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Rhizobia protect their legume hosts against soil-borne microbial antagonists in a host-genotype-dependent manner

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ABSTRACT

Microbial inhabitants of the rhizosphere can have substantial impacts on the fitness of their associated host plants, in both beneficial and detrimental ways. Soil-borne pathogens can impose severe fitness costs that can be mitigated or eliminated in many cases by co-occurring beneficial bacteria that directly or indirectly temper pathogens' antagonistic effects. Rhizobial bacteria are best known for their role as nitrogen fixing symbionts in the rhizobia-legume mutualism but there is growing evidence that they can also act as protective agents against microbial pathogens. This study examined the role of rhizobial bacterium *Ensifer medicae* in protecting the burclover, *Medicago polymorpha*, against antagonistic soil microbes in complex soil communities. Exposing plants to concentrated slurries of soil inoculum had an adverse effect on all aspects of plant fitness in *M. polymorpha.* However, inoculating plants with rhizobia increased plant survival in the presence of live soil inoculum from 18% to over 80%. In addition, the soil microbiome changed the symbiotic relationship between plants and rhizobia; plants that were co-inoculated with dilute soil inoculum and a beneficial rhizobium produced more nodules with a higher nodule biomass than plants inoculated with rhizobium alone. Finally, we found that the effects of soil microbes and rhizobia on root biomass, root:shoot ratio, and nodule number differed between host genotypes, indicating there is potential for complex plant-bacterial interactions to respond to selection and potentially contribute to the maintenance of both plant genetic variation and bacterial diversity.

1. Introduction

Plants have been continuously colonized by bacteria and fungi since their origin hundreds of millions of years ago and plant-microbe interactions were likely instrumental in plants' adaptation to land (Pirozynski and Malloch, 1975). The soil directly around and influenced by plant roots, known as the rhizosphere, is the crucial interface where plant-microbe interactions occur (Brimecombe et al., 2001). It is an extremely complex environment home to both beneficial and pathogenic microbes, which exert a large influence on both host plant traits as well as fitness (Brimecombe et al., 2001; Friesen et al., 2011; Raaijmakers et al., 2009). Reciprocally, plants shape the rhizosphere microbial community through production of root exudates and selective recruiting of microbes (Garbeva et al., 2004; Grayston et al., 1998). Soybeans, for example, secrete isoflavones that serve to attract beneficial microbes such as *Bradyrhizobium japonicum*, but may inadvertently attract pathogens (*Phytophthora sojae*) as well (Morris et al., 1998). Microbial density in the rhizosphere is higher than in bulk soil, yet microbial diversity is inversely proportional to proximity to the root surface, which is consistent with the view that the rhizosphere environment imposes strong selection and acts as a filter for microbial communities (Berendsen et al., 2012; Kerry, 2000; Wang and Zabowski, 1998). Recent work in maize, rice and *Arabidopsis* find the rhizosphere community structure is not only driven by host species identity, but also by the particular genotype of plant host within a species (Berg and Smalla, 2009; Edwards et al., 2015; Peiffer et al., 2013). Thus, natural selection could act on plant genetic variation to enhance plants' abilities to curate the microbes they interact with in ways that maximize plant fitness, such as by cultivating beneficial microbes and suppressing pathogens. However, natural selection is simultaneously acting upon the microbial populations in the rhizosphere and the traits that enhance microbial fitness may be those that help or hinder plant performance.

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While plant roots can produce antimicrobial compounds to directly thwart the antagonistic effects of pathogens (Baetz and Martinoia, 2014; Compant et al., 2005), associations with beneficial plant-growth promoting bacteria (PGPB) are responsible for many of the biocontrol processes that suppress pathogens in the rhizosphere (Weller, 1988). One of the pathways by which beneficial microbes protect plants is via competitive exclusion during root colonization and nutrient scavenging (Weller, 1988). This has been demonstrated for the beneficial fungus *Aureobasidium pullulans*, which outcompetes the fungal pathogen *Monilinia laxa* for both nutrients and colonization space, thus preventing brown rot in peaches (Di Francesco et al., 2017). One mechanism that can promote PGPB dominance over pathogens is the production of molecules that inhibit the growth of microbial competitors. Some beneficial bacteria can produce antibiotics effective against other microbes and secrete lytic enzymes that hydrolyze the cells walls of pathogens; they have also evolved mechanisms to degrade toxins produced by pathogenic microbes (Compant et al., 2005). For example, a gene was identified in the Gram-negative bacterium *Pantoea dispersa* that can detoxify albicidin, a toxin produced by the proteobacteria *Xanthomonas albilineans,* the causal agent of leaf scald on sugarcane crops (Zhang and Birch, 1997). Over 90% of sugarcane plants inoculated with *X. albilineans* were dead within six months, while all plants co-inoculated with *P. dispersa* were alive and showed no disease symptoms (Zhang and Birch, 1996). Plant defense responses can also be triggered by the initial colonization of beneficial microbes. This priming of plant defenses does not stimulate a full defensive response, but allows for a faster, stronger response when actually exposed to pathogens (Goellner and Conrath, 2008). While the importance of free-living PGPB is undisputed, there is relatively little work exploring the impact of endosymbiotic bacteria on modulating antagonistic plant-soil interactions.

