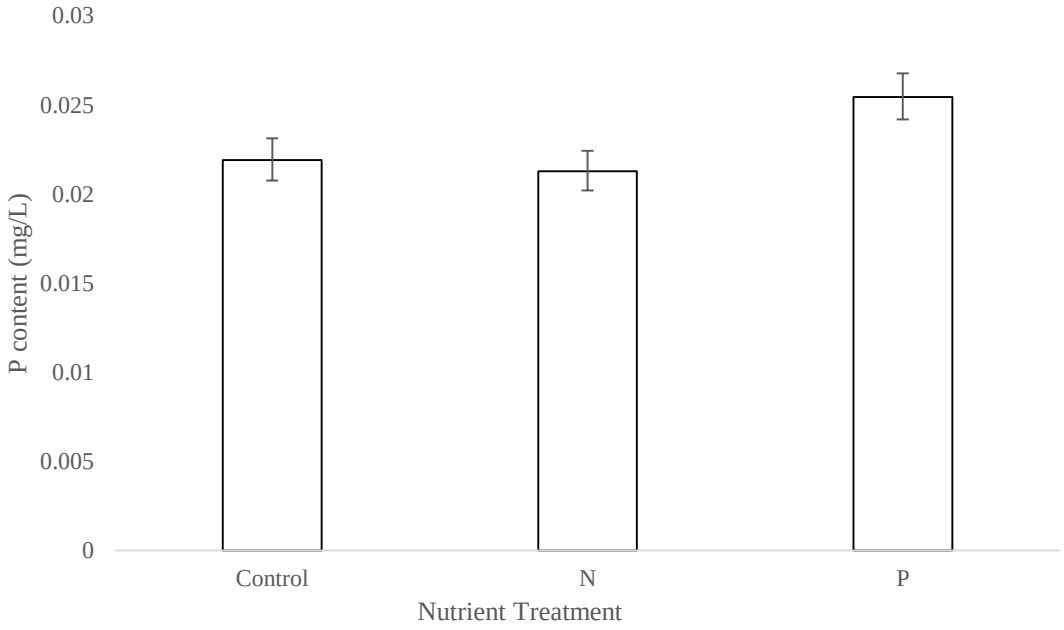

Figure 3: Mean shoot phosphorus content across nutrient treatments. Control = control, non-nutrient amended plants, N= nitrogen amended plants, and P= phosphorus amended plants. Error bars represent 1 ± SE.

Protease inhibitor (PI) activity was significantly greater in plants fertilized with P relative to other nutrient treatments (Table 2, Figure 4). There was a significant three-way interaction of treatments on POD activity (Table 2). Unfertilized plants inoculated with no symbionts had a greater POD activity than those inoculated with any symbionts (Figure 5). However, in N-fertilized plants, the greatest POD activity was observed for plants that were only inoculated with rhizobia, whereas all other treatment combinations were lower and similar to one another (Figure 5). In all P-fertilized plants, POD activity was low and unaffected by symbionts (Figure 5).

Table 2: Likelihood Ratio Test Analysis for Plant Defense and Herbivore Performance Response Variables.

	Larval Mass		PI		POD	
	LRT	*p*	*LRT*	*p*	*LRT*	*p*
AMF	0.30	0.583	1.61	0.210	2.44	0.123
Rhizobia	0.10	0.756	0.31	0.581	0.69	0.408
Nutrient	0.34	0.714	3.83	0.027	1.27	0.286
AMF x Rhizobia	0.60	0.440	0.05	0.828	0.12	0.735
AMF x Nutrient	2.41	0.096	2.62	0.081	0.02	0.976
Rhizobia x Nutrient	2.03	0.138	0.17	0.842	3.41	0.038
AMF x Rhizobia x Nutrient	0.42	0.661	1.56	0.219	3.15	0.049

Bold print indicates $p < 0.05$.

