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Microbial communities improve growth of slow-growing, uncommon legumes in a restored prairie

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Microbial communities improve growth of slow-growing, uncommon legumes in a restored prairie

Abstract

Restored prairies are attempts at restoring native prairies, most of which have been lost. However, restoration is often challenging because native prairie plants, such as native prairie legumes, often fail to establish in restored prairies. We hypothesized legume's failure to establish in restored prairies is due to changes in the microbial communities with which legumes have multi-mutualistic relationships: rhizobia and arbuscular mycorrhizal fungi (AMF). Additionally, we hypothesized that late successional legumes rely on multi-mutualistic relationships for establishment and growth more than early successional legumes, and would respond synergistically to rhizobia and AMF treatments. To test this, we inoculated eight native perennial legumes of varying life histories with rhizobia and/or AMF and transplanted them into a restored prairie. At the end of the growing season we measured the survival, health, above ground biomass, and height of the surviving legumes. Dual inoculation increased seedling survival, health, above ground biomass, and height compared to uninoculated controls. Averaged across all species, dual inoculation increased seedling height and above ground biomass synergistically (greater than the total effect of the mutualists' separate independent effects combined). On a species by species basis, mid and late successional species experienced synergistic effects on above ground biomass from dual inoculation while early successional species did not. However, the magnitude of the synergistic response did not increase with plant successional status. Our findings reinforce the importance of multi-mutualistic community interactions within prairie ecosystems for restoration success.

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MICROBIAL COMMUNITIES IMPROVE GROWTH OF SLOW-GROWING, UNCOMMON LEGUMES IN A RESTORED PRAIRIE

By

Alex Chumney

A Senior Project Submitted to the

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Honors College

In Partial Fulfillment of the Requirements for Graduation

with Departmental Honors in Biology

Approved in Ypsilanti, MI on April 20th, 2023

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ABSTRACT

Restored prairies are attempts at restoring native prairies, most of which have been lost. However, restoration is often challenging because native prairie plants, such as native prairie legumes, often fail to establish in restored prairies. We hypothesized legume's failure to establish in restored prairies is due to changes in the microbial communities with which legumes have multi-mutualistic relationships: rhizobia and arbuscular mycorrhizal fungi (AMF). Additionally, we hypothesized that late successional legumes rely on multi-mutualistic relationships for establishment and growth more than early successional legumes, and would respond synergistically to rhizobia and AMF treatments. To test this, we inoculated eight native perennial legumes of varying life histories with rhizobia and/or AMF and transplanted them into a restored prairie. At the end of the growing season we measured the survival, health, above ground biomass, and height of the surviving legumes. Dual inoculation increased seedling survival, health, above ground biomass, and height compared to uninoculated controls. Averaged across all species, dual inoculation increased seedling height and above ground biomass synergistically (greater than the total effect of the mutualists' separate independent effects combined). On a species by species basis, mid and late successional species experienced synergistic effects on above ground biomass from dual inoculation while early successional species did not. However, the magnitude of the synergistic response did not increase with plant successional status. Our findings reinforce the importance of multi-mutualistic community interactions within prairie ecosystems for restoration success.

INTRODUCTION

Native prairies provide an unique environment that supports endangered species such as Mitchell's satyr butterfly, leafy prairie-clover, and eastern prairie fringed orchid (Barton and Bach 2005, US Fish and Wildlife) (Fig. 1). Prior to European colonization, North American native prairies used to cover much of the Midwest and the Great Plains, but 96% of these ecosystems have been lost due to agriculture and development (White et al. 2000). Efforts have been made to restore these essential ecosystems, but restoration often comes with challenges. One such challenge is that many planted and sown native prairie plants fail to establish (Grman et al. 2015), likely in part due to changes in soil microbial communities (Jangid et al. 2010) and soil nutrient levels (MacDonald et al. 2012, Camill et al. 2004) within restored prairies due to legacy effects caused by agricultural use. Further research is necessary to deepen our understanding of community and ecosystem dynamics so that restored prairies can sustain native prairie diversity with varying life histories.

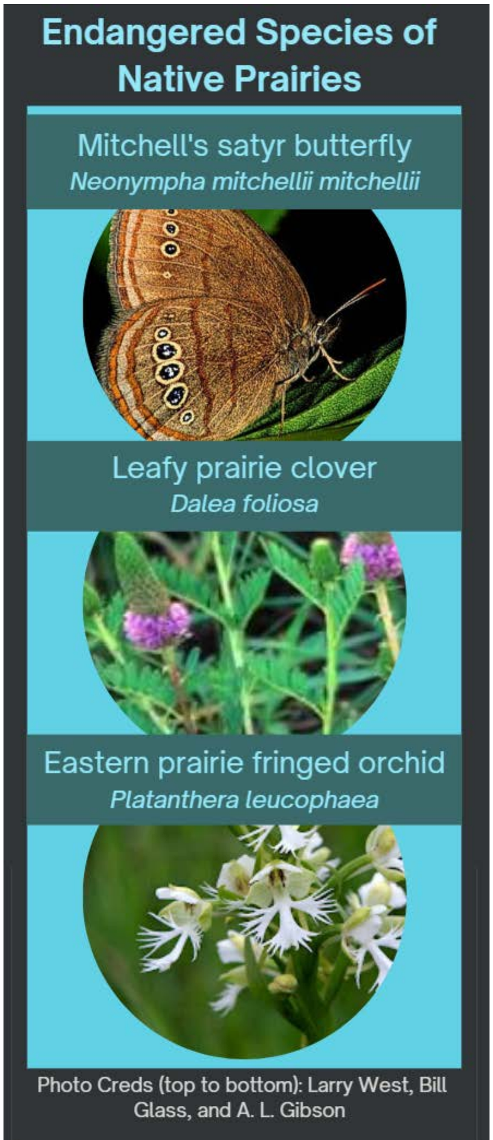

Photo Creds (top to bottom): Larry West, Bill Glass, and A. L. Gibson

Figure 1: Examples of endangered species of plants and animals that rely upon native prairies for their habitat.

Life history of prairie plants

A species life history traits include characteristics that influence the survival and reproduction of a species, which varies depending on their preferred environment and their relationships with the community around them. For example, early succession plant species typically are “weedy” plants with life history traits like rapid growth rates and reproduction and poor competitive ability. Early successional plants tend to be successful in environments that experience frequent disturbances (temporary changes that impact ecosystem functions). On the other hand, late successional plant species typically have slower growth and reproduction but have competitive advantages in diverse ecosystems that allow them to thrive in undisturbed ecosystems (Bauer et al. 2018). Variations in life history traits among species contribute to the diversity found in communities.

