Population level variation in host plant response to multiple symbionts

by

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A Thesis
presented to
the University of Guelph

In partial fulfillment of requirements
for the degree of
Master of Science
in
Integrative Biology

Guelph, Ontario, Canada

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ABSTRACT

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Multispecies symbiotic interactions are widespread in nature; however, few studies have explored the range of variation in symbiotic interactions within a species. One widespread multi-partner symbiosis takes place between legume plants and arbuscular mycorrhizal fungi (AM) fungi, and rhizobium bacteria. Because the nutrients provided by each symbiont are complementary, and the symbionts share a common genetic pathway to initiate the interaction, it is expected plant response to AM fungi and to rhizobia are positively correlated. I grew 35 populations of *Medicago truncatula* with or without the presence of each symbiont in a fully factorial experiment. I found populations varied in their growth response to AM fungi but not rhizobia, and the growth response to each symbiont was weakly positively correlated. The evolution of plant response to AM fungi may be more labile than plant response to rhizobia and not likely constrained by the evolution of the plant response to rhizobia.
ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Hafiz Maherali for his advice and guidance over the past two years. I would like to thank my advisory committee members Dr. Christina Caruso and Dr. Jonathan Newman for their valuable feedback; Tannis Slimmon and Mike Mucci for all their help with my experiment in the Guelph Phytotron. I would also like to thank all members of the Maherali-Caruso lab group, especially Josh Persi, Kendra Hockey, and Sarah Yepez who put in countless hours helping me collect my data.

I would to thank my parents Kim Drew and Ron Kilgour and all my brothers for their tremendous love and support. Finally, I would like to thank my partner Hayley Moriarity for always being there for me when I needed her during the ups and downs of this project.
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INTRODUCTION

Mutualistic interactions between species are widespread in nature. They are found in all ecosystems and include situations where resources and services are exchanged between partners, who each receive positive benefit (Douglas 2010). For example, flowering plants rely on insects and birds for pollination services in exchange for sugars from photosynthesis (Kiers et al. 2010). Similarly, many tree species depend on ants for protection from herbivores in exchange for shelter (Palmer et al. 2008). Because mutualistic interactions are common, they have evolutionary and ecological consequences (Douglas 2010). The eukaryotic cell, for instance, appears to have evolved from the cooperative interaction of two ancient prokaryotic cells (Bronstein 2015). Similarly, the association between mycorrhizal fungi and plants, allows plants to obtain normally inaccessible resources, and increases both individual and community productivity (Smith 2011).

Many of the most well studied mutualistic interactions take place between two partners (James et al. 1994; Nefdt and Compton 1996; Denison and Kiers 2011). Though these are often studied as pairwise interactions, a host can interact with multiple partners (Hunter 2006). These interactions can range from complementary to conflicting. Plants, for example, interact with mycorrhizal fungi in the soil as well as pollinators aboveground (Strauss and Irwin 2004). Specifically, colonization by mycorrhizal fungi increases flower number, size, and pollen production, which can increase pollinator visitation rates (Gange and Smith 2005; Wolfe et al. 2005). Not all interactions are complementary. Many plants rely on ants for defense from herbivores (Palmer et al. 2008), but this protection can be costly because the ants are frequently so aggressive that they attack potential pollinators as well as herbivores, which decreases pollination and seed production (Ohm and Miller 2014).
A special class of mutualisms are those that take place as symbiotic interactions. The relationship between plants and arbuscular mycorrhizal (AM) fungi for example, is considered a symbiotic interaction. Organism engaged in symbioses are more intimately linked than typical mutualisms. For example, when the host and symbiont are not symbiotic partners, one can allow the other partner to flee to escape conflict, whereas this may not be the case for a symbiosis (Bronstein 2015). While the ecological impact of many of these interactions is well understood, less is known about the evolution of multispecies symbiotic interactions (Bronstein et al. 2006).

The effects of multispecies symbiotic associations may conflict, be independent, or have a positive synergistic effect (Strauss and Irwin 2004). For instance, even though there may be conflict in multispecies interactions, natural selection may still favour a symbiosis because the benefits the host gains from these interactions are greater than the cost created by the conflicting interactions. As long as the ratio of the benefit to cost is positive, the interaction is under positive selection (Menzel et al. 2014). These symbiotic interactions may also be independent from one another. While a host can interact with multiple partners, these interactions may simply be two pairwise associations without influence on the other’s evolution (Nuismer and Doebeli 2004). If each association is independent, the effect of the multispecies symbiotic interaction would be equal to the sum of their individual effects (Strauss and Irwin 2004). Finally, multispecies symbiotic interactions can evolve because of positive synergistic effects. This means the effect of the multispecies interactions would be greater than the sum of their individual effects (Strauss and Irwin 2004).

One model system for studying the evolution of multispecies interactions is the tripartite interaction between legumes, arbuscular mycorrhizal fungi (AM fungi), and nitrogen fixing rhizobium bacteria (Denison and Kiers 2011). Legumes, like the majority of land plants, are able
to form symbiotic relationships with AM fungi (Wang and Qiu 2006). This symbiotic interaction enables legumes to obtain recalcitrant nutrients, such as phosphorus, from soils (Smith et al. 2009). In exchange for these nutrients, plants provide fungi with sugars from photosynthesis (Smith et al. 2009). Legumes are unique as they are also able to form symbioses with rhizobia. Approximately 90% of legume species form symbioses with rhizobia (Brewin 2004). These soil bacteria have the capacity to fix atmospheric nitrogen, and in return, legumes provide bacteria with sugars from photosynthesis which covers the energetic cost of nitrogen fixation (Zahran 1999). Globally, symbiotic interactions between cultivated legumes and rhizobia contribute approximately 25 Tg of N year\(^{-1}\) of the total 107 Tg N year\(^{-1}\) of biologically fixed nitrogen (Galloway et al. 2004).