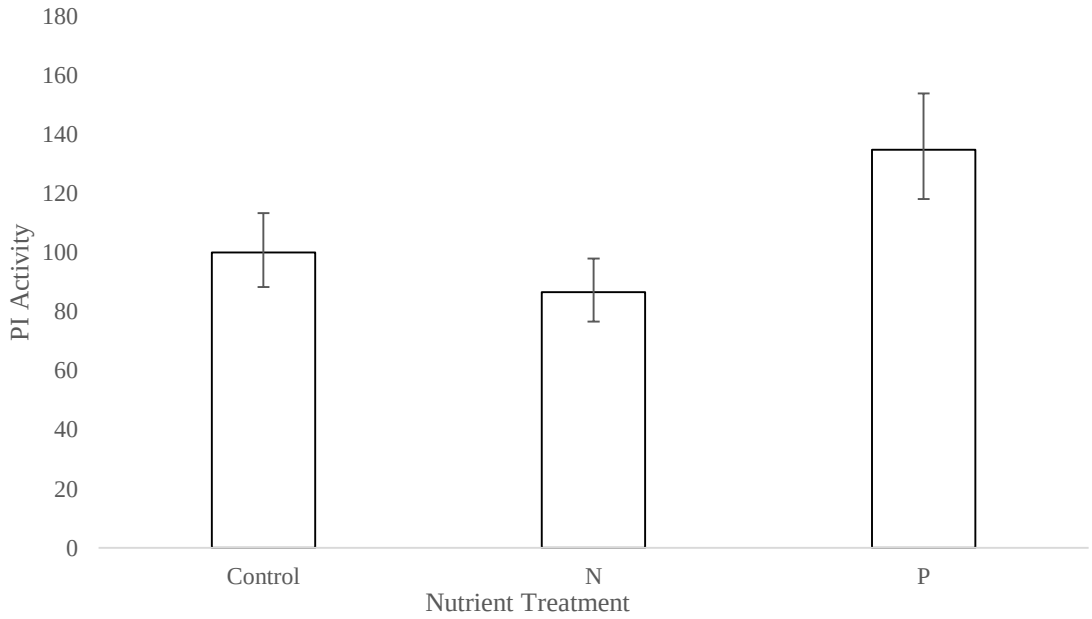

Figure 4: Mean foliar PI activity across nutrient treatments. Control = control, non-nutrient amended plants, N= nitrogen amended plants, and P= phosphorus amended plants. Error bars represent 1 ± SE.

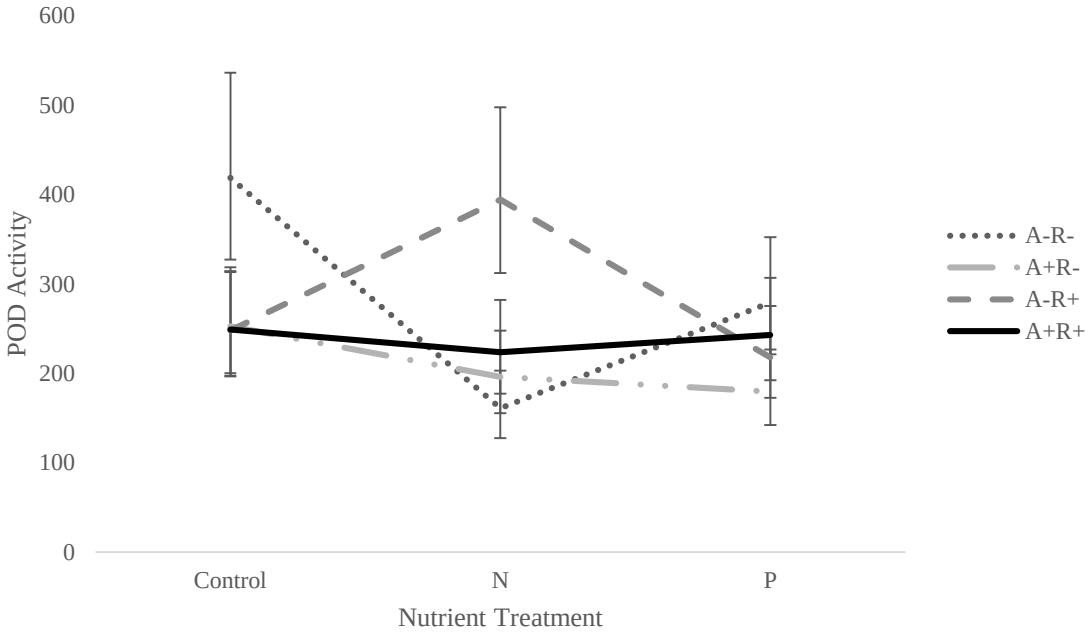

Figure 5: Mean POD activity for AMF x Rhizobia x Nutrient treatments. Treatment abbreviations are as follows: Control = control, non-nutrient amended plants, N= nitrogen amended plants, and P= phosphorus amended plants. A- = non-AMF inoculated, A+ = AMF inoculated, R- = non-rhizobia inoculated, and R+ =rhizobia inoculated treatments. Error bars represent 1 ± SE.

Effects of Nutrient Context on Symbionts

Plants inoculated with AMF across all treatments had significantly greater levels of colonization relative to non-inoculated plants (Figure 6), and this was unaffected by nutrient addition (Table 3). There was a significant interaction between nutrient, AMF and rhizobial treatments on nodule number (Table 3). In unfertilized plants, the nodule count was greater in non-symbiont plants than those inoculated with at least one symbiont and was reduced by nearly half in plants only inoculated with AMF (Figure 7A and B). In N-fertilized plants, there was no

significant difference in nodule number between dual-inoculation and rhizobia-only treatments (Figure 7A), but in plants inoculated only with AMF, there was a reduction in the number of nodules (Figure 7B). In P fertilized plants, those with no symbionts had the lowest number of nodules (Figure 7B), whereas plants inoculated with at least one symbiont had greater number of nodules relative to the control (Figure 7A and B).