Over time, communities tend to shift from early successional to late successional species (Fig. 2), however this trend does not hold for restored prairies. Rehabilitation of prairie communities includes sowing native prairie species onto post-agricultural fields in an attempt to restore it to its pre-developed state. However, even decades old restored prairies lack the diversity

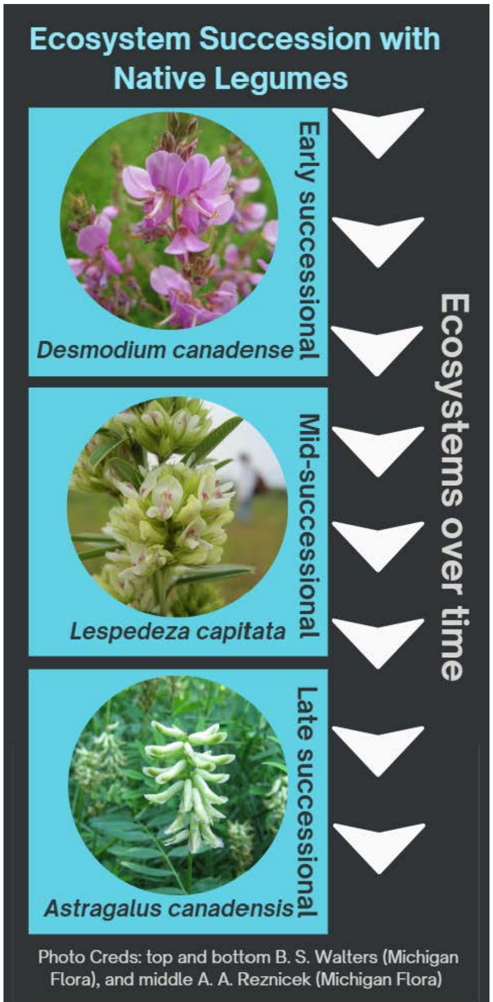

Photo Creds: top and bottom B. S. Walters (Michigan Flora), and middle A. A. Reznicek (Michigan Flora)

Figure 2: Diagram of legume species found in prairie ecosystems at varying stages of succession from early to late successional.

found in remnant prairies (remains of prairies left mostly undisturbed by development) (Martin et al. 2005, Middleton et al. 2010). Restored prairie communities are often dominated by native C_4 grasses that suppress diversity (Grman et al. 2020) and are missing critical late successional species (Grman et al. 2015). This may be due to changed soil microbial communities found in restored prairies.

Soil microbes within prairie ecosystems

Soil microbes are critical to prairie ecosystems, forming often mutualistic (mutually beneficial) relationships within the roots of plants. However, restored prairies are typically sown into post agricultural fields, which have a history of being tilled and fertilized. Due to these legacy effects, restored prairies often have diminished microbial communities (Kabir 1921; Grman et al. 2020). Inoculation with specific microbial communities can increase diversity, floristic quality, and total plant community (Koziol and Bever 2017). These studies imply that remnant prairies may have essential soil mutualists that are not present in restored prairies, hindering the establishment of prairie species such as native legumes.

Native prairie legumes are a diverse family of plants who form mutualistic relationships with two root dwelling organisms found within prairie microbial communities: rhizobia and arbuscular mycorrhizal fungi (AMF). Rhizobia are a group of nitrogen fixing bacteria, some of which live in root structures of legumes called nodules, and provide the plants with nitrogen in exchange for carbohydrates (Fig. 3A). The other soil mutualist, AMF, is a group of fungi that uptakes soil phosphorus and exchanges it for carbohydrates with the partner plant (Fig. 3B). Rhizobia and AMF are critical to prairie ecosystems and legume growth (Primieri et al. 2022). When both rhizobia and

AMF form mutualistic relationships with legumes, this forms a multi-mutualistic relationship.

Multi-mutualistic relationships tend to benefit legumes, their mutualistic partners, and the surrounding ecosystems (Afkhami et al. 2021, Larimer et al. 2010, Primieri et al. 2022). For example, legumes inoculated with AMF can facilitate the transfer and uptake of nitrogen in neighboring plants (Ingraffia et al. 2021) while increasing plant community diversity (Bauer et al. 2012, Klabi et al. 2014). Rhizobia presence can increase legume survival rate (van der Heijden et al. 2016) and overall legume biomass (Grman et al. 2020). This in turn benefits restored prairies; legumes have been shown to increase species recruitment, plant community productivity, and available soil nitrogen (Li et al. 2015). In general, mutualists such as rhizobia and AMF benefit legume growth, but understanding the extent to which rhizobia, AMF, and legumes interact and impact one another may offer critical insight into restoration success.

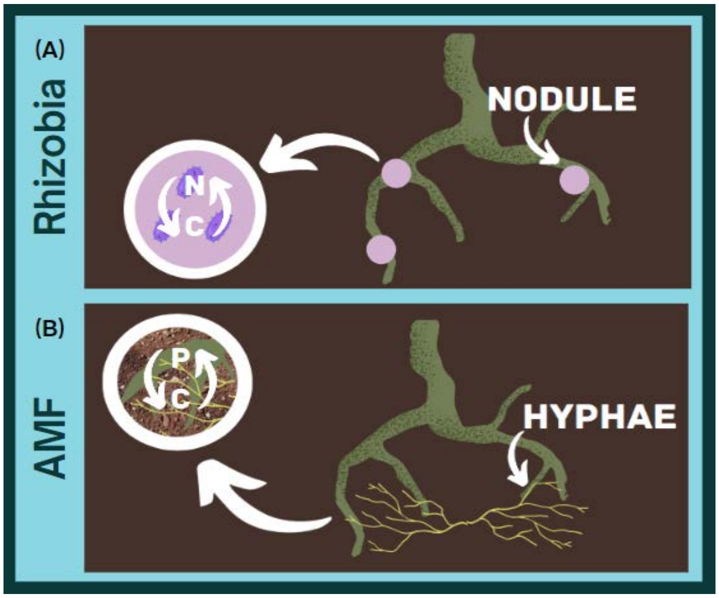

Figure 3: A simplified diagram of how (A) rhizobia exchange nitrogen (N) for carbohydrates (C) in nodules along the root of a legumes and (B) AMF in the soil exchanging phosphorus (P) for carbohydrates through the use of hyphae (filaments that make up the vegetative part of the fungus).

Synergistic effects of mutualists

Multi-mutualistic relationships of legumes, rhizobia, and AMF may provide additional benefits beyond the single mutualistic relationships, potentially providing synergistic effects (greater than the total effect of their separate independent effects combined) rather than additive (Fig. 4). Results of studies focusing on potential synergistic effects between AMF and rhizobia inoculations thus far have been contradictory. AMF can positively impact the fitness of rhizobia and legumes (Afkhami et al. 2021) and inoculation with both AMF and rhizobia can offer a synergistic benefit for legumes, exceeding expected growth (Zhou et al. 2022). A meta-analysis showed that while rhizobia and AMF did positively impact plant growth, they did not have synergistic effects (Larimer et al. 2010); yet another noted perennial legumes (typically late successional species) did in fact experience synergistic growth when inoculated with both rhizobia and AMF while annual legumes did not (Primieri et al. 2022).