The tripartite interaction between legumes, AM fungi, and rhizobia may have evolved because it enables plants to obtain two complementary resources, phosphorus and nitrogen, which are often growth limiting (Sardans et al. 2011; Ågren et al. 2012; Ossler et al. 2015). Resource-based symbiotic interactions can also enhance plant demand for other resources. For example, the symbiosis with AM fungi, by alleviating phosphorus limitation, can cause plant growth to become nitrogen limited (Johnson et al. 2010). To alleviate this nitrogen limitation, the evolution of the symbiotic interaction between AM fungi and legumes may have caused natural selection for legumes to form a symbiotic relationship with the nitrogen-fixing bacteria, rhizobia. If the evolution of this multispecies interaction is due to the complementarity of the nutrients AM fungi and rhizobia provide, there may be simultaneous selection on each symbiont. One possible way rhizobia could have begun interacting with legumes is by utilizing the pre-existing symbiotic pathway AM fungi use to establish the symbiotic interaction (Oldroyd 2013). Multiple genetic studies of legumes have found the establishment of both symbiotic relationships utilize
many of the same genes (Ané et al. 2004; Oláh et al. 2005; Horváth et al. 2011). The shared genetic pathway suggests the interaction between the two symbionts may be genetically correlated. If natural selection was to act upon one trait the genetically correlated trait would also evolve.

Despite evidence for the nutritional complementarity of the two symbiotic partners (Ossler et al. 2015), a quantitative meta-analysis by Larimer et al. (2010) found no overall positive synergistic effect between AM fungi and rhizobia. Even though the overall effect size was non-significant, Larimer et al. (2010) note that the studies that reported a positive synergistic effect occurred most frequently in environments where both nutrients were limiting. More recently published studies have also reported that co-infection by these microbes has a positive synergistic impact on plant growth (Abd-alla et al. 2014; Larimer et al. 2014; Afkhami and Stinchcombe 2016). If the symbionts have no impact on each other and have an additive effect on the legume, the evolution of these pairwise interactions are expected to be independent of one another. However, if plant growth is greater than the sum of the individual effects when simultaneously infected by both AM fungi and rhizobia due to the nutrient complementarity of symbionts alleviating the stoichiometric nutrient limitations, then the evolution of plant response to AM fungi and rhizobia may be correlated.

The objective of this study was to identify factors that could influence the evolution of plant response to multispecies symbiotic interactions. To do this, I used the Mediterranean annual legume *Medicago truncatula* (Barrel medic), that interacts with AM fungi and rhizobia (Javot et al. 2007; Fox et al. 2011). Because of its short life cycle and small genome, this legume has become a model system for studying plant-microbial symbiotic interactions (Young and Udvardi 2009). The evolution of these interactions may be due to a complementarity of the
nutrients the two symbionts provide or a common genetic pathway. To determine if plants simultaneously interacting with both symbionts have an additive or positive synergistic growth response I surveyed 35 populations of *M. truncatula*. Each population is quite genetically homogenous, therefore when I refer to population I am referring to a genotype. Due to the complementarity of the nutrients provided by the two microbes, I predicted plants simultaneously interacting with AM fungi and rhizobia would have positive synergistic effect on the growth of the legumes. To test the hypothesis that plant response to AM fungi is correlated with the response to rhizobia, I surveyed the multispecies symbiotic responsiveness of the same 35 populations of *M. truncatula*. Due to the shared genetic pathway, I predicted populations that have a positive growth response to one symbiotic partner will respond positively to the other symbiotic partner. Finally, I tested the hypothesis that rhizobia root colonization is correlated with AM fungi root colonization. Due to the rhizobia providing excess nitrogen and therefore increasing the demand for phosphorus, I predicted plants inoculated with rhizobia would have a greater proportion of AM root colonization than plants only inoculated with only AM fungi.
METHODS

To determine if simultaneously interacting with AM fungi and rhizobia had a positive synergistic growth effect on *M. truncatula*, as well as determine if there was a correlation between the benefits AM fungi and rhizobia provide, I grew 35 populations of *M. truncatula* in a greenhouse at the University of Guelph Phytotron (Guelph, ON, Canada). All 35 populations were randomly selected and obtained from the United States Department of Agriculture (USDA) GRIN system (http://www.ars-grin.gov). Each population was grown with or without the presence of each symbiont in a 2×2 fully factorial experiment. The plants were grown in 5 temporal blocks, one week apart. The total number of plants grown in this experiment was (35 populations × 4 treatments combinations × 5 blocks) = 700 plants.

Field soil used throughout this experiment was collected from the Guelph Arboretum (Guelph, Ontario 43°32’08.5”N, 80°12’41.9”W). The positive synergistic effect of co-inoculation is found most frequently in environments where nitrogen and phosphorus are limiting (Larimer et al. 2010). Soil from the Guelph Arboretum naturally has a low concentration of phosphorus (2.1 mg P/kg soil), nitrate (3.3 mg NO3/kg soil), and ammonium (2.9 mg NH4 /kg soil) (Sherrard and Maherali 2012). Low nutrient soil was used because it had previously been shown to induce increased plant response to AM fungi and rhizobia (Fox et al. 2011; Watts-Williams et al. 2015).

To set up the experiment, 12 seeds per population were scarified with 220 grit sandpaper, to encourage germination. Next, the seeds were surface sterilized by placing them in 50% ethanol for 30 seconds followed by 3 washes in DI H₂O. The seeds were germinated in Petri plates in the dark at 4 °C for 48 hours and then at 23°C for 24 hours. Three seedlings from each population were selected haphazardly and transplanted to 656ml Deepots (D40 Stuewe & Sons,
Oregon, USA), and thinned to one plant per pot after one week of growth. The Deepots were filled with 600 mL of 1:1 non-calcareous granitic sand (Hutcheson Sand and Mixes, Huntsville, ON, Canada) and field soil. To remove any microbial contaminants, the mixture was autoclaved (121°C for 90 minutes) twice. To ensure the pots retained the mixture, the pots were lined with 3cm² window screen, sterilized in 5% bleach for 10 minutes. To increase aeration, this medium was mixed 4:1 with steam sterilized perlite (Therm-O-Rock West, Inc). Finally, the pots were randomly distributed onto two greenhouse benches.