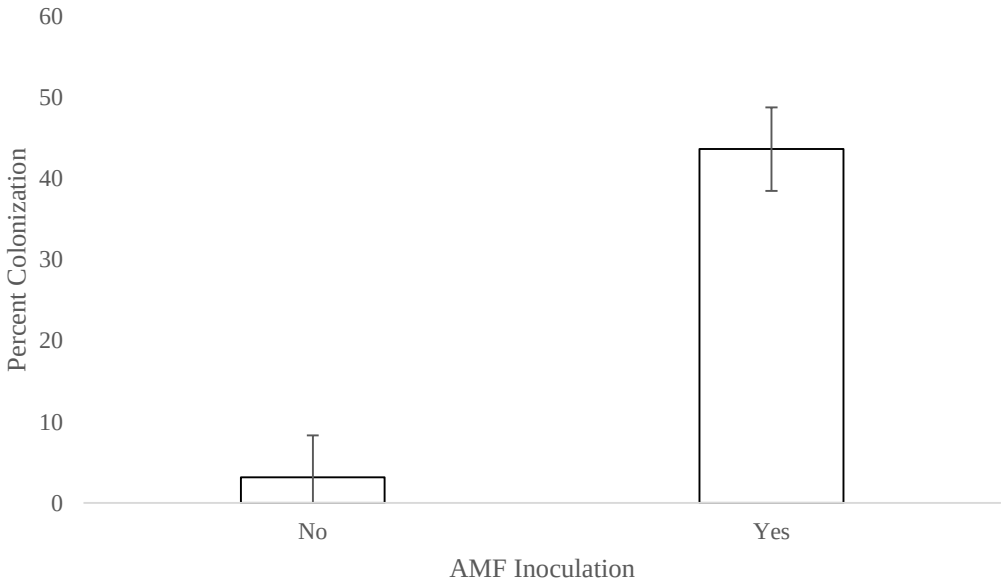

Figure 6: Mean percent colonization of AMF between AMF treatments. Error bars represent 1 ± SE.

Table 3: Chi-Square and Likelihood Ratio Test Analysis for Rhizobia and AMF Abundance, Respectively.

	Nodule Number		AMF % Colonization	
	χ^2	*p*	*LRT*	*p*
AMF	0.78	0.376	132.06	**<.0001**
Rhizobia	3.02	0.082	0.01	0.937
Nutrient	2.18	0.336	0.02	0.978
AMF x Rhizobia	3.04	0.081	0.50	0.480
AMF x Nutrient	3.95	0.139	0.55	0.580
Rhizobia x Nutrient	0.79	0.675	0.02	0.979
AMF x Rhizobia x Nutrient	6.29	**0.043**	1.07	0.349

Bold print indicates $p < 0.05$.