The biocontrol properties of symbiotic bacteria have recently become more appreciated, notably those from the Rhizobiaceae clade that form symbiotic nitrogen-fixing relationships with legumes where they inhabit root nodules (Fischer, 1994). The nitrogen fixed by rhizobia may be their most important and well-characterized contribution to plant growth (Das et al., 2017), and enhanced plant nutrition could indirectly impact plant-pathogen interactions if healthier plants are more capable of resisting pathogens (Dordas, 2009; Huber and Graham, 1999). However, rhizobia may also enhance plant growth by directly protecting plants against microbial pathogens using the mechanisms described in free-living PGPB systems (Das et al., 2017; Gopalakrishnan et al., 2015). Rhizobia act as biocontrol agents in a multitude of ways, including by outcompeting pathogens or by inducing plant defenses (Das et al., 2017). Rhizobia can directly counteract pathogens by producing antibiotics, siderophores, and enzymes that degrade fungal cell walls (Arora et al., 2001; Bardin et al., 2004; Chandra et al., 2007), similar to non-symbiotic bacteria. For example, several strains of *Ensifer meliloti* have been found to use mycolytic enzymes to suppress the fungal pathogen *Fusarium oxysporum* and enhance growth of fenugreek (Kumar et al., 2011). Rhizobia can also exert priming effects. It has been shown that pretreating chickpea seedlings with *Rhizobium* isolates induced expression of plant defense-related enzymes prior to inoculation with the pathogen *Fusarium oxysporum* f. sp. *ciceris* (Arfaoui et al., 2007). These examples with isolated pathogens do not typically account for interactions within complex microbial communities, which could further influence the effect of beneficial bacteria on plant fitness. Exploring microbial interactions between all soil inhabitants and their effects on plants is crucial for understanding plant-microbe co-evolution and functionality in complex natural and agricultural systems.

To explore the role of rhizobia in protecting plants against soil antagonists, we used a model wild legume-rhizobium system. *Ensifer medicae* is a nitrogen-fixing alpha-proteobacterium whose host range includes multiple members of the genus *Medicago* (Reeve et al., 2010). A saprotroph when alone in soil, it nodulates with compatible medics (*Medicago* species) and fixes nitrogen in root nodules. *Medicago polymorpha*, one of its partners, is an annual burr medic that has expanded world-wide from its native habitat around the Mediterranean basin (Bena et al., 1998; Paredes et al., 2002; Small and Jomphe, 1989). It is most commonly found in disturbed sites such as old fields, grazed grasslands, and agricultural fields (de Haan and Barnes, 1998) where it competes with native grasses (Lau and Strauss, 2005). *M. polymorpha* associates primarily with *E. medicae* as well as, less frequently, the closely related species *E. meliloti* (Bena et al., 2005; Bender et al., 1987; Lu et al., 2017; Maróti and Kondorosi, 2014; Porter et al., 2011). In the North American invaded range, both *M. polymorpha* and *E. medicae* have colonized from the European range (Helliwell et al., 2018; Porter et al., 2017).

In this study, we used a series of controlled inoculation experiments manipulating the presence of a single beneficial rhizobium strain (*E. medicae* strain WSM 419) factorially with live soil inoculum across multiple genotypes of *M. polymorpha* to address three general hypotheses:

H1: Rhizobia will enhance plants' ability to grow with a complex soil microbial community containing antagonistic microbes.
H2: The interactive effect of exposure to both rhizobia and soil microbial communities differs across plant genotypes.
H3: Complex soil microbial communities influence the symbiosis between plants and rhizobia.

2. Materials and methods

2.1. Medicago polymorpha germplasm, germination, and growth

M. polymorpha seeds from mature pods were scarified on one side with 600-grit sandpaper and incubated dry at 4 °C overnight. Seeds were surface sterilized in 6% sodium hypochlorite for three minutes and rinsed six times in sterile deionized water. The seeds imbibed for another three hours before being sterilized again using 0.6% bleach for one minute. After imbibing for approximately 4 hours, seeds were plated on 1% water agar and put in the dark at 4 °C for one week to synchronize germination.

In order to assess the influence of soil microbes from bulk soil on plant fitness, we compared mortality of plants inoculated with soil slurries to mortality when inoculated with either rhizobia or buffer. We used six genotypes of *M. polymorpha* from their native range (France: W6–5326; Italy: W6–5595; Portugal: PI-493291 & PI-493293; Spain: PI-319034 & W6–5325;) and six genotypes from their invaded range (Australia: PI-197336 & W6–5527; Chile: PI-368958; US-California: Mt. Wilson-4; US-Florida: Polatka-3 & St. Augustine-13). The plants were divided into 10 treatments (8 soil inoculants, 1 *E. medicae* WSM 419 inoculant, 1 buffer inoculant). Each genotype was replicated three times per treatment. Single healthy seedlings were planted individually into microcosms constructed of 48 oz. Square HDPE boxes (US Plastic #82077) sealed with a 1" x 2" ventilation portal covered with Breatheeasier membrane. Microcosms were filled with triple-autoclaved vermiculite mixed with 500 ml of 0.5x Fahraeus solution (0.5 mM $MgSO_4$*$7H_2O$, 0.7 mM KH_2PO_4, Na_2HPO_4*$2H_2O$, 50uM Fe-EDTA, 6.62uM $MnSO_4$, 6.27uM $CuSO_4$, 6.19uM $ZnSO_4$, 16.17uM H_3BO_3, 4.86uM Na_2MoO_4) with sterile perlite on top.

2.2. Bacteria growth and inoculum preparation

Ensifer medicae strain WSM 419 was grown for 48 h at 30 °C in sterile TY broth (0.6% tryptone, 0.3% yeast extract, 0.038% $CaCl_2$). The culture was centrifuged at 4000 × *G* for 10 min and resuspended in sodium phosphate buffer (pH 7.0) at a final concentration of 10^7 cfu/ml. Inoculated plants received 1 ml of resuspended cells. Control plants received 1 ml of sodium phosphate buffer (pH 7.0).