Plants were grown in 16 hours of light at a daytime temperature of 20.5-23.5°C, and 8 hours of dark at a nighttime temperature of 19.5-20.5°C. Artificial lights were used in the morning, evening, and on cloudy days when the light intensity dropped below 300 umol/m²/s. By week two, many plants began to show signs of nutrient stress. To ensure plants had adequate nutrients, they were fertilized with 50 mL of ¼ strength fertilizer, 200ppm 18/9/18, on weeks 3, 5, and 7. Plants were watered every day; alternating between completely saturating the soil (~50mL) and misting each plant, except for days when fertilizer was applied. Throughout the experiment 6 plants died, and were not replaced.

Legumes in the genus Medicago symbiotically interact with the same species of rhizobia, *Ensifer medicae* (Garau et al. 2005). *Medicago lupulina* is closely related to *M. truncatula* and both species are compatible with *Ensifer medicae* (Larrainzar et al. 2014; Simonsen et al. 2015). I isolated rhizobia from root nodules of *M. lupulina*. To obtain nodules I grew 10 *M. lupulina* plants from seeds collected in Southern Ontario, Canada. The seeds were grown in soil collected from Kortright Rd W, Guelph, Ontario (43°31’05.4”N, 80°12’52.2”W), known to have *M. lupulina* with established rhizobia nodules on their roots. This soil was mixed 1:1 with autoclaved non-calcareous granitic sand in order to reduce the nutrient concentration and
encourage nodulation. Plants were grown in the University of Guelph Phytotron (Guelph, ON, Canada) for 67 days under the same conditions previously mentioned.

The bacteria were isolated from the root nodules using the method found in (Somasegaran and Hoben, 1994). The root nodules were cut open and spread on a yeast mannitol agar plate. Once distinct colonies were established, they were used to inoculate tryptone yeast media (TY media). This media was constructed using tryptone, yeast extract, and calcium chloride from (BioShop Canada Inc, Burlington, ON, Canada) and was constructed using the instructions described by Somasegaran and Hoben, 1994. The inoculated media was incubated at 28°C for 48 hours, and rotated at 120 RPM. Before inoculating plants, inoculum was diluted with sterile DI water to a cell density of ~8 × 10^6 cells (based on a OD_{600}). Plants which received the rhizobia treatment were inoculated with 1 mL of the inoculum 48 hours and one week after germination. Plants which did not receive the rhizobia inoculum were given two treatments of 1 mL sterile TY media.

To identify the isolated strain of rhizobia I inoculated TY media with a single colony of rhizobia and incubated it at 28°C for 48 hours, rotating at 120 rpm. Bacterial cells were isolated by centrifuging 1 mL of media at 10,000 rpm for 2 minutes. DNA was isolated from the resulting pellet using the NucleoSpin Plant II kit (Macherey-Nagel). The extracted DNA was PCR amplified at the Advanced Analysis Centre Genomics Facility (http://www.uoguelph.ca/aac/). Two housekeeping genes, dnaK and pnp, were amplified using the primers dnaK 1466F (5’-AAGGARCANCAGATCCGCATCCA-3’), dnaK1777R (5’-TASATSGCCTSRCRAGCTTCAT-3’), pnp913F (5’-AAGRTCGBKGCCTGGAAC-3’), and pnp1473R (5’-ACCTTGAAGTCCATRTCG-3’) respectively (Martens et al, 2007, 2008). PCR conditions were 2 minutes at 96°C, followed by 30 cycles of 96°C for 30 seconds, 56°C for 15
seconds for dnaK and 45°C for 15 seconds for pnp, 60°C for 4 minutes, and held at 10°C. The sequences were compared to the available public nucleotide database using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The isolated strain of rhizobia was *Ensifer medicae* WSM419.

The isolate of AM fungi used throughout the study was *Rhizophagus intraradices*. The commercial inoculum (Myke Pro Greenhouse WP) with a spore count of 1600 spores per gram, was obtained from a commercial supplier (Premier Tech Agriculture, Rivere-du Loup, QC, Canada). Two days before planting the seeds, the two treatments receiving AM fungi had the top 300 mL of soil mixed with 1.5 g of the inoculant powder. Mixing the powder with the top layer of soil reduced the chance of the spores being disturbed during watering. Non-AM fungi inoculated treatments did not receive additional soil because the volume of AM fungi inoculant was negligible.

After 8 weeks, the plants were harvested. Plant aboveground biomass was separated from the root system, placed into a 3l paper bag (Guelph Paper Company, Guelph, ON, CANADA), dried at 60°C for 48 hours, and then weighed. To determine if rhizobia inoculation affected leaf nitrogen content I measured leaf chlorophyll content, which is associated with leaf nitrogen content (Chapman and Barreto 1997). Before drying the plants, I randomly selected three leaves from each plant and measured their chlorophyll content using a SPAD-502 leaf chlorophyll meter. Finally, plant roots were washed of the sand soil mixture and preserved in 50% ethanol for later colonization measurements.

To evaluate root colonization by *E. medicae* I counted the number of nodules per plant. The root systems of plants in rhizobia only treatment were small enough that the total number of nodules per plant could be counted. However, the root systems of AM fungi and rhizobia treated
plants were large and the number of nodules too high to count, therefore, 3 randomly selected secondary roots were chosen and the total number of nodules of these sections were counted. The total root length of these root systems was determined using a high-resolution scanner (6,400 dpi). Root images were analyzed with WinRhizo software (version 2009a; Reagent Instruments 2009) at a dpi of 600. The number of nodules per unit length of root was then calculated. Using nodules per length of root allowed me to compare the total number of nodules of whole plants to sub-selected root sections. Control plants and those treated with only AM were checked for nodules and scanned to determine if any rhizobia contamination occurred. Like rhizobia treated plants, the entire root system of the control plants was scanned while 3 randomly selected secondary roots of AM only treated plants were checked and scanned. Population 49 was removed from the analyses due to a high level of contamination of control and AM fungi inoculated only plants.