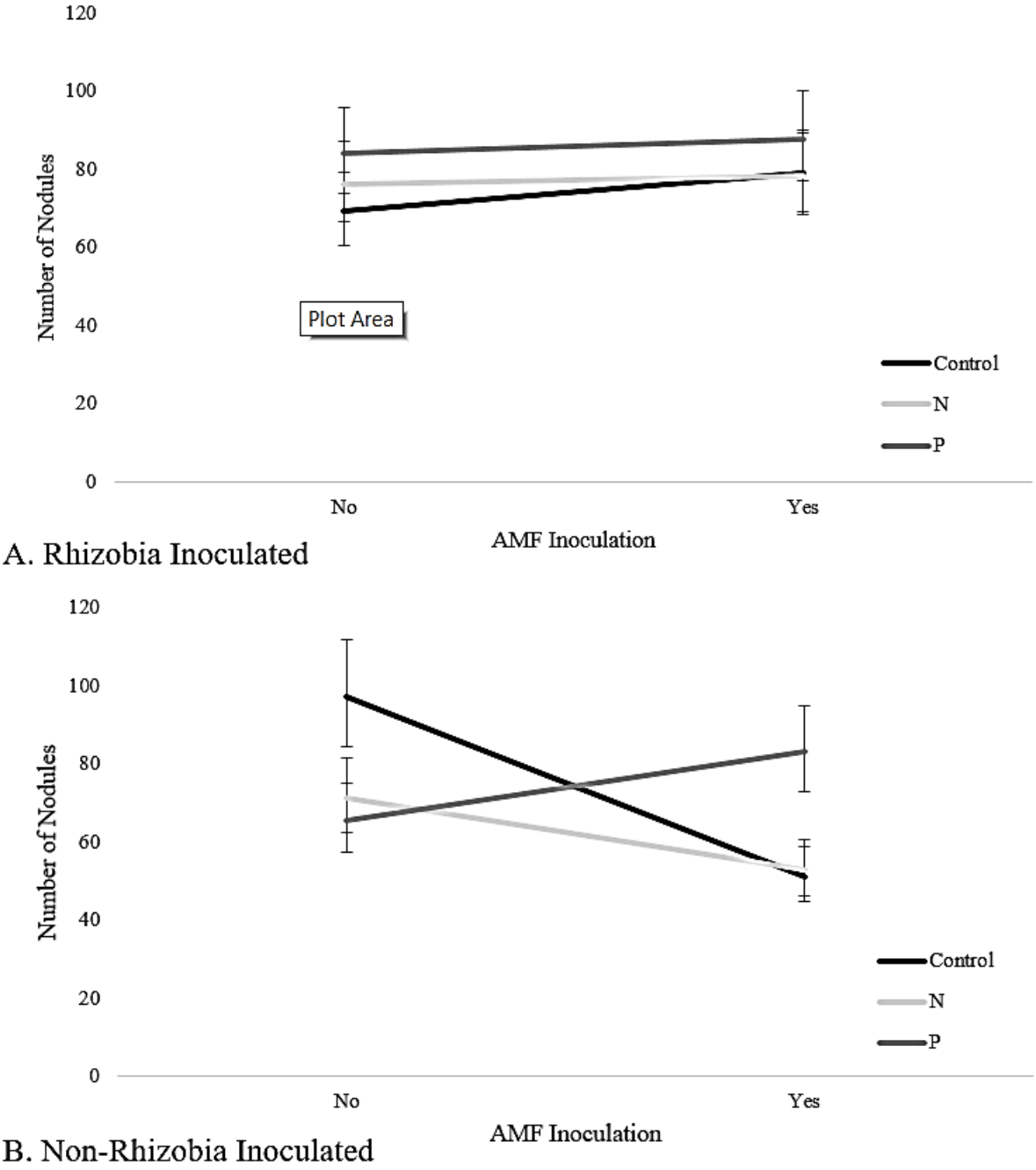

Figure 7: Mean nodule counts for a) rhizobia inoculated plants and b) non-rhizobia inoculated plants across AMF x Nutrient treatments. Treatment abbreviations are as follows: Control = control, non-nutrient amended plants, N= nitrogen amended plants, and P= phosphorus amended plants. Error bars represent 1 ± SE.

There was no significant effect of treatments on bioassay larval mass, although there was a marginal interaction between AMF and nutrient treatments (Table 2), with caterpillars tending to grow larger on plants with AMF, but only P fertilization (data not shown).

DISCUSSION

The rate of fertilizer application for agricultural and domestic uses has been growing since the Industrial Revolution. This may have negative impacts on the relationships plants have with their microbial root symbionts and may lead to detrimental effects on drought tolerance and defense chemical expression. In this study, I investigated how AMF and rhizobia interact in different nutrient contexts to influence each other, plant growth and performance, and defense against herbivores. I also assessed whether or not previous claims that dual inoculations protect symbionts from nutrient loading are credible. Results illustrated that abiotic and biotic components, and in certain cases interactions between the two, influenced several plant growth and defense traits, such as biomass production, differential allocation to above- and belowground plant tissues, as well as expression of the defense chemical peroxidase. Nutrient treatment did not negatively influence AMF colonization measures or nodulation in rhizobial-inoculated plants and thus did not provide support for the putative amelioration effect for dual-inoculation treatments. Furthermore, the influence of nutrient and symbiont treatments did not have a significant effect on herbivore performance.

Plant Growth and Performance

Inoculation with rhizobia was associated with an increase in aboveground and total biomass across all nutrient and AMF treatments, which is in agreement with the notion that

rhizobia ameliorate nitrogen limitation that increases biomass (Bauer et al. 2012, Kempel et al. 2009, Larimer et al. 2010, Tajini et al. 2012). There was also a significant interaction between AMF and nutrient treatments. Plants inoculated with AMF that were amended with nitrogen had 14% greater shoot mass relative to uninoculated, nitrogen-treated plants, yet in the phosphorus-amended treatment, plants not inoculated with AMF had greater biomass than inoculated ones. The AMF, phosphorus-amended plants had a shoot biomass similar to their AMF, non-fertilized counterparts. The lack of response to phosphorus amendment may be explained by alfalfa not being limited in phosphorus when inoculated with AMF, thereby not responding with increased biomass. A meta-analysis conducted by Hoeksema et al. (2010) illustrated that one of the greatest predictors of plant response to AMF inoculation was nitrogen availability, not phosphorus availability. Their explanation highlighted the importance of balancing of N:P ratios, whereby AMF relieves a plant of phosphorus limitation, and therefore any further supplementation of phosphorus will most likely not induce a response, as the plant is not phosphorus limited (Hoeksema et al. 2010). Their results are congruent with the results observed in this study, whereby plants increased aboveground biomass when amended with nitrogen and inoculated with AMF, as well as when not inoculated with AMF but amended with phosphorus. Interestingly, aboveground biomass was not greater in non-amended AMF plants relative to controls, but this may be explained by root:shoot allocation differences influenced by the averaging of rhizobial treatments. Also interesting was the that only a slight difference between AMF treatments in non-amendment plants was observed, which deviates from the expectation that the greatest benefits imparted by AMF occur in low-nutrient environments (Collins and Foster 2009, Grman and Robinson 2013, Shantz et al. 2016, van der Heijden 2010).