However, both meta-analyses found only a limited number of field experiments testing synergistic effects. Most studies were conducted in greenhouses, which are ideal for highly controlled experiments but cannot reliably imitate natural ecosystem conditions. Additionally, previous meta-analyses could not distinguish between early and late successional legume species, and instead focused on factors such as annual versus perennial. A field experiment can help sort out the relationship between perennial legumes with varying life histories and their mutualists, providing a greater understanding on the impact of rhizobia and AMF in restored prairies.

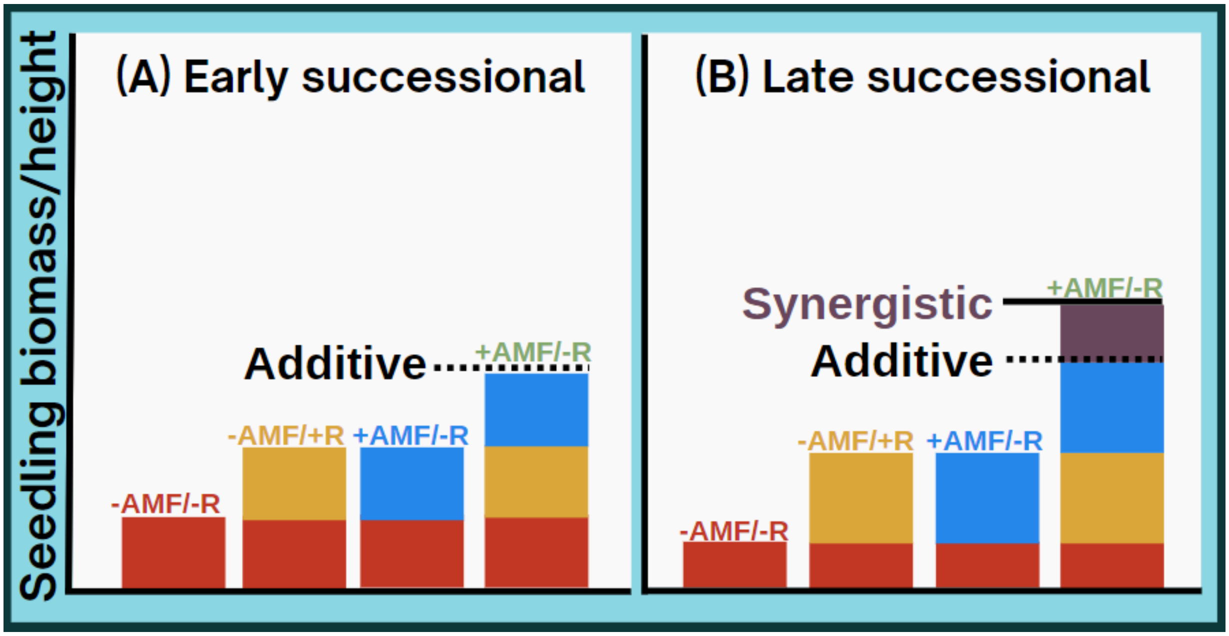

Figure 4: Prediction figures of additive versus synergistic effects on plant growth and establishment for (A) early successional species and (B) late successional species. Treatments include -AMF for no added AMF and -R for no added rhizobia, and +AMF and +R for added AMF and rhizobia, respectively. Red is used for the expected growth with control treatments, with the additional yellow and blue bars for the expected growth from +R and +AMF treatments. Additive effects are the combined sum of differences of the mutualists effects during dual inoculations (combined yellow and blue bars), while synergistic is greater than the combined sum effect (the combined yellow and blue bars, plus the “extra” purple bar).

To understand the impact of multi-mutualistic soil microbes, rhizobia and AMF, on survival and growth of legumes with varying life histories (from late successional to early successional), we conducted a field experiment on eight different legume species grown with rhizobia and AMF. We asked (1) how late successional species’ reliance on absent mutualists prevent them from establishing in prairies, (2) how inoculation of soils with AMF and rhizobia impact early successional legumes compared to late successional legumes, and (3) whether inoculating with AMF and rhizobia has positive synergistic effects on late successional legumes growth and not early successional. We hypothesized:

1. If rhizobia and/or AMF inoculation and availability promotes legume seedling survivorship and growth, then independent or dual inoculated

seedlings would have greater survivorship, health, above ground biomass, and height than uninoculated seedlings regardless of successional status.

2. If dual inoculation of rhizobia and AMF has additive but not synergistic effects on early successional legumes seedling survivorship and growth, then dual inoculation will have greater survivorship, health, above ground biomass, and height than single inoculation but not more than the sum of effects of single inoculation on early successional species.
3. If inoculation of both AMF and rhizobia synergistically promotes growth in late successional legumes, then dual inoculation of AMF and rhizobia on late successional legume species would have a greater total effect on plant biomass than the combined sum of singular inoculations.

To test these hypotheses, we selected eight legume species with varying life histories (early successional to late successional), inoculated them with four treatments (with or without rhizobia and/or AMF), transplanted them into a restored prairie, and harvested them after a single growing season. Our goal from this study is to improve the chances of restoration success through greater understanding of prairie community interactions, thus increasing biodiversity and restoring prairies as closely as possible to their native state where critical and endangered organisms can find refuge.

METHODS

We tested the effect of AMF and/or rhizobia on eight different legume species comparing the seedling survival, health, above ground biomass, and height within a field experiment. We selected legume species with varying life histories, from early successional to late successional based on data collected from Michigan and Indiana prairies and their corresponding coefficient of conservatism values (Fig. 5). Coefficient of conservatism values are numbers (0-10) assigned to plant species that indicate the likelihood a plant species is to be found in unaltered habitat and can be used to connect species to life history traits (Bauer et al. 2018). Low numbers (closer to 0) tend to be early successional species while high numbers (closer to 10) tend to be late successional species.

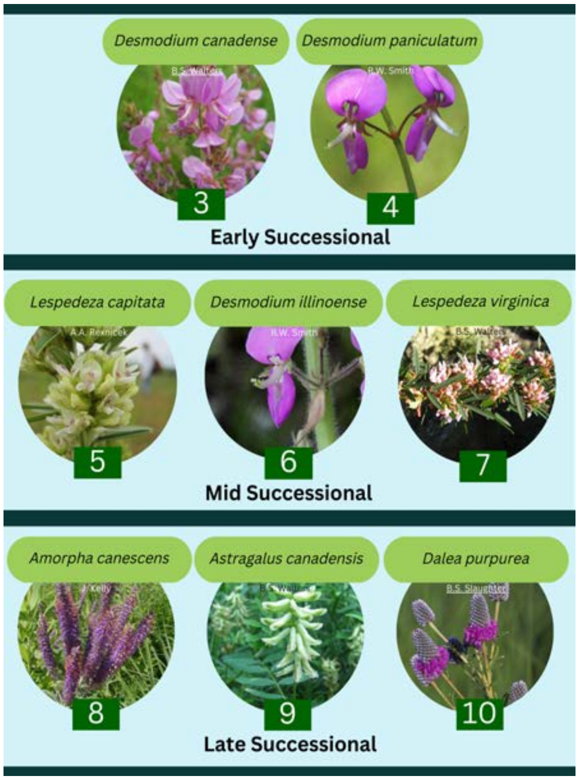

Figure 5: The list of focal legumes in order of increasing coefficient of conservatism (number in green box below each image) (images source: Michiganflora.net).