I measured percent root colonization by AM fungi of 3 randomly selected blocks of AM and AM/rhizobia inoculated plants and 10 randomly selected plants from each non-AM inoculated treatment. To measure colonization, I randomly selected 15 1-cm segments of root per plant and then cleared them in 10% KOH 90°C for 5 minutes. The roots were stained with an ink/vinegar mixture and then mounted on glass microscope slides (Vierheilig et al. 1998). The percentage of colonization was estimated using presence/absence of colonization using a gridline intersect method (McGonigle et al. 1990). The fungal structures used to determine the percentage of colonization were arbuscules and vesicles.

There were low levels of rhizobia contamination found on control and AM fungi inoculated plants. On average control plants had 1.75 nodules per plants while plants inoculated with AM fungi had 3.77 nodules per plants. For comparison, plants inoculated with rhizobia had
14.14 nodules per plant while plants inoculated with AM fungi and rhizobia had 55.80 nodules/plant. No AM fungal contamination was detected throughout this experiment.

To evaluate whether aboveground biomass responded to the presence or absence of rhizobia, the presence or absence of AM fungi, and population as factors, I used a three-way ANOVA. Aboveground biomass was log transformed to meet the homogeneity of variance assumptions of ANOVA. I reported unadjusted means but used adjusted means to determine if populations differed. To determine whether the response to AM fungi or rhizobia varied among populations I examined the Population × AM fungi and Population × Rhizobia interactions in the three-way ANOVA. To evaluate if the aboveground biomass of each population differed with and without the presence of AM fungi I compared them using 95% confidence intervals.

The responsiveness of *M. truncatula* to each symbiotic interaction and their combined effects was calculated as the log response ratio of inoculated plants / non-inoculated plants (ln (inoculated plant’s biomass / non-inoculated plant’s biomass)). If the response ratio was greater than zero, the plant responded positively to the inoculation (Maherali 2014). I used a Spearman’s correlation to determine if log response ratio of plants inoculated with AM fungi was positively correlated with the log response ratio of plants inoculated with rhizobia. Finally, to determine whether the biomass of plants in the absence of AM inoculation is correlated with the biomass of plants inoculated with AM I used a spearman rank correlation.

To evaluate if leaf chlorophyll was affected by the presence of either symbiont I used a three-way ANOVA with the presence of each symbiont and population as factors. Plant biomass was used as a covariate in this analysis to determine if any differences in leaf chlorophyll was simply the result of plant size. To determine if the presence of one symbiont affects the colonization of the other I used a two-way ANOVA with the presence of the symbiont and
population as the factors. Nodule/cm was log transformed to meet homogeneity of variance assumptions of ANOVA. I reported unadjusted means but used adjusted means to determine if populations differed. To evaluate whether the number nodules, arbuscules, or vesicles varied among populations I used a two-way ANOVA with the presence of the symbiont and population as the factors. Linear regression was used to determine if there was a relationship between aboveground shoot biomass and nodules/cm, proportion of roots colonized by arbuscules or vesicles. Finally, linear regression was used to determine whether there was a relationship between rhizobia colonization and AM fungi colonization. While ANOVAs were performed on natural log transformed data, values in figures were back-transformed.
RESULTS

The aboveground biomass of *M. truncatula* increased with the inoculation of rhizobia and AM fungi. Aboveground biomass increased 18.8x in response to AM fungi colonization but only 1.2x in response to rhizobia colonization. The aboveground biomass of plants inoculated with both symbionts increased 20.1x in response to colonization. However, the biomass of AM fungi and rhizobia inoculated plants did not differ from the biomass of plants inoculated with only AM (Fig. 1a). While both symbiotic partners had a positive effect on the aboveground biomass of *M. truncatula*, the effect of rhizobia depended on the level of AM inoculation. Inoculation with rhizobia increased aboveground biomass 21%. However, when plants were also inoculated with AM fungi the effect of rhizobia only increased aboveground biomass by 7% (Fig. 1a).

The magnitude of the aboveground biomass response to AM fungi inoculation differed among populations (Fig. 1b, Table 1). However, there was more variation in the mean aboveground biomass among populations in the absence of AM fungi than in the presence of AM fungi. For example, aboveground biomass of plants without AM fungi ranged from 0.021 g to 0.069 g (Fig. 2a), while the aboveground biomass of plants with AM fungi ranged from 0.419 g to 0.946 g (Fig. 2b). Aboveground biomass response ratio of plants inoculated with AM fungi ranged from 8.09 to 30.75 (Fig. 3a); data in figure was natural log transformed. The coefficient of variation of aboveground biomass of plants inoculated with AM was 19.92 while plants without AM had a coefficient of variation of 29.78. I found biomass in the absence of AM weakly predicts the response to AM ($r = 0.365$, $P = 0.034$, Fig. 3b). While the response of aboveground biomass to AM fungi inoculation varied among populations, the response of aboveground biomass to inoculation with rhizobia did not differ among populations (Fig. 1c, Table 1). Aboveground biomass response ratio of plants inoculated with rhizobia ranged from
0.88 to 1.65 (Fig. 3a). Finally, my results indicate there was a weak positive correlation between the response to AM fungi and rhizobia ($r = 0.376$, $P = 0.028$, Fig. 3a).