Biomass allocation to either shoot or root tissues was dependent upon nutrient and symbiont treatments. Phosphorus-amended plants did not differ in biomass allocation across all symbiont treatments, nor did nitrogen-amended plants inoculated with at least one symbiont differ in allocation ratio. This may illustrate that the abiotic context is a greater predictor of allocation ratios rather than biotic context. Whereas Larimer et al. (2014) observed that symbiont treatments determined plant responses more so than nutrient additions, the plants in this study, when amended with nitrogen or phosphorus, did not alter allocation responses among symbiont treatments. The decrease in root:shoot variation between symbiont treatments amended with nitrogen or phosphorus supports that in this study system the abiotic context mediates allocation strategy more so than the biotic environment. Interestingly, while symbiont treatments did not differ in biomass allocation under phosphorus amendment, aboveground biomass declined under phosphorus and AMF-inoculated treatments. This may suggest a reason why total biomass declined under these treatments and may indicate an antagonistic interaction between AMF and phosphorus fertilization on shoot biomass. Similar results have been observed for phosphorus-amended plants (Bethlenfalvay et al. 1982) and may be due to the fact that high phosphorus uptake by plants may create a nitrogen-limiting environment (Yang et al. 2014). Fertilization with nitrogen or phosphorus has been observed to induce limitation in the other (Tao and Hunter 2012), as this releases the plant from being limited by the one nutrient yet causes plants to be limited by the other nutrient. Wang et al. (2011) observed that in soybeans nodulation was much more sensitive to nitrogen and phosphorus availability than AMF, perhaps illustrating that nutrient loading with fertilizers may reduce rhizobial nutrient contribution, and in the case of high-phosphorus contexts induce a nitrogen-limiting environment even when plants are

inoculated. In terms of non-inoculated plants, on average they had similar allocation ratios between non-amended and phosphorus-amended treatments, yet interestingly, these plants allocated more biomass belowground when fertilized with nitrogen. This may perhaps reinforce the evidence that alfalfa is not as dependent on phosphorus as nitrogen and that nitrogen acquisition is paramount to alfalfa performance (Undersander et al. 1991). Non-nutrient-amended plants displayed the greatest variation in biomass allocation ratios across symbiont treatments, with dual-inoculated plants allocating more biomass to shoots and single-symbiont plants allocating the most biomass belowground. The greatest allocation to aboveground tissues in dual-inoculation treatments is in agreement with other studies that find synergistic interactions between AMF and rhizobia on plant biomass in low-nutrient conditions (Abd-Alla et al. 2013, Ferrari and Wall 2008, Jia et al. 2004, Oliveira et al. 2005, Wang et al. 2011), illustrating perhaps the benefits to plants that associate with symbionts for nutrient acquisition. Relative to nutrient-amended plants, the single-symbiont, non-amended plants allocated more to root tissues, perhaps due to less nutrient receipt in comparison to amended counterparts and therefore leading to more investment in symbionts and/or root systems for sufficient nutrient acquisition. A fertilization experiment performed on field plantings of alfalfa found that in unfertilized conditions, plants allocated more belowground than their fertilized counterparts (Holechek 1982). Perhaps increased allocation belowground in the presence of one or no symbionts is a positively selected life history strategy of alfalfa.