Establishment of seedlings in greenhouse

In February of 2022, we planted eight different legume species in Eastern Michigan University's greenhouse. To plant the seeds, first we sterilized seedling pots and prepared individual species seeds. We scarified *L. capitata* by rubbing the seeds with fine sandpaper. All species were soaked in a 5% bleach solution for three minutes before being rinsed with distilled water five times immediately before being submerged in sterile water for 3 days in a fridge before planting. The seeds were then planted in a sterilized 80% sand/soil mix. The sand was all-purpose sand purchased from a nearby retailer and the topsoil was collected from an old field. To sterilize the sand/soil mixture, it was autoclaved at 120ºC for 50 minutes before planting. These steps were taken to minimize the chance of microbial contamination.

Treatments were applied in a 2 by 2 factorial design: ¼ of seedlings were left uninoculated, ¼ were inoculated with 1 mL with a mix of available species-specific beneficial rhizobia strains (Table 1), ¼ were inoculated with a similar diverse mix of beneficial AMF strains from four remnant prairies in Michigan, and the final ¼ were inoculated with both rhizobia mixes and AMF mixes (Fig 6). To inoculate with AMF, we covered the seeds with a teaspoon of AMF inoculum (prepared in trap cultures of Michigan prairie soil in a greenhouse), half of which was autoclaved to kill all AMF for control treatments. A week later, 6 species specific rhizobia strains that had been streaked on TYME plates 3 weeks ahead were suspended in DI water. To ensure equal densities of rhizobia bacteria across legume species, each suspended rhizobia species was then measured using a spectrophotometer and diluted as necessary to ensure that optical density (measured at 600 nm) was 0.1. Then 6 species specific rhizobia strains were combined in equal parts before inoculation.

Legume Species	Rhizobia Species
Amorpha canescens	*Bradyrhizobium elkanii*
Coefficient of conservatism: 8	*Bradyrhizobium japonicum*
	Bradyrhizobium viridifuturi
	Mesorhizobium amorphae
	Mesorhizobium newzealandense
	Mesorhizobium sophorae
Astragalus canadensis	*Bradyrhizobium elkanii*
Coefficient of conservatism: 9	*Bradyrhizobium elkanii*
	Bradyrhizobium viridifuturi
	Mesorhizobium erdmanii
	Mesorhizobium newzealandense
	Rhizobium indigoferae
Dalea purpurea	*Bradyrhizobium cajani*
Coefficient of conservatism: 10	*Bradyrhizobium elkanii*
	Bradyrhizobium erythrophlei
	Bradyrhizobium viridifuturi
	Rhizobium cauense
	Rhizobium indigoferae
Desmodium canadense	*Bradyrhizobium elkanii*
Coefficient of conservatism: 3	*Bradyrhizobium japonicum*
	Bradyrhizobium liaoningense
	Bradyrhizobium viridifuturi
	Rhizobium pisi
	Rhizobium rosettiformans
Desmodium illinoense	*Bradyrhizobium elkanii*
Coefficient of conservatism: 6	*Bradyrhizobium japonicum*
	Bradyrhizobium viridifuturi
	Neorhizobium galegae
	Rhizobium jaguaris
	Rhizobium lusitanum

Legume Species	**Rhizobia Species**
Desmodium paniculatum	*Bradyrhizobium cajani*
Coefficient of conservatism: 4	*Bradyrhizobium japonicum*
	Bradyrhizobium liaoningense
	Bradyrhizobium viridifuturi
	Bradyrhizobium viridifuturi
	Bradyrhizobium viridifuturi
Lespedeza capitata	*Rhizobium giardinii*
Coefficient of conservatism: 5	*Rhizobium giardinii*
	Rhizobium tibeticum
	Rhizobium tibeticum
	Rhizobium tibeticum
	Rhizobium tibeticum
Lespedeza virginica	*Bradyrhizobium japonicum*
Coefficient of conservatism: 7	*Bradyrhizobium japonicum*
	Bradyrhizobium japonicum
	Bradyrhizobium japonicum
	Neorhizobium huautlense
	Rhizobium cauense

Table 1: List of focal legume species with their six focal rhizobia species. The rhizobia species were mixed to create a “slurry” of different species before application.

About 480 legume seedlings in total were planted (8 species x 10 replicates = 80 plants per treatment x 4 treatments = 320 plants, plus 50% extra for potential premature deaths). Treatments were spaced apart from one another to minimize cross-contamination, with the control furthest away from high traffic areas. In the end, we had higher than expected mortality rates and ended up with fewer replicates of some species and treatments (Table 2). In particular, late successional species *A. canadensis* and *D. purpurea* lacked enough transplantable seedlings to draw meaningful conclusions.

Species	Treatment	Transplanted Seedlings
Desmodium canadense	-AMF/-R	10
	-AMF/+R	10
	+AMF/-R	10
	+AMF/+R	10
Desmodium paniculatum	-AMF/-R	10
	-AMF/+R	10
	+AMF/-R	3
	+AMF/+R	3
Lespedeza capitata	-AMF/-R	10
	-AMF/+R	10
	+AMF/-R	9
	+AMF/+R	10
Desmodium illinoense	-AMF/-R	9
	-AMF/+R	10
	+AMF/-R	10
	+AMF/+R	9
Lespedeza virginica	-AMF/-R	10
	-AMF/+R	10
	+AMF/-R	10
	+AMF/+R	10
Amorpha canescens	-AMF/-R	10
	-AMF/+R	10
	+AMF/-R	10
	+AMF/+R	10
Astragalus canadensis	-AMF/-R	10
	-AMF/+R	10
	+AMF/-R	2
	+AMF/+R	9
Dalea purpurea	-AMF/-R	10
	-AMF/+R	9
	+AMF/-R	0
	+AMF/+R	0

Table 2: The number of replicates per treatment for each legume species. Species are in the first column, while the second column is the treatment (color coordinated as well), with the number of seedlings that could be transplanted into the field in the final column.

Transplanting into a restored prairie

Fieldwork took place at a restored prairie with permission from Legacy Land Conservancy at the Lloyd and Mabel Johnson Preserve in Washtenaw County, Michigan (Fig. 7A). The field site was originally an agricultural field until 2017 when restoration efforts began, and thus was a fairly young restored prairie with legacy effects from agricultural practices (fertilization and tilling). The site was dominated by C_4 grasses *Andropogon gerardii* (Big bluestem) and *Sorghastrum nutans* (Indian grass). The site underwent a prescribed burn in early May 2022 (Fig. 7B).