The relationship between nodule/cm and aboveground biomass depended on the presence of AM fungi. There was no significant relationship between the number of nodules/cm and aboveground biomass for plants only inoculated with rhizobia ($F_{1, 32} = 0.313$, slope = 0.118, $P = 0.580$, Fig. 4a). However, there was a significant negative relationship between nodules/cm and aboveground biomass for plants inoculated with both rhizobia and AM fungi ($F_{1, 32} = 8.596$, slope = -1.043, slope = $P = 0.006$, Fig. 4b). While the number of nodules/cm had a negative association with aboveground biomass, plants inoculated with AM fungi and rhizobia had 3.74x nodules/cm than plants only inoculated with rhizobia (Fig. 5a, Table 2). The nodules/cm of plants inoculated with AM fungi differed among populations (Fig. 6a, Table 2). There was more variation in the mean number of nodules/cm among populations in the presence of AM fungi than in the absence of AM fungi. The number of nodules/cm of plants without AM ranged from 0.007 nodules/cm to 0.06 nodules/cm (Fig. 7a), while the number of nodules/cm of plants inoculated with AM fungi ranged from 0.02 nodules/cm to 0.25 nodules/cm (Fig. 7b). Finally, the leaf chlorophyll content of plants inoculated with AM fungi and rhizobia was 13.4% greater than plants inoculated with only AM fungi (Fig. 8, Table 3).

The proportion of roots colonized by arbuscules was 15.6% higher in plants inoculated with rhizobia and AM fungi than plants inoculated with only AM fungi (Fig. 5b, Table 4). The presence of rhizobia did not affect whether the proportion of roots colonized by arbuscules differed among populations (Fig. 6b). The proportion of roots colonized by arbuscules without rhizobia ranged from 29% to 75% (Fig. 9a), while the proportion of roots inoculated with rhizobia ranged from 34% to 71% (Fig. 9b). While the roots of plants inoculated with both
symbionts had a greater proportion of arbuscules, I found no relationship between aboveground biomass and the proportion of arbuscules in the absence of rhizobia (F_{1,32} = 2.885, slope = 0.005, \( P = 0.099 \), Fig. 10a) or in the presence of rhizobia (F_{1,32} = 0.316, slope = 0.002, \( P = 0.0578 \), Fig. 10b).

The proportion of roots colonized by vesicles did not differ between plants inoculated with rhizobia and AM fungi and plants inoculated with only AM fungi (Table 4). The proportion of roots colonized by vesicles did not differ among populations (Fig. 6c). No significant relationship was detected between aboveground biomass and the proportion of roots colonized vesicles without (F_{1,32} =2.465, slope = 0.003, \( P = 0.126 \), Fig. 10c) or with rhizobia (F_{1,32} =0.469, slope = 0.002, \( P = 0.498 \), Fig. 10d). There was no relationship between the proportion of arbuscules and nodules/cm (F_{1,32} = 2.463, slope = 38.154, \( P = 0.126 \), Fig. 10e). There was no relationship between the proportion of vesicles and nodules/cm (F_{1,32} = 0.297, slope = 12.763, \( P = 0.589 \), Fig. 10f).
Table 1: Three-way ANOVA on natural log transformed aboveground biomass of *Medicago truncatula* using the factors: AM fungi (AM), Rhizobia (Rhz), Population (Pop), and Block. Table includes degrees of freedom (df), mean square (MS), F-statistic ($F$), and $P$-value.

<table>
<thead>
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<th>Trait Term</th>
<th>df</th>
<th>MS</th>
<th>$F$</th>
<th>$P$</th>
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<td>1385.107</td>
<td>9967.855</td>
<td>&lt; $1 \times 10^{-99}$</td>
</tr>
<tr>
<td>Rhz</td>
<td>1</td>
<td>2.873</td>
<td>20.675</td>
<td>7.000 $\times 10^{-06}$</td>
</tr>
<tr>
<td>Pop</td>
<td>33</td>
<td>0.822</td>
<td>5.914</td>
<td>1.520 $\times 10^{-20}$</td>
</tr>
<tr>
<td>AM * Pop</td>
<td>33</td>
<td>0.396</td>
<td>2.851</td>
<td>4.692 $\times 10^{-07}$</td>
</tr>
<tr>
<td>AM * Rhz</td>
<td>1</td>
<td>0.673</td>
<td>4.843</td>
<td>0.028</td>
</tr>
<tr>
<td>Rhz * Pop</td>
<td>33</td>
<td>0.076</td>
<td>0.548</td>
<td>0.982</td>
</tr>
<tr>
<td>AM * Rhz * Pop</td>
<td>33</td>
<td>0.056</td>
<td>0.406</td>
<td>0.999</td>
</tr>
<tr>
<td>Block</td>
<td>4</td>
<td>0.984</td>
<td>7.08</td>
<td>1.500 $\times 10^{-05}$</td>
</tr>
<tr>
<td>Error</td>
<td>534</td>
<td>0.139</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2: Two-way ANOVA on natural log transformed nodules/cm of *Medicago truncatula* roots using the factors: AM fungi (AM), Population (Pop), and Block. Table includes degrees of freedom (df), mean square (MS), F-statistic (F), and P-value.

<table>
<thead>
<tr>
<th>Trait Term</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM</td>
<td>1</td>
<td>145.53</td>
<td>438.606</td>
<td>$4.762 \times 10^{-58}$</td>
</tr>
<tr>
<td>Pop</td>
<td>33</td>
<td>1.547</td>
<td>4.661</td>
<td>$4.053 \times 10^{-13}$</td>
</tr>
<tr>
<td>Pop * AM</td>
<td>33</td>
<td>1.09</td>
<td>3.285</td>
<td>$4.671 \times 10^{-08}$</td>
</tr>
<tr>
<td>Block</td>
<td>4</td>
<td>2.175</td>
<td>6.556</td>
<td>$4.800 \times 10^{-05}$</td>
</tr>
<tr>
<td>Error</td>
<td>264</td>
<td>0.332</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3: Three-way ANOVA on leaf chlorophyll of *Medicago truncatula* using the factors: AM fungi (AM), Rhizobia (Rhz), Population (Pop), and Block. Aboveground biomass was used as a covariate. Table includes degrees of freedom (df), mean square (MS), F-statistic (F), and P-value.