Interestingly, plant phosphorus levels were only significantly meditated by phosphorus fertilization, while there was no significant influence of AMF on plant phosphorus content. Although greater plant phosphorus uptake under fertilization is expected, the absence of

significant effects of AMF on plant acquisition of phosphorus is in disagreement with many other studies that have shown the fungal symbiont to be beneficial to plant phosphorus content levels (Abd-Alla et al. 2013, Biro et al. 2000, Jia et al. 2004, Tajini et al. 2012, Wang et al. 2011, Xavier and Germida 2002). Also unexpected was the insignificant influence that symbiont and nutrient treatments had on plant protein content. While other studies have found that AMF and rhizobia, separately or inoculated together, increase plant nitrogen content (Grman and Robinson 2013, Tajini et al. 2012, Vazquez et al. 2001, Xavier and Germida 2002), or even nitrogen amendment in the presence of rhizobial-increased plant protein content (Vasileva and Athar 2013), my study did not find evidence of this. The study performed by Dean et al. (2014) observed that nitrogen fertilization did decrease plant dependence on rhizobia for nitrogen, but overall nitrogen content in the plant did not change. This was also supported by Oliveira et al. (2004), with their greenhouse-grown alfalfa showing no difference in total nitrogen content of plants between nitrogen-fertilized and non-fertilized plants. These results may illustrate that the domestication of alfalfa to a crop species may have reduced its dependency on microbial symbionts for nutrient acquisition. Several studies have purported that legume domestication has selected for decreased dependency of legumes on their symbiotic partners, as evidenced in reduced fixed N_2 rates by rhizobia and nitrogen assimilation from their symbiotic partners in comparison to wild genotypes or congeners (Provorov 1996, Provorov 2013, Provorov and Tikhonovich 2003, Rengel 2002). This is also congruent with the findings of Larimer et al. (2010), who illustrated via meta-analysis that only additive (not synergistic) effects were observed in dual-inoculation treatments, which they purported to be a result of over-representation of domesticated crop species in their analysis.

Expression of plant defense chemicals were significantly influenced by nutrient context. Protease inhibitor activity was found to be greater in phosphorus-amended plants compared with nitrogen- and non-amended plants. In contrast, POD expression was most influenced by nitrogen availability. In plants that were not inoculated with symbionts, non-amended and phosphorus-fertilized plants displayed greater POD activity in comparison to their AMF-inoculated counterparts. In addition, non-rhizobial, nitrogen-amended plants did not drastically alter their POD activity across AMF treatments. This may be indicative of a prophylactic response to nitrogen limitation. Similar findings have been observed for *Arabidopsis* under potassium limitation, as well as other response mechanisms to abiotic stress (Aremengaud et al. 2004, Aremengaud et al. 2010, Santino et al. 2013). Previous studies have shown that AMF also marginally function in nitrogen acquisition (Denison and Kiers 2011, Grman and Robinson 2013), so that inoculation with AMF may lead to reduced nitrogen limitation and subsequent decrease in POD activity. In contrast, non-amended and phosphorus-fertilized plants that were inoculated with rhizobia did not differ considerably in their POD activities between AMF treatments, highlighting that nitrogen acquisition was the main determinant in POD activity, which supports the putative nitrogen stress response. Interestingly, rhizobial plants that were amended with nitrogen had much lower POD activity upon inoculation with AMF relative to their non-AMF counterparts. This response does not seem in agreement with the purported prophylactic response hypothesis, but it may be explained by the fact that heightened nitrogen acquisition by plants causes nutrient imbalance and ultimately results in phosphorus limitation, eliciting an abiotic stress response. This response has been observed elsewhere (Tao and Hunter 2012). Further research utilizing a range of nutrient treatment concentrations, rather than just one

set concentration for each nutrient, should be conducted to determine if an interaction between symbiont and nutrient treatments is a significant predictor of foliar defense chemical expression.

Effects of Nutrient Context on Symbionts

There was no influence of nutrient treatment on AMF colonization, which deviated from expectations and previous studies as well (Collins and Foster 2009, Cornwell et al. 2001, Larimer et al. 2014, van der Heijden 2010). The observed result in this study may furthermore support that this study system is not wholly dependent upon AMF for optimal performance of an agricultural legume. Other studies have shown that biotic dependence on symbionts for nutrient acquisition is a highly selected character in prairie legumes (Larimer et al. 2014), and therefore plants do not decrease allocation to symbionts even when fertilized. In contrast, the use of alfalfa in this study, which exhibited no significant influence of symbiont treatments on plant nutrient content, perhaps further supports that domesticated crops have the unintended consequence of selecting for reduced dependency on microbial symbionts (Provorov 1996, Provorov 2013).

In plants that were inoculated with rhizobia, control-nutrient plants had a slightly greater number of nodules when also inoculated with AMF. The increase in nodule number under low-nutrient conditions with AMF coinfection has been observed in other studies (Abd-Alla et al. 2013, Larimer et al. 2014, Oliveira et al. 2005, Tajini et al. 2012, Wang et al. 2011) and can be explained by AMF supplementation to the high phosphorus demand that nodules have (Barea et al. 2005, Denison and Kiers 2011). This may also explain why the greatest number of nodules