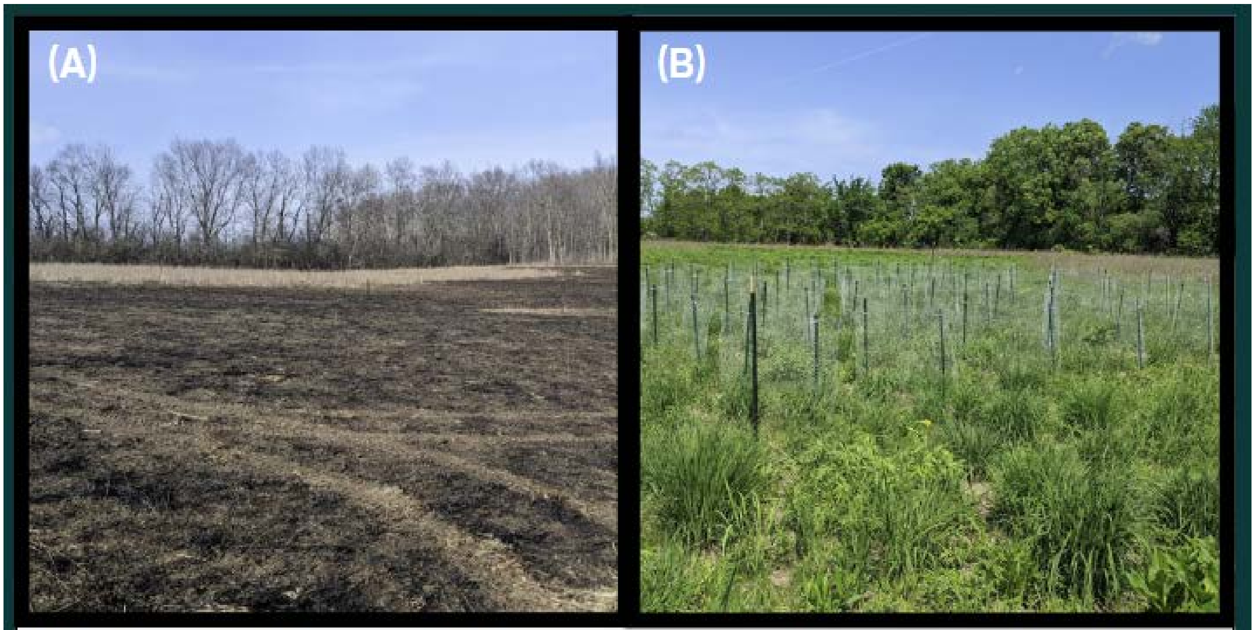

Figure 6: Figure showing field site (a) after the prescribed burn (early May, 2022) and (b) a few weeks following plot set up and plantings (late May, 2022).

About a week after the prescribed fire, we prepared the site for transplanting. First, we set up the 10 replicate blocks for the blocked experimental design, with each block containing 4 plots, one for each treatment (40 plots in total). We constructed a cage of chicken wire (2 ft by 5 ft) around each plot to deter large herbivores. We planted one seedling of each species of the appropriate inoculation treatment in each plot, for a total of eight seedlings per plot. Seedling species were randomly arranged within each plot. After each seedling was planted, initial height measurements were recorded and each plot was watered. We returned after 3 days and watered one final time.

We monitored the legumes throughout the summer, taking four height measurements throughout the growing season and rating the overall health of the legumes twice. We measured the health of the legumes by color as well as number of healthy leaves and stems without knowledge of treatment (Table 3). At the end of the growing season (September 2022), we clipped the above ground biomass of the legumes before drying them at 60°C for 48 hours before weighing them.

Score	Description
0	Dead plants, no living leaves/stems
1	Almost dead with significant browning, little to no living leaves/stems
2	Sickly, less green than yellow leaves/stems
3	Mostly green plants, with little yellow, mostly healthy leaves/stems
4	Green plants with all green leaves/stems
5	Vibrant green plants with lots of healthy leaves/stems

Table 3: System for scoring seedling health by color and abundance of healthy leaves and stems.

Statistical analysis

We used R (v. 4.0.4; R Core Team 2021) for data analysis. We analyzed seedling survival using generalized linear mixed models with binomial errors (function glmer in library lme4; Bates et al. 2015). We combined the species into one model, using species and blocks as random effects. Coefficient of conservatism and single inoculation by rhizobia and AMF were fixed effects, used to test for synergistic interactions between AMF and rhizobia as well as to see if the rhizobia and AMF interaction depended on the coefficient of conservatism. We repeated this analysis to test the effect of AMF without rhizobia (removing all seedlings inoculated with rhizobia), rhizobia without AMF (removing all seedlings inoculated with AMF), and dual inoculation (removing all single-inoculated seedlings). We analyzed seedling health using similar analyses with binomial errors. We coded seedlings with a health score of 0, 1, or 2 as “dying” and seedlings with a health score of 3, 4, or 5 as “living” from measurements taken in September (Table 3).

To analyze seedling growth across all species, we analyzed seedling biomass and height from September independently. Using generalized least square models, which allow variances to increase with means (function gls in library nlme, weights=varPower, Pinheiro et al. 2021) without allowing for random effects. To avoid pseudoreplication while also testing for the effects of single and dual inoculation interactions across species, values from all replicates were averaged for each treatment for each species before using the species as replicates in a model with inoculation by rhizobia and AMF as fixed factors. Additionally, we used species averages to test for the overall effects of rhizobia without AMF, AMF without rhizobia, and dual inoculation as implemented above.

To test individual species’ growth responses to inoculation, we implemented separate generalized least squares analyses for each legume species separately. We tested the effect of rhizobia by excluding all plants inoculated with AMF, and similarly tested AMF by excluding all plants inoculated with rhizobia. Finally, to test for the synergistic effect, we tested for the significance of the interaction p-value in a two-factor analysis with both AMF and rhizobia treatments.

Finally, to determine if the effects of mutualists depended on species coefficients of conservatism, we calculated log response ratios (LRR) to quantify the response of each species to AMF, rhizobia, and their synergistic interaction (Fig. 7, LRR modified from Primieri et al. 2022). From there we used linear regressions with the coefficient of conservatism as a predictor of the mutualist LRR.