<table>
<thead>
<tr>
<th>Trait Term</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf chlorophyll</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>1</td>
<td>1255.47</td>
<td>34.379</td>
<td>$7.836 \times 10^{-09}$</td>
</tr>
<tr>
<td>Rhz</td>
<td>1</td>
<td>2772.708</td>
<td>75.926</td>
<td>$3.481 \times 10^{-17}$</td>
</tr>
<tr>
<td>Pop</td>
<td>34</td>
<td>362.524</td>
<td>9.927</td>
<td>$1.292 \times 10^{-38}$</td>
</tr>
<tr>
<td>AM * Pop</td>
<td>34</td>
<td>104.685</td>
<td>2.867</td>
<td>$2.778 \times 10^{-07}$</td>
</tr>
<tr>
<td>AM * Rhz</td>
<td>1</td>
<td>147.705</td>
<td>4.045</td>
<td>$0.045$</td>
</tr>
<tr>
<td>Rhz * Pop</td>
<td>34</td>
<td>49.906</td>
<td>1.367</td>
<td>$0.084$</td>
</tr>
<tr>
<td>AM * Rhz * Pop</td>
<td>34</td>
<td>56.482</td>
<td>1.547</td>
<td>$0.027$</td>
</tr>
<tr>
<td>Block</td>
<td>4</td>
<td>391.459</td>
<td>10.72</td>
<td>$2.263 \times 10^{-08}$</td>
</tr>
<tr>
<td>Aboveground biomass</td>
<td>1</td>
<td>497.57</td>
<td>13.625</td>
<td>$2.450 \times 10^{-04}$</td>
</tr>
<tr>
<td>Error</td>
<td>549</td>
<td>36.518</td>
<td></td>
<td></td>
</tr>
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</table>
Table 4: Two-way ANOVA on AM fungi colonization traits of *Medicago truncatula* using factors: Rhizobia (Rhz), Population (Pop), and Block. Table includes degrees of freedom (df), mean square (MS), F-statistic (F), and P-value.

<table>
<thead>
<tr>
<th>Trait Term</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhz</td>
<td>1</td>
<td>2715.523</td>
<td>17.718</td>
<td>4.700 × 10^{-5}</td>
<td>1</td>
<td>0.058</td>
<td>0</td>
<td>0.985</td>
</tr>
<tr>
<td>Pop</td>
<td>33</td>
<td>302.911</td>
<td>1.976</td>
<td>0.004</td>
<td>33</td>
<td>389.864</td>
<td>2.405</td>
<td>2.400 × 10^{-4}</td>
</tr>
<tr>
<td>Rhz * Pop</td>
<td>33</td>
<td>166.473</td>
<td>1.086</td>
<td>0.361</td>
<td>33</td>
<td>187.337</td>
<td>1.156</td>
<td>0.279</td>
</tr>
<tr>
<td>Block</td>
<td>2</td>
<td>172.488</td>
<td>1.125</td>
<td>0.328</td>
<td>2</td>
<td>788.002</td>
<td>4.861</td>
<td>0.009</td>
</tr>
<tr>
<td>Error</td>
<td>132</td>
<td>153.26</td>
<td></td>
<td></td>
<td>132</td>
<td>162.091</td>
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Figure 1: The response of mean (± 1 SE) aboveground biomass to AM fungal and rhizobium inoculation, and their interaction (A), reaction norms depicting the effect of AM fungal inoculation (B), and rhizobia inoculation (C) on aboveground biomass of 34 populations of *Medicago truncatula*. Letters indicate differences in aboveground means (P-value <0.05).
Figure 2: The response of mean (± 1 SE) aboveground biomass of 34 populations of *Medicago truncatula* to all AM fungal inoculation treatments (A) and non-AM fungal inoculation treatments (B). Horizontal line indicates mean aboveground biomass. Note differences in scales between A and B.
Figure 3: Scatterplot of the plant growth response ratios to AM fungal inoculation vs plant growth response ratios to rhizobium inoculation (A) and aboveground biomass and the relationship between plant growth in the absence of AM fungi vs with AM inoculation (B). Each data point represents one population of *Medicago truncatula*. A line denotes a significant relationship.
Figure 4: Scatterplot of nodules/cm vs aboveground biomass of rhizobia only inoculated plants (A), AM fungi and rhizobia inoculated plants (B). Each data point represents one population of *Medicago truncatula*. A line denotes a significant relationship.
Figure 5: The response of mean (± 1 SE) nodules/cm to AM fungal inoculation (A). The response of mean (± 1 SE) of proportion of roots colonized by arbuscules to rhizobium inoculation (B).
Figure 6: Reaction norm depicting the effect of AM fungi on number of nodules/cm (A), the effect of rhizobia inoculation on proportion of roots colonized by arbuscules (B), and vesicles (C) of 34 populations of *Medicago truncatula*. 
Figure 7: The response of mean (± 1 SE) nodules/cm of 34 populations of *Medicago truncatula* to rhizobium inoculation (A) and AM fungal and rhizobium inoculation (B). Horizontal line indicates mean aboveground biomass. Note differences in scales between A and B.
Figure 8: The response of mean (± 1 SE) leaf chlorophyll content of *Medicago truncatula* to AM fungal inoculation and AM fungal and rhizobia inoculation.
Figure 9: The response of mean (± 1 SE) roots colonized by arbuscules of 34 populations of *Medicago truncatula* to AM fungal inoculation (A) and AM fungal and rhizobium inoculation. Horizontal line indicates mean aboveground biomass. Note differences in scales between A and B.
Figure 10: Scatterplot of aboveground biomass vs proportion of roots colonized by arbuscules of plants inoculated with only AM fungi (A) and AM fungi and rhizobia (B). Aboveground biomass vs proportion of roots colonized by vesicles of plants inoculated with only AM fungi (C) and AM fungi and rhizobia (D). Nodules/cm vs proportion of roots colonized by arbuscules (E) and vesicles (F). Each dot represents one population of *Medicago truncatula*.
DISCUSSION