were present in phosphorus-treated plants. Interestingly, phosphorus- and nitrogen-amended plants did not differ in nodule number between AMF treatments; also more nodules were present in nitrogen-amended plants than in non-amended ones. These results are in disagreement with previous findings that have purported that nitrogen fertilization negatively influences nodule abundance (Dean et al. 2014, Fujikake et al. 2002, Larimer et al. 2014, Sodek and Silva 1996, Vazquez et al. 2001) and that AMF can ameliorate negative effects of nitrogen fertilization on nodule abundance (Larimer et al. 2014). Heath et al. (2010) argued that the response of rhizobia to nitrogen fertilization is not a fixed and discretely negative response; rather, it falls along a spectrum and is influenced by multiple factors, such as nutrient context, partner identities, and even genotypes of the partners. In addition, while many studies utilize nodule counts as a proxy for rhizobial abundance and fitness (Biro et al. 2000, Dean et al. 2014, Heath and Tiffin 2009, Kempel et al. 2009, Larimer et al. 2014, Tajini et al. 2012), nodule number does not take into account the nitrogen fixing activity, and therefore the level of nitrogen provisioning, of nodules. With these speculations in mind, perhaps other measures should be employed to assess nutrient and AMF treatment influence on rhizobial fitness.

Surprisingly, there was also nodulation on non-rhizobial-inoculated plants. Other studies investigating legumes have also had nodulation in negative rhizobial controls as well (Kempel et al. 2009, Larimer et al. 2014, Wang et al. 2011). This may be an artifact of spontaneous nodulation, whereby nodules form despite the lack of rhizobia inoculant (Joshi et al. 1991, Joshi et al. 1993, Truchet et al. 1989) and have been observed not only in alfalfa, but other leguminous species as well (Blauenfeldt et al. 1994, Tirichine et al. 2006). Electron microcopy has confirmed spontaneous nodule structures to be identical in ultrastructure and histology to rhizobial-induced

nodules (Joshi et al. 1991, Joshi et al. 1993, Truchet et al. 1989), as well as display expression of nodulating genes such as ENOD2 (Bauer et al. 1994, de la Pena et al. 1997, Pichon et al. 1994, Truchet et al. 1989), which indicates plant influence over nodulation as well. One major putative theory behind nodulation in the absence of rhizobia in alfalfa purports that it is a vestigial trait, as evidenced by expression of genes and factors in alfalfa that occur without rhizobia infection (de la Pena et al. 1997, Joshi et al. 1991). Because spontaneous nodules often display large accumulation of starch granules (Blauenfeldt et al. 1994, Joshi et al. 1991, Joshi et al. 1993), proliferation of metabolically active vascular tissue into the nodules (Blauenfeldt et al. 1994, Joshi et al. 1993), such as are often exhibited under nitrogen-limiting conditions (Joshi et al. 1991, Pichon et al. 1994, Truchet et al. 1989), many have hypothesized that nodules are an ancestral form of carbon storage under times of abiotic stress (Blauenfeldt et al. 1994, Joshi et al. 1991, Joshi et al. 1993, Pichon et al. 1994, Truchet et al. 1989). This is also more often seen in small-seeded legumes, such as alfalfa or clovers, which, unlike large-seeded soybeans, do not cache carbon in foliar tissues (Blauenfeldt et al. 1994). In terms of molecular mechanisms of spontaneous nodules, recent studies have highlighted the role of receptor-like kinases and intracellular kinases in spontaneous nodulation. The overexpression of the epidermal receptors NFR1 and NFR5 on root cells (Ried et al. 2014), as well as overexpression of the intracellular domain of the SYMRK (Ried et al. 2014, Saha et al. 2014), have been shown to spurn hyper-activation of nodule organogenesis in the absence of rhizobia, regardless of the binding or presence of a signaling event.

Gram staining of a subset of non-rhizobial, nodulated plants found no evidence of Gram-negative bacteria and therefore indicated lack of rhizobial infection (Burdon 1946, Dreyfus et al.

1988). Interestingly, there was nodulation even under nitrogen amendment, which has been debated as an inhibitor of spontaneous nodulation in alfalfa (Hirsch et al. 1989, Tirichine et al. 2006, Truchet et al. 1989). In this experiment, nodules were observed in all non-rhizobial-treatment plants, and nodule counts in non-amended and nitrogen-amended plants were much lower in comparison when they were inoculated with AMF as well. This pattern was not observed in plants inoculated with rhizobia and perhaps illustrates that supplemented nutrient acquisition by AMF decreased the need for nodules as storage organs, since nutrient limitation was reduced. In contrast, nodule number was greater in AMF phosphorus-amended plants relative to non-AMF-inoculated ones. This may be indicative of nitrogen limitation as a consequence of high phosphorus availability, as has been illustrated in previous studies (Tao and Hunter 2012). This may therefore counter the hypothesis of nodules in alfalfa roots serving as storage organs under abiotic stress.