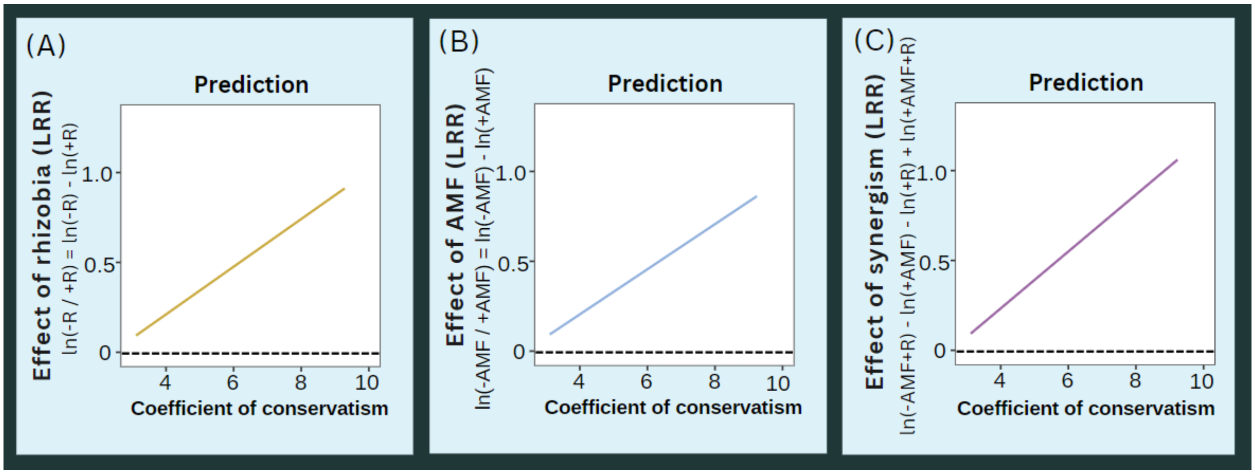

Figure 7: Equations and predictions for calculating log response ratio (y-axis) against increasing coefficient of conservatism (x-axis) for the effect of (A) rhizobia, (B) AMF, and (C) synergism. Formulas for rhizobia and AMF were modified from Primieri et al. 2022 to remove all plants inoculated with AMF and rhizobia, respectively.

RESULTS

Seedling survival response to microbial treatments

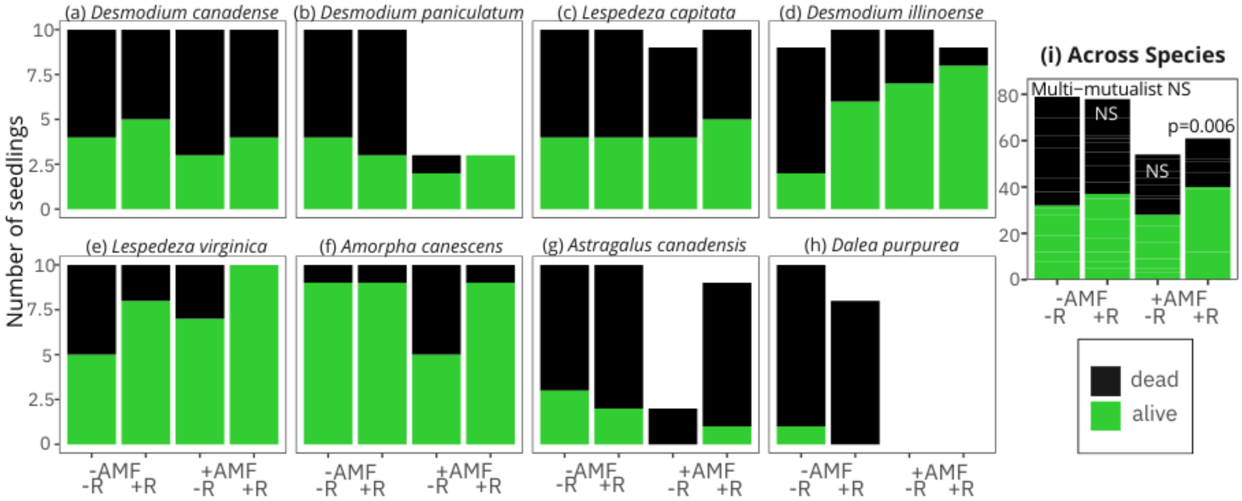

Figure 8: Individual species survival at the end of the growing season in order of increasing coefficient of conservatism (a-h) and summed survival of seedlings across species (i). Treatments include -AMF for no added AMF and -R for no added rhizobia, and +AMF and +R for added AMF and rhizobia, respectively. Green indicates living plants and black indicates dead plants. Across all species (i), statistical significance compared to control, uninoculated seedlings is indicated on each treatment and in the top left corner for synergistic effects. We lacked the power to test significance per individual species (a-h).

Dual inoculation of rhizobia and AMF significantly increased seedling survival across species, while single inoculation of AMF and rhizobia did not (Fig. 8i). By the end of the growing season, only 41% of control seedlings had survived across species, compared to an increase to 47% of those inoculated with only rhizobia (p=0.4) and 52% of those inoculated with only AMF (p=0.3). Overall dual inoculation had a significant increase for survival rate, increasing from 41% to 66% (p=0.006), averaged across species. We found no evidence of synergistic multi-mutualist effects (p=0.287). Due to the lack of power, we were unable to perform statistical tests on a species-by-species basis; however dual inoculation did appear to increase survival for mid to mid-late successional species *L. capitata*, *D. illinoense*, and *L. virginica* (Fig. 8c, 8d, and 8e, respectively). This was not always the case, as dual inoculation appeared to decrease survival of late successional species *A. canadensis* (Fig. 8g). Mid-late successional species *A. canescens* (Fig. 8f) saw no change with survival for dual inoculation compared to the control treatment or single inoculation of rhizobia.

Seedling health response to microbial treatments

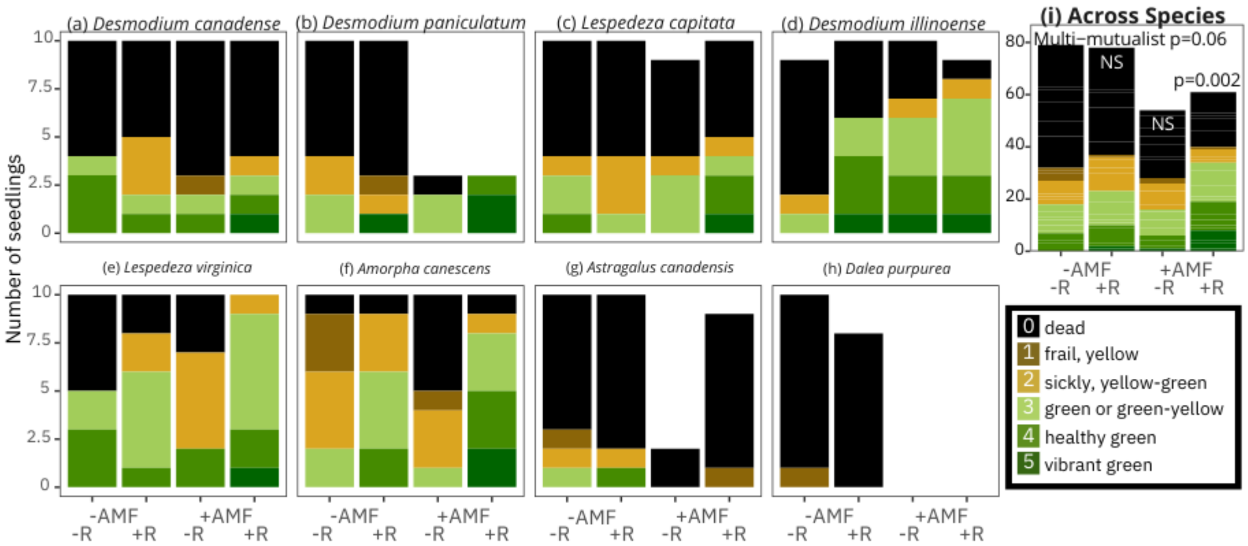

Figure 9: Individual species health by color and abundance of leaves and stems from 0-5 at the end of the growing season (Table 3) in order of increasing coefficient of conservatism (a-h) and summed across species (i). Treatments include -AMF for no added AMF and -R for no added rhizobia, and +AMF and +R for added AMF and rhizobia, respectively. Across all species (i), statistical significance compared to control, uninoculated seedlings is indicated on each treatment and in the top left corner for synergistic effects. We lacked the power to test significance per individual species (a-h).