Table 1
Soils used to prepare inoculum for *M. polymorpha*.

Location	Short Name	Long Name	Lat	Long
Florida	FL.1	University of Florida, Gainesville	29 °38'03.1"N	82 °22'06.0"W
Florida	FL.2	Polatka	29 °38'46.1"N	81 °37'41.9"W
Florida	FL.3	Rivercrest Park, Tampa	27 °59'22.1"N	82 °27'56.9"W
Florida	FL.4	St. Augustine	29 °55'00.3"N	81 °19'36.4"W
Portugal	PT.1	Manta Rota	37 °09'45.4"N	7 °31'14.0"W
Portugal	PT.2	Castro Marim	37 °13'35.1"N	7 °26'19.9"W
Portugal	PT.3	Gilberto	37 °06'37.7"N	7 °39'01.5"W
Portugal	PT.4	Fuzeta	37 °03'15.0"N	7 °44'33.6"W

2.3. Soil inoculum preparation

Eight different soil slurries were made from field soil collected in 2013 from sites that contained *M. polymorpha*. Four of the soils came from the native range (Portugal, Table 1) and four soils were from the invaded range (US, Florida, Table 1). Soil slurries were created by shaking soil in sodium phosphate buffer for 10 min, passing the suspension through sterile cheesecloth and diluting to a concentration of 10^{10} culturable cfu/ml with sodium phosphate buffer, based on dilution plating the soil inoculum onto TY agar. Soil treatments were inoculated with 1 ml of the soil slurry. After inoculation, plants grew for 7 weeks. After this time, living plants were harvested, dried for 10 days at 60 °C, and measured biomass of shoots, roots. Total nodule biomass was measured, and nodules were counted to calculate mean nodule mass for each plant.

We also assessed how the density of microbes in soil changes the outcomes of plant-rhizobia interactions using two genotypes of *M. polymorpha* (Portugal: PI-493292; US-Florida: St. Augustine-2). The plants were divided into a 2 ×2 ×2 factorial design (Soil Concentration: Low, High; Inoculant: *E. medicae* WSM 419, Sodium phosphate buffer; Soil Origin: Florida (FL.2, Polatka), Portugal (PT.1, Manta Rota)). High concentration soil inoculum was prepared as in the previous experiment, while low concentration soil inoculum was diluted with sodium phosphate buffer by 10^{-5}. There were two additional controls of *E. medicae* WSM 419 without soil and buffer without soil for a total of ten different treatments, but we did not include a sterile soil control in this set of experiments. Each genotype was replicated six times per treatment. Plants were grown in 16 oz. Square HDPE containers (US Plastics #82073) with triple-autoclaved vermiculite moistened with Fahraeus nutrient solution and a top dressing of sterile perlite as above. Plants were grown with autoclaved micro-perforated 8 × 21 in. polypropylene bags (PJP Marketplace #361015) placed on top of containers to maintain sterility; this modification was done to reduce humidity within the growth environment and allow more airflow. Plants were inoculated according to their treatments and were grown for 6 weeks. Biomass was measured as above.

2.4. Statistical analyses

Data were analyzed using R version 3.4.3 using the stats package (R Core Team, 2018). Mortality data were analyzed using a generalized linear model with a binomial link. All other data were analyzed using a linear model. Post hoc comparisons were carried out using the emmeans package in R. All figures were created using ggplot2 2.2.1 and cowplot 0.9.2 (Wickham, 2009; Wilke, 2015) and show means plus standard error. All data and code used for analysis is archived and available upon request.

3. Results

Hypothesis 1. Rhizobia enhance plants' ability to grow with a complex soil microbial community containing antagonistic microbes

3.1. Natural soils contain antagonistic microbes that cause high mortality when grown in controlled conditions

Less than half of the *M. polymorpha* plants inoculated with a concentrated soil inoculum comprising a soil microbial community collected from the rhizosphere of wild plants were alive 7 weeks post-inoculation, with an 18% average rate of survival (Fig. 1A). Mortality significantly differed among inoculation treatments ($X^2_{(9)}$ = 198.124,