My results did not support the hypothesis that AM fungi and rhizobia would have a positive synergistic effect on plant growth. The effect of rhizobia depended on the level of AM inoculation, but the effect was not in the predicted direction. Rhizobia alone increased plant growth by 21%, whereas when plants were also inoculated with AM fungi, rhizobia only increased plant growth by 7%. Thus, AM fungi did not enhance the effect of rhizobia on plant growth. While I found no synergism between the two microbial symbionts, growth response to AM fungi and rhizobia was weakly positively correlated among populations. Thus, despite the evidence for the common genetic pathway (Oldroyd 2013), the association between AM fungi and rhizobia is weak. In addition, I observed the interactions between the three partners in this symbiosis were asymmetric. Both microbial partners had a positive effect on plant growth, but plant growth increased by 1780% when inoculated with AM fungi, whereas plant growth increased by only 21% when inoculated with rhizobia. The presence of rhizobia had a positive effect on AM fungal colonization, increasing the proportion of roots colonized by arbuscules by 15.6%. However, the presence of AM fungi had a larger effect on rhizobia root nodulation, increasing nodules/cm by 274%.

The lack of synergism is consistent with a recent meta-analysis on the same tripartite system which found that while both AM fungi and rhizobia increase plant growth, the overall effect of dual inoculation is not synergistic (Larimer et al. 2010). However, a recent study by Afkhami and Stinchcombe (2016) found these microbial partners interact to increase plant growth of *M. truncatula*. The synergistic effects detected in this previous study may not be representative of the entire species, and may instead be caused by the plant genotype used in the study. The previous study on the synergistic effects of these symbionts only used a single plant
genotype. While several populations used in my study appeared to respond synergistically to dual inoculation, many other populations did not respond this way, and the overall effect was not significant. For example, rhizobia inoculation alone did not significantly affect aboveground biomass of population PI660383, however when plants were also inoculated with AM fungi, rhizobia increased aboveground biomass by 53% (AM*Rhz $P=0.046$). It is possible if I had only selected population PI660383 I would have erroneously concluded; the AM fungi and rhizobia are synergistic.

Another explanation for the lack of synergism is AM fungi may not specialize solely in phosphorus acquisition. AM fungi can be responsible for the transfer of approximately 21% to 75% of nitrogen to plants depending on the species (Tian et al. 2010). Therefore, it is possible the strong growth effect of AM fungi was caused by AM fungi providing nitrogen and phosphorus. However, plants inoculated with AM fungi and rhizobia had higher leaf chlorophyll content than plants only inoculated with AM fungi. Leaf chlorophyll has been shown to be positively correlated with leaf nitrogen (Chapman and Barreto 1997). The greater chlorophyll content of plants inoculated with both symbionts indicates the rhizobia were providing the plant with additional nitrogen. This increase in nitrogen did not translate to a significant increase in aboveground biomass for plants inoculated with AM fungi and rhizobia compared to plants inoculated with AM fungi alone. Other indicators of plant performance such as pollen grain production, are very nitrogen demanding (Lau and Stephenson 1993). It is possible I would have seen a synergistic effect if I had measured this parameter as well.

Overall, *M. truncatula* responded weakly to rhizobia inoculation. Though it is possible that the strain I used, *Ensifer medicae*, WSM419, was incompatible with *M. truncatula*, this is unlikely because it has been previously shown to be highly effective at N$_2$ fixing with *M.*
truncatula (Terpolilli et al. 2008; Larrainzar et al. 2014). While this strain may be effective at fixing \( \text{N}_2 \) with *M. truncatula*, the low concentration of phosphorus in the soil may have reduced the effect rhizobia had on plant growth. Previous studies have shown plants inoculated with rhizobia in soil with low levels of phosphorus have smaller and fewer nodules with a decreased ability to fix nitrogen (Hogh-Jensen et al. 2002; Rotaru and Sinclair 2009; Cabeza et al. 2014).

My plants, which were grown in soil with a low concentration of phosphorus, had reduced root nodulation compared to plants inoculated with AM fungi and rhizobia. Nodulation increased 274% when plants were also inoculated with AM fungi, suggesting the rhizobia are acquiring some of the phosphorus supplied to the plants by the AM fungi. If rhizobia are acquiring some of the phosphorus I expect if plants only inoculated with rhizobia were fertilized with phosphorous this would be accompanied by an increase in nodulation. Furthermore, my results suggest in low phosphorous soil, rhizobia are much more dependent on the presence of AM fungi.

Despite this increase in nodulation in the presence of AM fungi, the effect of rhizobia on plant growth in this treatment was weaker than in the absence of AM fungi. In addition, when plants were also inoculated with AM fungi I found a negative relationship between nodules/cm and aboveground biomass. This negative correlation is consistent with previous studies on *M. truncatula* and rhizobia which also found a negative correlation between nodule number and plant fruit number (Heath and Tiffin 2007; Heath 2010). Plants inoculated with AM fungi and rhizobia were smaller when they were heavily nodulated, implying a cost to associating with both partners. Furthermore, if plants with smaller aboveground biomasses have lower fitness, then this negative correlation suggests over time populations may evolve to reduce colonization of rhizobia.
Although the inoculation of AM fungi positively affected plant growth of every population, I detected a significant amount of variation in plant population response to AM fungi inoculation. Expressed on a log scale, population-level variation in the response ratio for *M. truncatula* encompassed 16% of the range found among plant species (Fig. 11, Chaudhary et al. 2016). While multiple studies have explored population level variation of other species responses to AM fungi (Linderman and Davis 2004; Gao et al. 2007; An 2010; Ramos-Zapata et al. 2010), to my knowledge this is one of the first studies to investigate the degree to which *M. truncatula* populations vary in their growth response to AM fungi. A previous study which investigated variation in response to AM fungi inoculation found no variation among 8 populations of *M. truncatula* (Schultz et al. 2010). However, it should be noted Schultz et al (2010) only grew their plants for four weeks which may not have been enough time to see a response to AM fungi.