Dual inoculation did show significant positive effects on seedling health (Figure 9i; p=0.002), with seedling health being higher across all species regardless of life history. Due to lack of power, we were unable to perform statistical tests on each species; however, an apparent positive effect of dual inoculation was strongest in *D. illinoense*, *L. virginica*, and *A. canescens* where 78%, 90%, and 80% of dual inoculated seedlings were healthy compared to 10%, 50%, and 20% of uninoculated seedlings, respectively (Fig. 9d, 9e, and 9f, respectively. We did see marginally significant synergistic effects of dual inoculation across species (Fig. 9i; p=0.06), with overall greater than additive increases in health. Synergistic effects seem most apparent in mid- to late-successional *L. virginica* and *A. canescens* (Fig. 9e and 9f, respectively).

Seedling above ground biomass response to microbial treatments

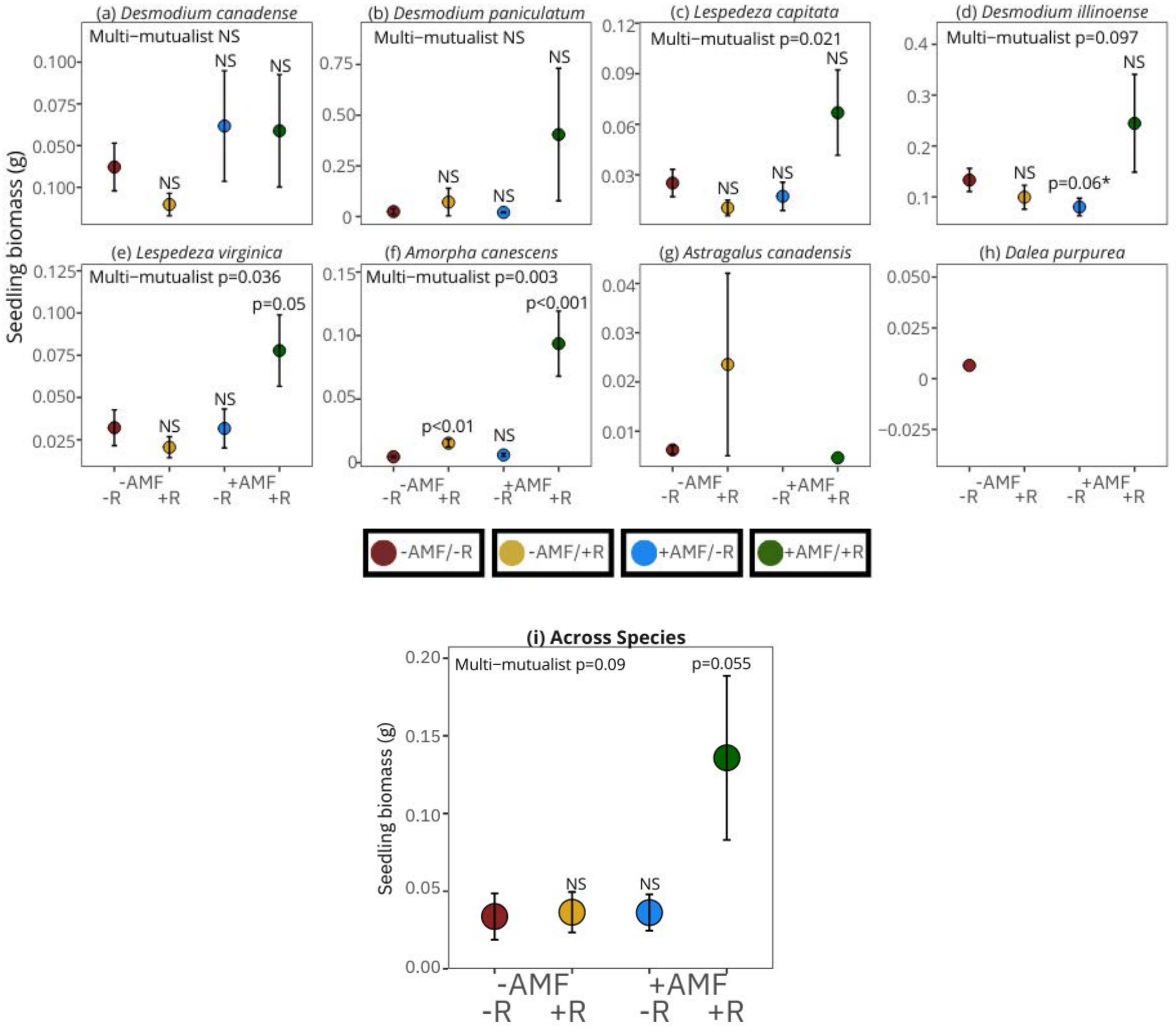

Figure 10: Above ground biomass at the end of the growing season by species in order of increasing coefficient of conservatism (a-h) and across all species (i). Treatments include -AMF for no added AMF and -R for no added rhizobia, and +AMF and +R for added AMF and rhizobia, respectively. * indicates a negative effect of microbial treatment (parasitic).

Across species, dual inoculation did have marginally significant positive effects (Fig. 10i, p=0.055) and synergistic effects (Fig. 10i, p=0.09) on above ground biomass. No individual species saw significant increases in above ground biomass from inoculation of AMF or rhizobia (Fig. 10), with the exception of *A. canescens* with single inoculant rhizobia (Fig. 10f, p<0.001). *D. illinoense* saw a negative (parasitic) marginally significant effect from single inoculation of AMF on biomass (Fig. 10d, p=0.06). Dual inoculation significantly increased biomass of *L. virginica* (Fig. 10e, p = 0.05) and *A. canescens* (Fig. 10f, p<0.001). Effects of dual inoculation were significant or marginally significantly synergistic for *L. capitata* (Fig. 10c, p=0.097;), *D. illinoense* (Fig. 10d, p=0.097)*, L. virginica* (Fig. 10e, p=0.036)*,* and *A. canescens* (Fig. 10f, p=0.003). Two early successional species, *D. canadense* and *D. paniculatum*, showed no significant response to any inoculation treatment (Fig. 10a-b).