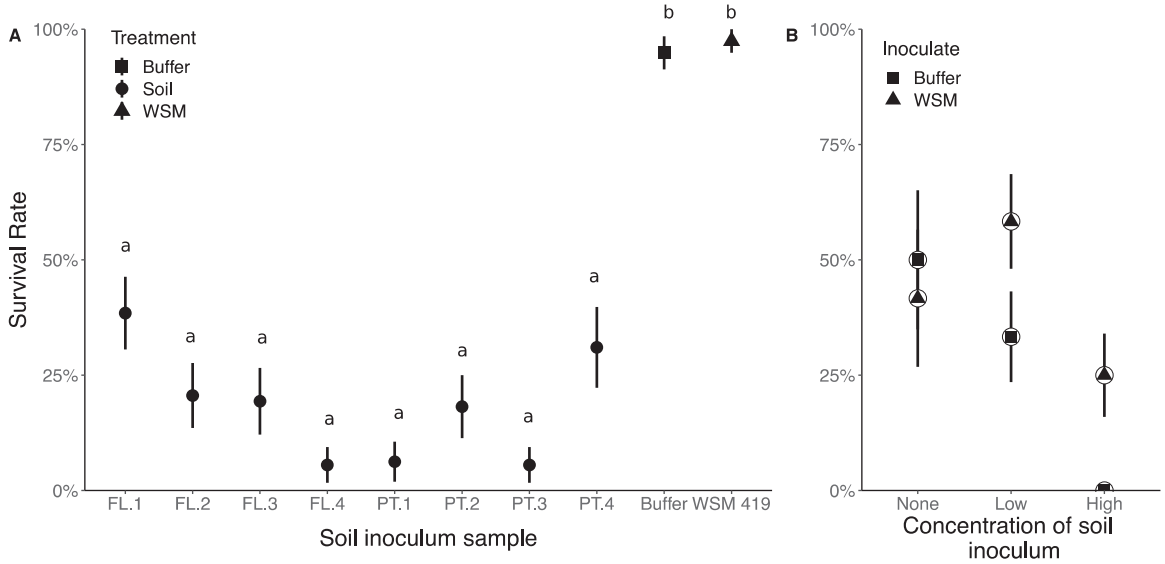

Fig. 1. *Medicago polymorpha* survival decreased when inoculated with microbial communities from diverse natural soils. A) Survival rate of *M. polymorpha* inoculated with soil from 8 different locations, four from Florida (FL) and four from Portugal (PT) compared to survival when inoculated with rhizobia (*E. medicae* WSM 419) and to a negative control (buffer). Each data point is an average survival of six *M. polymorpha* genotypes per treatment. B) *M. polymorpha* inoculated with WSM 419 plus buffer (None), dilute soil (Low), or concentrated soil slurries (High). Survival rates for St. Augustine-2 and PI-493292 were averaged together. Shapes represent the different treatment types showing standard error of the mean. Post hoc significance between treatments is indicated by different letters if $p < 0.05$. In B, post hoc testing between all groups and between Inoculate levels within soil concentrations were tested.

$p < 0.001$), driven mainly by survival rates being significantly higher when the plants were inoculated with buffer or pure rhizobia compared to when they were inoculated with any of the live soil slurries.

3.2. Rhizobia increase survival overall in the presence of soil microbes

Further evidence of antagonistic microbes in the live soil slurries came from the comparison of concentrated ($\sim 10^{10}$ cfu/mL) to dilute ($\sim 10^{5}$ cfu/mL) soil slurries; decreasing the concentration of soil microbes increased plant survival, which suggests that these soils contained microbes with a net detrimental effect on plant hosts. When plants were inoculated with pure rhizobia and live soil slurry, the overall rate of survival increased compared to plants that were grown only with the soil slurry inoculum (Soil Concentration: $X^2_{(2)} = 15.86$, $p < 0.001$; Inoculated: $X^2_{(1)} = 5.304$, $p = 0.0212$; Soil Conc x Inoculated: $X^2_{(2)} = 7.0967$, $p = 0.02878$; Fig. 1B).

Hypothesis 2. The interactive effect of exposure to both rhizobia and soil microbial communities differs across plant genotypes

3.3. Rhizobia increase plant biomass in the presence of dilute soil inoculum in a plant-genotype-dependent manner

Root biomass was significantly influenced by the concentration of the soil inoculum ($F_{2,19} = 4.323$, $p = 0.028$), which suggests rhizobia are only beneficial to host plants when plants are not overwhelmed by the presence of other microbes in concentrated soil slurries. Root biomass also showed significant growth differences between the two plant genotypes ($F_{1,19} = 42.970$, $p < 0.001$; Fig. 2A). *M. polymorpha* PI-493292 had larger root systems than *M. polymorpha* St. Augustine-2 when both genotypes were grown in the presence of pure rhizobia (*EMM* = 0.0629, *t ratio* = 3.701, $p = 0.0015$) and when they were co-inoculated with rhizobia and a dilute soil inoculum (*EMM* = 0.0578, *t ratio* = 5.803, $p < 0.001$). Post hoc comparison of each genotype indicated that plant genotypes responded differently to the soil inoculum concentration. *M. polymorpha* PI-493292 roots were smaller when grown with a high concentration of the soil inoculum compared to the dilute treatment (*EMM* = 0.0414, *t ratio* = 3.224, $p = 0.012$) and growth with only pure rhizobia (*EMM* = 0.0478, *t ratio* = 3.145, $p = 0.014$). Conversely, none of the soil inoculum treatments significantly altered root growth for *M. polymorpha* St. Augustine-2.

Root:shoot ratios behaved similarly to root biomass data (Fig. 2B), indicating that the responses we observed are due to shifts in allocation rather than differences in overall plant size. Root:shoot ratio was significantly influenced by the concentration of soil inoculum ($F_{2,19} = 4.696$, $p = 0.022$) and differed between the two genotypes ($F_{1,19} = 6.197$, $p = 0.022$). *M. polymorpha* PI-493292 had a larger mean root:shoot ratio compared to *M. polymorpha* St. Augustine-2, but only when both genotypes were grown with pure rhizobia and no soil (*EMM* = 0.246, *t ratio* = 2.625, $p = 0.017$). Post hoc comparison of each genotype indicated that plant hosts responded differently to soil slurry treatments. *M. polymorpha* PI-493292 root:shoot ratios were larger when grown without soil compared to growth in either concentrated (*EMM* = 0.246, *t ratio* = 2.946, $p = 0.022$) or dilute (*EMM* = 0.21, *t ratio* = 2.963, $p = 0.021$) soil inoculum. None of the soil or rhizobia treatments significantly altered root:shoot ratios for *M. polymorpha* St. Augustine-2.

Unlike the belowground measurements, aboveground growth was only affected by genetic differences between the plants ($F_{1,19} = 14.176$, $p = 0.0013$; Fig. 2C). *M. polymorpha* PI-493292 grew bigger shoots than *M. polymorpha* St. Augustine-2 overall but post hoc tests of genotype differences within each treatment were only significant within the rhizobia plus dilute soil inoculum co-inoculation treatment (*EMM* = 0.223, *t ratio* = 3.691, $p = 0.0016$), which was also the treatment in which *M. polymorpha* PI-493292 had the highest growth.