The high degree of variation among populations indicates natural selection or genetic drift has shaped the *M. truncatula*'s response to AM fungi at a population level.

The high degree of variation in AM fungal colonization among populations may be associated with variation in phosphorus availability among populations. This is because increased phosphorus concentration has been found to reduce AM fungal colonization (Smith and Smith 2011). Previous studies on *M. truncatula* found the addition of phosphorus significantly reduced AM fungal colonization (Balzergue et al. 2013; Watts-Williams et al. 2015). It is possible populations of *M. truncatula* from phosphorus poor soils may have evolved to be more dependent on AM fungi than those from nutrient rich soils. If this is the case then populations from phosphorus poor soil should also have higher AM fungal colonization than populations from phosphorous rich soil.
Populations of *M. truncatula* also significantly varied in the proportion of roots colonized by AM fungi, with a range in colonization of 29%-75%. My results are similar to previous studies, which have found variation root colonization by AM fungi in other species of plants. For example, the colonization of 6 genotypes of rice (*Oryza sativa*) ranged from 28%-58% (Gao et al. 2007) while another study which looked at 139 cultivars of subterranean clover (*Trifolium subterraneum*) found the percentage of root length colonized ranged from 12%-68% (Ryan et al. 2016). Like these studies, I did not find a relationship between root colonization and plant growth. Ossler et al. (2015) hypothesized that there may be a cost to high levels of colonization. However, if this were the case I would expect the relationship between colonization and biomass to fit a quadratic model, which it did not. The high degree of variation in the proportion of roots colonized by arbuscules among all 34 populations suggests there is some genetic control over AM fungi colonization. Despite detecting a high degree of genetic variation in both AM fungi colonization and plant growth response I did not find a link between AM fungi colonization and plant growth performance. One explanation is the proportion of root colonized by arbuscules may be a poor predictor of phosphorus transfer to plants, as the rate of nutrient exchange could vary among arbuscules.

Although a small amount of research has explored the evolution of the interactions between plants and rhizobia (Porter and Simms 2014) and AM fungi (Rúa et al. 2016), even fewer studies have been conducted on how these multispecies symbiotic interactions may alter the evolution of the interacting partners. To my knowledge, this is the first study to investigate if there is a correlation between the AM fungi response and rhizobia. While I did find a correlation between plant growth response to AM fungi and rhizobia, it was weak; suggesting the evolution
of one symbiosis is unlikely constrained by the evolution of the other. For example, if there was selection for greater AM fungi growth response in phosphorous poor soil, this would not be accompanied by selection for greater rhizobia growth response.

While the presence of one symbiont increased the colonization of the other, the overall effect they had on one another was asymmetric. While rhizobia nodulation increased significantly in the presence of AM fungi, the effect of rhizobia inoculation was much more moderate, increasing AM colonization by only 15.6%. This asymmetry in effect size of both symbionts has been reported before in other species (Larimer et al. 2014; Meng et al. 2015). However, other studies have found that while the symbionts do influence the colonization of the other, the overall effects are not asymmetric (Jia et al. 2004; Abd-Alla et al. 2014). Although I found each symbiont increased the colonization of the other, I found no relationship between rhizobia colonization and AM fungi colonization. The lack of relationship and the asymmetry in colonization suggests evolution of AM fungi is not affected by the presence of rhizobia. For example, if there was selection for greater rhizobia colonization, AM fungi colonization would not be selected.

In conclusion, my findings suggest that M. truncatula’s responses to AM fungi and rhizobia are asymmetric. The asymmetry in variation of the growth responses to each symbiont suggests the evolution of M. truncatula’s response to AM fungi may be more labile than the evolution of its response to rhizobia. Furthermore, the weak correlation between AMF response and rhizobia response suggests that evolution in one symbiosis is unlikely to be constrained by evolution in the other. Finally, I found a high degree of variation in the growth response to AM fungi among the 34 populations of M. truncatula. Future research on the interactions between AM fungi plant interactions should consider natural variation among different populations before
making broad assumptions about a species response to these symbiotic partners. One avenue that should be explored is whether the degree to which a species is geographically distributed affects the amount of variation in plant growth response to AM fungi. I used *M. truncatula* which is a widely geographically distributed species. I predict a species that is narrowly geographically distributed would have a much smaller degree of variation in its response to AM fungi among populations.
Figure 11: Violin plot depicting interspecific variation of AM fungi ln log response ratio (blue) and *M. truncatula* intraspecific variation among all 34 populations (pink). Data for interspecific variation was taken from Chaudhary et al. (2016).
REFERENCES


Myke Pro Mycorrhizal Inoculant. Premier Tech Biotechnologies.


APPENDIX

The locations of populations PI 577639 and PI 660403 are likely incorrect, given that *Medicago truncatula* is a Mediterranean annual. When these two populations were omitted, the significance of the correlation between the response to AM fungi and rhizobia changed from ($r = 0.376$, $P = 0.028$, Fig. 3a) to ($r = 0.346$, $P = 0.052$). The significance of AM * Rhz $P = 0.045$ (Table 3) changed to AM * Rhz $P = 0.101$. The removal of these two populations did not change any other statistical results.
Figure A1: Reaction norm depicting the effect of AM fungal inoculation (± 1 SE) on aboveground biomass of 34 populations of *Medicago truncatula.*
Figure A2: Reaction norm depicting the effect of rhizobia inoculation (± 1 SE) on aboveground biomass of 34 populations of *Medicago truncatula*.