Spontaneous nodulation is an underappreciated phenomenon in legume biology and perhaps further indicates that nodule counts alone are not accurate representations of rhizobacteria abundance and fitness. Although nodule counts have been used by numerous other studies to quantify rhizobia abundance and performance (Biro et al. 2000, Dean et al. 2014, Heath and Tiffin 2009, Kempel et al. 2009, Larimer et al. 2014, Tajini et al. 2012), other quantitative methods, such as nodule mass (Abd-Alla et al. 2013, Bethlenfalvay et al. 1982, Ferrari and Wall 2008, Tajini et al. 2012), acetylene reduction (Abd-Alla et al. 2013, Barea et al. 2005, Ferrari and Wall 2008, Fujikake et al. 2002, Vazquez et al. 2001) and foliar amide and ureide content (Dean et al. 2014, Herridge et al. 1984, Sodek and Silva 1996), should perhaps be used instead.

There was no significant effect of nutrient treatment or symbiont treatments on larval mass. Although there were responses of constitutive defense chemicals to symbiont and/or nutrient treatments, there was no response of herbivores when feeding on foliar tissues of plants among these treatments. The findings of this experiment are in disagreement with previous studies, whereby microbial symbiont treatments were observed to have either negative effects on chewing-insect herbivore performance (Dean et al. 2014, Hartley and Gange 2009, Koricheva et al. 2009, Kula et al. 2005, Rabin and Pacovsky 1985) or positive effects on herbivore performance (Kempel et al. 2009, Vanette and Hunter 2013). However, differences in the influence of microbial mutualists on herbivore performance often depended upon the specialization of the feeding guild of the herbivore (Hartley and Gange 2009, Koricheva et al. 2009). While the meta-analysis conducted by Hartley and Gange (2009) observed generally negative influences of symbionts on herbivores, only about 54% of studies illustrated that generalist chewing herbivores were actually negatively affected. Koricheva et al. (2009) also observed that chewing herbivores were combined across different levels of specialization: chewers experienced no changes in mass, growth rate or development due to AMF influence, nor did these parameters change AMF influence on chewing herbivores due to manipulation of nitrogen and phosphorus fertilization, which is congruent with the results of this study.

Other studies have highlighted that the outcome of plant-insect interactions is highly species specific. For instance, Vanette et al. (2013) inoculated different milkweed species with the same community of AMF, which in turn elicited different responses in terms of plant

biomass and root and foliar defense chemical profiles. Similar findings were observed in the study by Roger et al. (2013), in which different genotypes of AMF mediated plant resistance and insect preference by a congener of *Spodoptera frugiperda*, and the meta-analysis by Koricheva et al. (2009) noted species-specific interactions as well. Others have postulated that pairwise interactions between mutualists may not always elicit net positive effects (Stanton 2003), as well as that microbial priming of induced or constitutive foliar defenses may not be great enough to deter insect predators (Koricheva et al. 2009), rather than pathogens (Dean et al. 2014, Pozo et al. 2008, Shantz et al. 2016). Therefore, perhaps the combination of symbionts in this study may not have acted in a synergistic or antagonistic manner to influence herbivore performance.

Conclusions

Results of this study indicated no support for the negative influence of nutrient loading on symbiont abundance and fitness, and as a corollary of that, no evidence of ameliorating effects of one symbiont on the other in dual-inoculation treatments. In addition, there was evidence of nodulation in the absence of rhizobial inoculation, which has been illustrated to be present in small-seeded legumes and indicative of possible spontaneous organogenesis under nutrient stress (Joshi et al. 1991, Truchet et al. 1989). This calls attention to the need to perhaps change the standard methods of quantifying rhizobial fitness and abundance by other means to avoid bias. Symbiont treatments did interact with nutrient treatments to influence differential biomass allocation across treatments, although these interactions were largely driven by non-nutrient-amendment plants receiving supplementation of nutrients via symbionts. Plants that were

amended with nutrients displayed very little change in allocation of biomass among symbiont treatments and may illustrate that the domestication of alfalfa has reduced its dependence on belowground mutualists for nutrient acquisition (Provorov 1996). Furthermore, plant phosphorus and protein content were found not to be dependent upon symbiont treatment, perhaps supporting the evolutionary loss of maintaining strong symbiotic relationships with belowground mutualists via domestication. Interesting future research should perhaps investigate the influence of host plant domestication on plant-microbial interactions. Last, there was no influence of nutrient and symbiont treatments on herbivore performance, despite significant differences in foliar defensive chemistry across treatments. Several studies have purported that microbial mediation of plant defensive chemistry may be species specific (Roger et al. 2013, Vanette et al. 2013), as well as herbivore response (Hartley and Gange 2009, Koricheva et al. 2009), therefore meriting further investigation into specific combinations of symbionts, plant hosts and insect herbivores to determine discrete ecological patterns.

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