Hypothesis 3. A complex soil microbial community influences the symbiosis between plants and rhizobia

3.4. Soil inoculum concentration influences the relationship between nodulation and plant performance (Table 1 and Fig. 3)

Under the dilute soil inoculum treatment, there was an overall positive relationship between nodule number and shoot biomass (Fig. 3A) and between nodule biomass and shoot biomass (Fig. 3B). There was not a significant relationship for either comparison at high soil concentration. Although the Pearson correlations were significant for the dilute soil inoculum but not the high soil inoculum, the trends observed

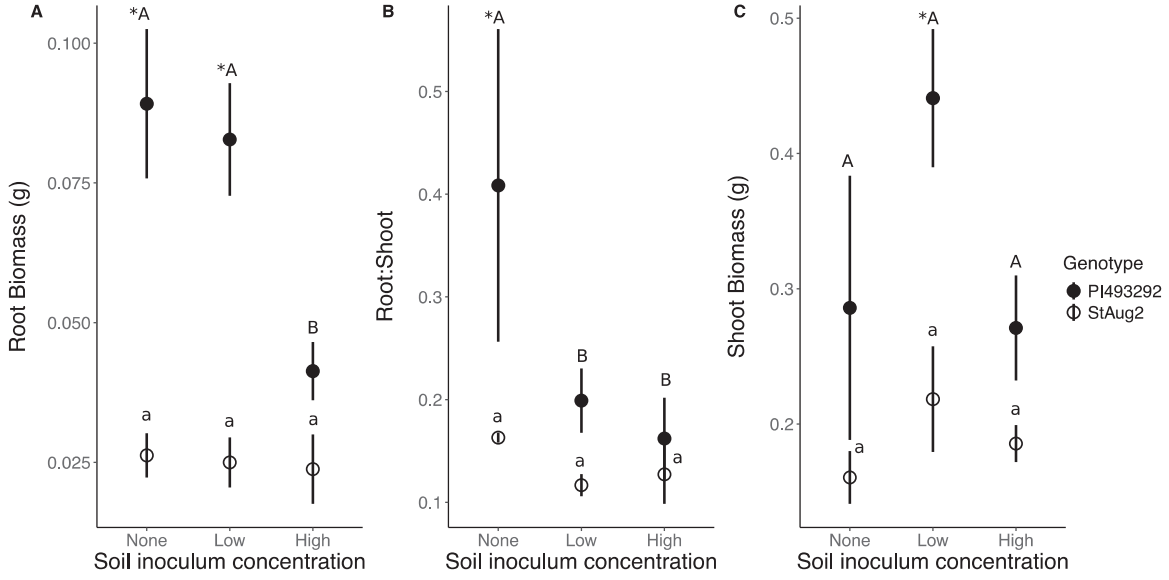

Fig. 2. Plant genotype and soil inoculum concentration influenced host plant growth. A) Root biomass B) Root:Shoot ratio C) Shoot biomass data is shown for each genotype, St. Augustine-2 (open circles) and PI-493292 (closed circles) when co-inoculated with WSM 419 plus soil. Post hoc comparisons were done 1) between genotypes within a soil inoculum concentration, and 2) soil inoculum concentrations within each genotype. An asterisk over top of a closed circle indicates that the genotypes were significantly different from each other ($p < 0.05$) for that soil concentration. Capital letters for PI-493292 and lower-case letters for St. Augustine-2 were used to represent post hoc comparisons respectively, with significant comparisons indicated by different letters.

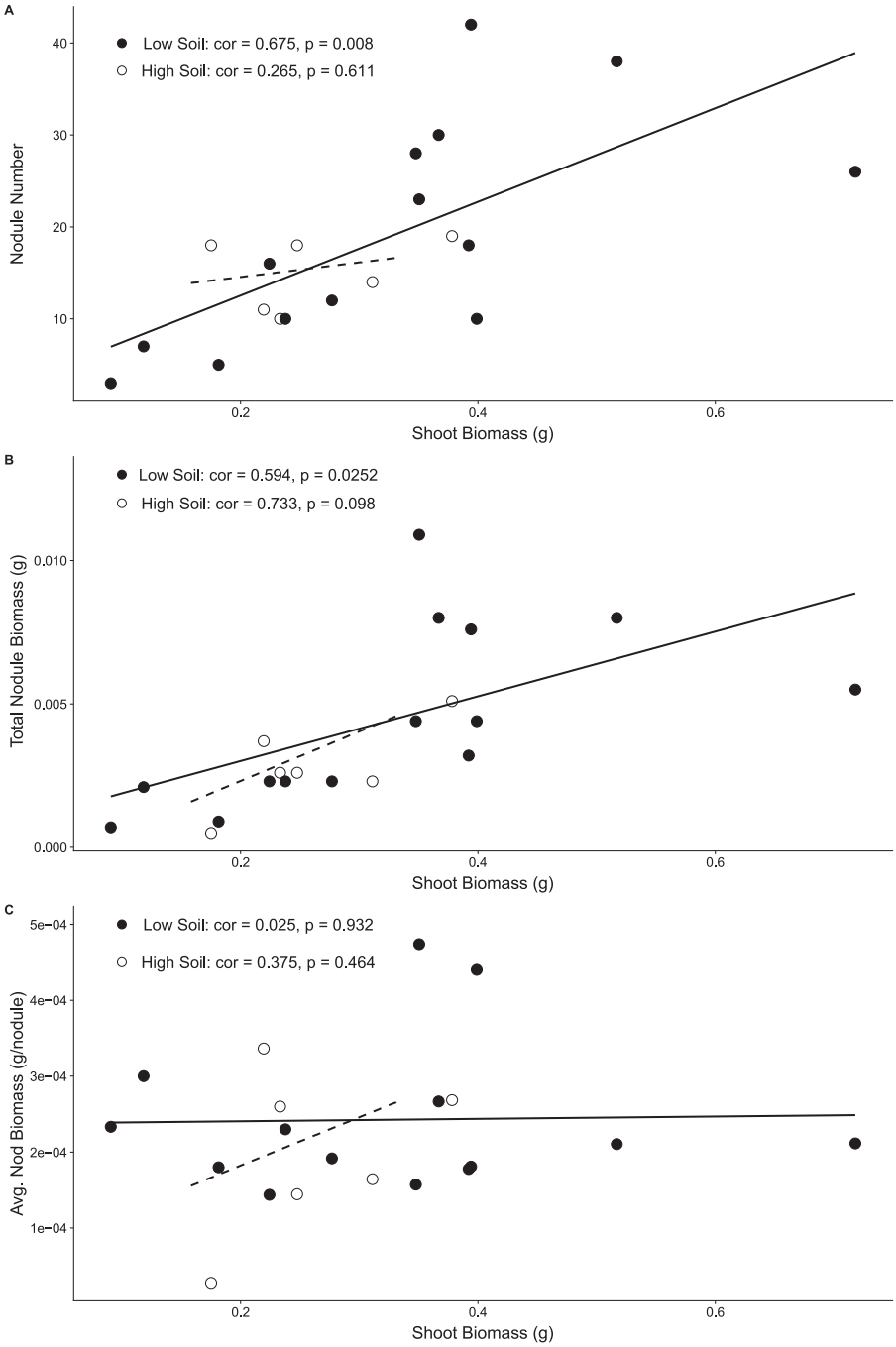

Fig. 3. Host plant fitness and rhizobia fitness had greater alignment under the low soil treatment. A) Correlation between plant total biomass and rhizobia nodule number for high and low soil inoculum treatments. B) Correlation between plant total biomass and rhizobia nodule biomass for high and low soil treatments. C) Correlation between plant total biomass and average rhizobia nodule biomass for high and low soil inoculum treatments. For total nodule number and total rhizobia biomass, rhizobia fitness and *Medicago polymorpha* fitness have a greater alignment under the low soil inoculum treatment but there was not alignment if we used the average nodule biomass. Panels show Pearson correlation coefficients along with p values. Filled circles and the solid line represent the low soil inoculum treatment and open circles and the dotted line are the high soil inoculum treatment.

Table 2
Pearson correlations between shoot biomass and nodule measurements at low and high soil inoculum concentration.

	Shoot Biomass	
	Low soil inoculum	High soil inoculum
Total nodule number	$r = 0.74$; $p < 0.001$	$r = 0.30$; $p = 0.562$
Total nodule biomass	$r = 0.59$; $p < 0.001$	$r = 0.73$; $p = 0.0978$
Avg. nodule biomass	$r = 0.025$; $p = 0.932$	$r = 0.38$; $p = 0.464$

among correlation coefficients were not significantly different between the soil treatments (Total Nod Biomass: $p = 0.865$; Total Nod Number: $p = 0.328$). We also analyzed the relationship between the average nodule biomass and shoot biomass but did not find a significant relationship in either soil treatment (Fig. 3C). (Table 2)

3.5. *Rhizobium* fitness variation depends on genetic differences between host plants

M. polymorpha PI-493292 produced more nodules than *M. polymorpha* St. Augustine-2 ($F_{1,19} = 27.459$, $p < 0.001$; Fig. 4A). Post hoc testing found that the nodule number for plants co-inoculated with rhizobia and dilute soil inoculum differed significantly between the two genotypes ($EMM = 20.286$, $t\ ratio = 6.024$, $p < 0.001$). Although soil inoculum concentration was not significant as a main effect influencing nodule number, the interaction of soil slurry concentration and genotype was significant ($F_{2,19} = 5.0014$, $p = 0.018$). Post hoc comparison of each plant genotype indicated that plants responded differently to the soil slurry treatments. *M. polymorpha* PI-493292 produced fewer

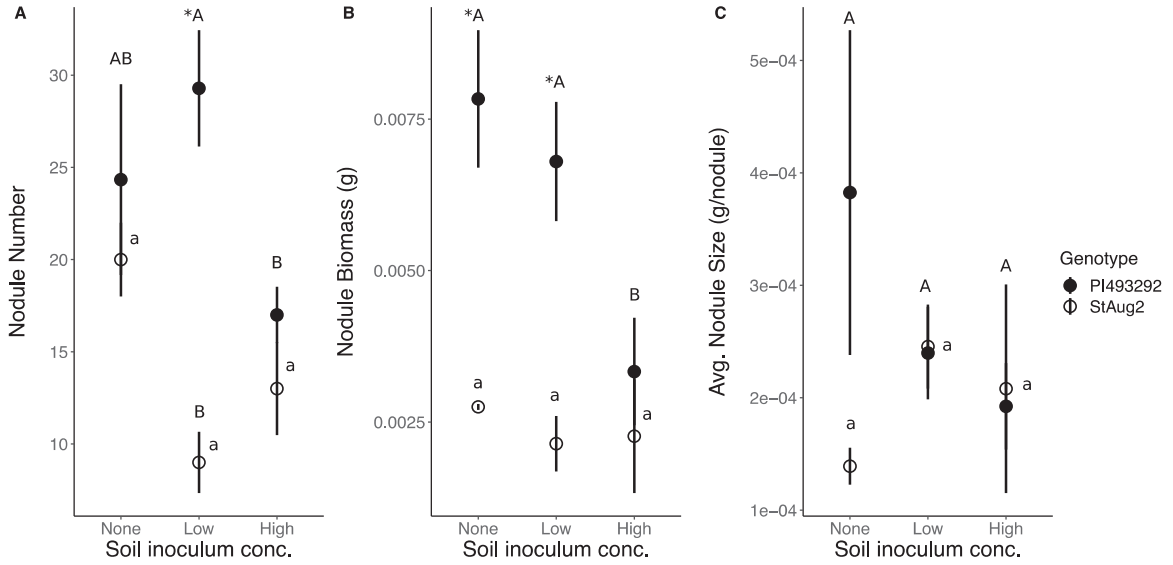

Fig. 4. Rhizobia fitness varied depending on soil treatment and depended on the identity of the associated host plant. A) Nodule number and B) Nodule Biomass were measured for each host plant genotype, St. Augustine-2 (open circles) and PI-493292 (closed circles) when co-inoculated with WSM 419 plus soil. C) Average nodule size was calculated but did not vary between genotypes or soil inoculum concentrations. An asterisk over top of a closed circle indicates that the genotypes were significantly different from each other ($p < 0.05$) for that soil concentration. Capital letters for PI-493292 and lower-case letters for St. Augustine-2 were used to represent post hoc comparisons respectively, with significant comparisons indicated by different letters.

nodules when grown with a high soil inoculum concentration compared to the low concentration treatment (*EMM* = 12.286, *t ratio* = 2.826, $p = 0.0278$). None of the treatments significantly altered nodule production for *M. polymorpha* St. Augustine-2.

Soil inoculum concentration ($F_{2,19} = 3.548$, $p = 0.0491$) and host plant identity ($F_{1,19} = 26.235$, $p < 0.001$) impacted the total biomass of nodules that were produced (Fig. 4B). Post hoc comparisons revealed that nodule biomass significantly differed between genotypes when plants were grown with only rhizobia (*EMM* = 0.0051, *t ratio* = 2.959, $p < 0.001$) and when they were co-inoculated with rhizobia and a dilute soil inoculum (*EMM* = 0.0047, *t ratio* = 4.630, $p < 0.001$). *M. polymorpha* PI-493292, when grown with a concentrated soil inoculum, produced less total nodule biomass than when grown with rhizobia and dilute soil inoculum (*EMM* = 0.00347, *t ratio* = 2.670, $p = 0.0385$) or when grown with only rhizobia (*EMM* = 0.00450, *t ratio* = 2.929, $p = 0.022$). The average nodule biomass (Fig. 4C) did not vary according to any of our treatments, nor did the ratio of shoot to nodule biomass.

4. Discussion

4.1. Plant performance was hindered by the presence of the soil microbial community but was rescued by the addition of a beneficial symbiont

Diverse root-associated bacteria have been documented to act as biocontrol agents, which when co-inoculated with antagonistic microbes prevent plant disease (Compant et al., 2005; Das et al., 2017). Inoculation of *Medicago polymorpha* with concentrated soil microbes from both native and invasive regions resulted in massive legume mortality independent of the genotype of the host plant, with an average rate of survival of 18%. Plant mortality was reduced by diluting the soil inoculum, indicating that there are either density-dependent effects of antagonistic microbes as observed in plant pathogens like *Ralstonia solanacearum, Pectobacterium carotovorum, Pseudomonas syringae pv. syringae* (Helman and Chernin, 2015; von Bodman et al., 2003), or that there are rare antagonistic taxa that are diluted to extinction (Smith and Snyder, 1971). More importantly, our study found that co-inoculating plants with a single strain of beneficial rhizobium (*Ensifer medicae* WSM 419 (Reeve et al., 2010; Terpolilli et al., 2008)) moderated the negative effects of the soil slurry and returned survival rates to levels equal to those of plants without soil slurries. This demonstrates that in addition to its well-known role as a symbiotic nitrogen-fixer, in our system rhizobia also acts as a protective agent.

4.2. Potential mechanisms of plant performance rescue through association with rhizobia

We currently do not know the mechanism of protection provided by *Ensifer medicae* to *Medicago polymorpha*. One hypothesis is that rhizobia directly inhibit or outcompete phytopathogens, as has been found in soybeans and alfalfa (Aeron et al., 2017; Chakraborty and Purkayastha, 1984; Gao et al., 2018). For example, Arfaoui and colleagues (2007) found that rhizobia act as an effective phytoprotector of chickpea by preventing some of the deleterious effects caused by the *Fusarium* wilt in growth chamber trials. An alternative hypothesis is that the presence of rhizobia may alter the overall microbial community composition (Lu et al., 2017; Schlaeppi and Bulgarelli, 2015). If the composition of the microbial community changes, this could shift microbe-microbe interactions and attenuate the effect of pathogens present (Hamel et al., 2005). A final hypothesis is that rhizobia could influence plant physiology and/or immune functioning in a manner that acts to alleviate the effects of pathogens (Cao et al., 2017; Gourion et al., 2015; Tóth and Stacey, 2015). This could occur through the enhancement of host nutrition, or through interactions with the host immune system. Disease resistance often comes at the cost of plants diverting resources away from growth (Bergelson and Purrington, 1996; Ward et al., 2010) so a lack of essential nutrients such as nitrogen or phosphorus could negatively influence plant allocation towards defense (Baldwin et al., 1998; Van Dam and Baldwin, 1998). Soil-borne pathogens usually infect root systems decreasing the plant's ability to obtain nutrients and water, which can lead to nutrient deficiency (Dordas, 2009). Application of fertilizers can reduce disease severity by compensating for reduced root growth (Mur et al., 2017), thus nutritional mutualists such as rhizobia could affect host disease status purely through improved host nutrition. However, there may be additional advantages to using rhizobia over mineral fertilizer to achieve this reduction in pathogen harm. In particular, the feedback system between plant and symbiont may make it easier to modulate the amount of nitrogen transferred to the plant to compensate for reduced uptake by roots (Dordas, 2009).

4.3. Soil slurries affected the symbiotic relationship between the rhizobia and their host plants

We found that plant biomass was positively correlated with nodule number and nodule biomass in the low concentration soil slurry treatment, which indicates that fitness between the two species is aligned (Friesen, 2012). Plant biomass is often used as a proxy for plant fitness because the time needed to quantify all measures of fitness is prohibitive (Younginger et al., 2017). Recent studies have shown that nodulation increases rhizobium fitness in *M. truncatula* and that nodule size could also serve as a good proxy for rhizobium fitness because nodule size and CFU/nodule were correlated in *M. polymorpha* (Heath and Tiffin, 2009; Porter and Simms, 2014). Thus, comparing fitness between the two species can indicate whether there is a selective benefit for both to cooperatively interact. However, in the concentrated soil treatment, these fitness traits were no longer aligned. This is important because plants control the number of nodules that are formed through a combination of autoregulation of shoot-derived signals and control of ethylene signaling (Ferguson et al., 2010; Reid et al., 2011). Nodule biomass depends on both the available carbon resources provided by the plant, as well as overall host plant health (Friesen, 2012; Penmetsa and Cook, 1997). In one plant genotype, both nodule biomass and nodule number were reduced when co-inoculated with concentrated soil slurry (Fig. 4). There are two hypotheses that may explain why rhizobial fitness, in terms of nodule number and biomass, was lower in the high soil treatment. It may be that *M. polymorpha* is allocating more resources towards pathogen defense thus diverting carbon away from nodules. An alternative hypothesis is that other soil microbes are outcompeting rhizobia prior to nodule formation, resulting in the plant being rhizobia-limited in the number of nodules it can form. Our evidence that *M. polymorpha* co-inoculated with the low soil treatment allocated more resources towards nodule biomass than nodulating plants in the high soil treatment supports the former hypothesis.

4.4. Plant genotype modulates responses to soil and rhizobia co-infection

Unlike motile organisms that migrate to avoid detrimental environmental conditions, the sessile nature of plants require that they evolve response mechanisms to cope with challenges such as pathogen infestation (Pigliucci, 2005). These responses are genotype-specific and range from showing plasticity with high variability in plant traits to more robust trait response, where there is little variation despite change in the environment (Lachowiec et al., 2015). High plasticity allows plants to adapt to many environments and is beneficial when conditions are unpredictable. However, plasticity may carry a cost in more stable environments and presenting the same phenotype may increase fitness. In the latter cases, a constitutive response to plant pathogens may be preferred over an inducible system, as inducibility carries a cost (Heil, 2001). There may also be variation in response to pathogens, with some plant genotypes having a higher tolerance to pathogens (Simms, 1993). As host plant fitness is influenced by the microbial community, containing both antagonistic and beneficial members (Parker et al., 2017; Parker, 1995; Raaijmakers et al., 2009), plants may cultivate genotype-specific microbes to mediate the effects of antagonistic microbes (Berg et al., 2002; Goh et al., 2013; Marques et al., 2014; Schweitzer et al., 2008; Van Overbeek and Van Elsas, 2008), which can also alter phenotype plasticity and plant fitness. For many of the plant traits that we analyzed, there was significant variation between the two *M. polymorpha* genotypes. This was driven by differences in how each genotype responded to the soil treatments. St. Augustine-2 showed less variation in response to the presence of soil slurry and rhizobia compared to PI-493292, where host plant traits were more dependent on soil microbial density. Association with *E. medicae* WSM 419 not only decreased mortality but decreased phenotypic plasticity in St. Augustine-2. This raises several questions for future work. Is the response shown by both plant genotypes independent of rhizobium identity, or is plasticity determined by an interaction between plant genotype and symbiont genotype? Although it is difficult to generalize based on two genotypes, our work highlights the importance of including the influence of microbial genotype x host plant genotype x environment (G x G x E) interactions in order to gain a more complete understanding of plant fitness (Gallart et al., 2018; Parker et al., 2017; Wagner et al., 2016). These complex interactions could contribute to the maintenance of variation in the legume-rhizobium mutualism (Heath and Stinchcombe, 2014), as selection on host and symbiont would vary depending on the broader microbial community.

5. Conclusion

Our study adds to the growing literature documenting that individual microbial symbionts can have multiple beneficial effects on their plant hosts. This work shows that *Ensifer medicae* also acts as a biocontrol agent that enhances survival of plants challenged with live soil slurry containing antagonistic agents. We also document effects of soil microbes on the legume-rhizobium symbiosis and find that these plant-microbe-microbe interactions vary in a host-genotype-dependent manner. One of the genotypes studied showed differences in response depending on soil slurry concentration, while the other genotype's growth was not perturbed by changing soil slurry concentration. Taken together, these results suggest that the broader soil microbiome may contribute to the maintenance of variation in host-symbiont interactions and highlight the importance of measuring plant-microbe mutualisms in complex biotic contexts.

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