Seedling height response to microbial treatments

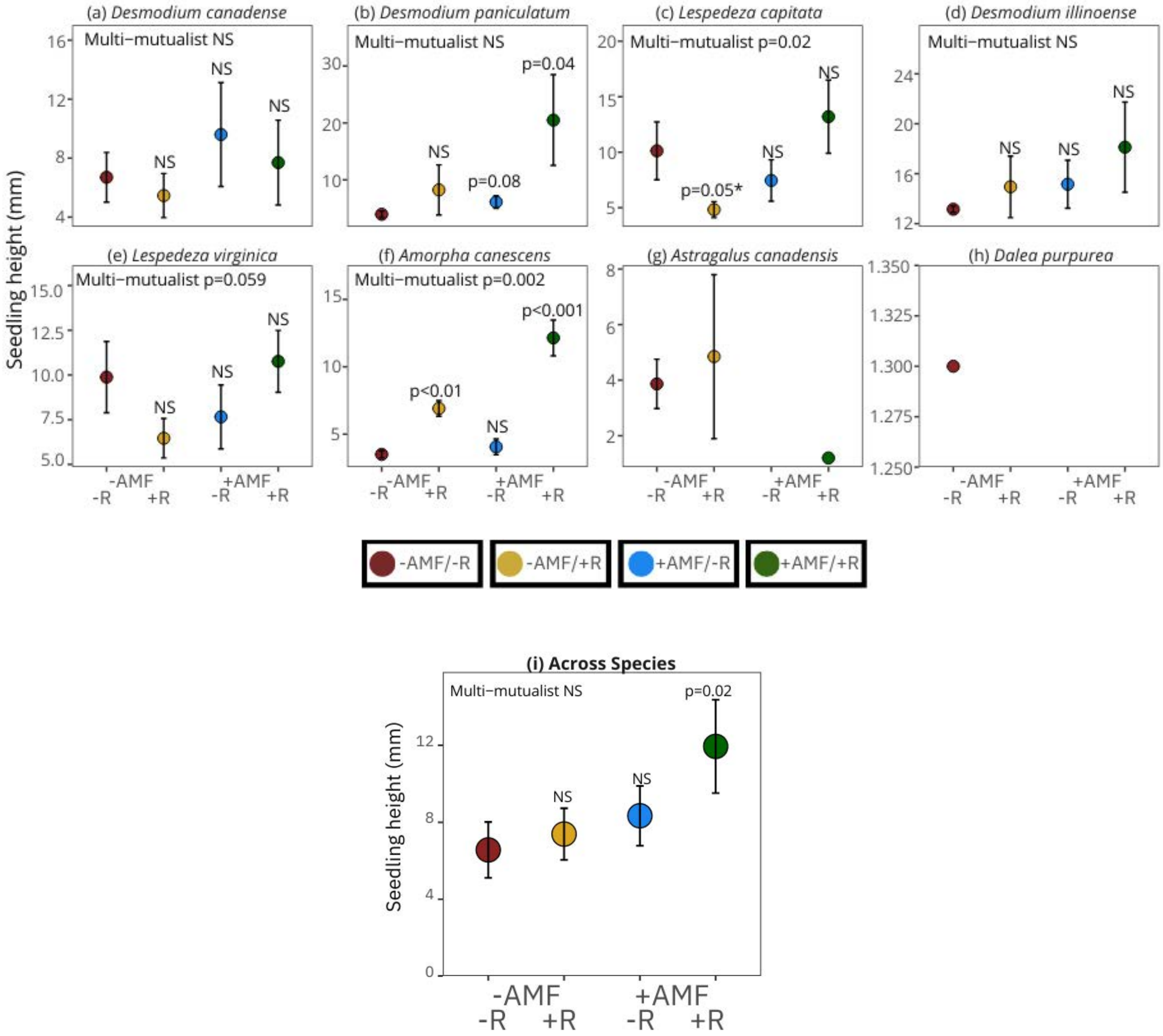

Figure 11: Height at the end of the growing season by species in order of increasing coefficient of conservatism (a-h) and across all species (i). Treatments include -AMF for no added AMF and -R for no added rhizobia, and +AMF and +R for added AMF and rhizobia, respectively. * indicates a negative effect of microbial treatment (parasitic).

Across species, dual inoculation increased seedling height compared to uninoculated control seedlings (Fig. 11i, p=0.02). Single inoculations by AMF or R did not increase seedling height with the exception of *A. canescens*, which grew taller with rhizobia (Fig. 11f, p<0.01). AMF marginally significantly increased height of *D.*

paniculatum (Fig. 11b, p=0.08). Rhizobia inoculation decreased *L. capitata* height (Fig. 11c, p=0.05). Dual inoculations significantly increased height for *D. paniculatum* (Fig. 11b, p=0.04) and *A. canescens* (Fig. 11f, p<0.001). *L. virginica* had a marginally significant synergistic effect (Fig. 11e, p=0.059); *L. capitata* and *A. canescens* had significant synergistic effects in height (Fig. 11c, p=0.02; Fig. 11f, p=0.002, respectively).

Impact of microbial treatments on species with varying life histories

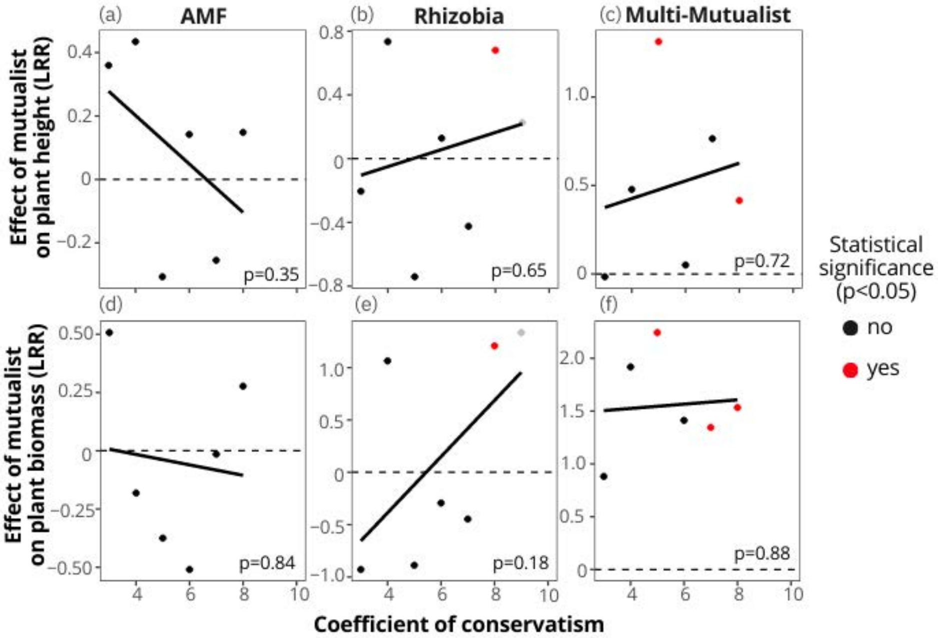

Figure 12: Natural log of the response ratio (LRR) for legume height (a-c) and biomass (d-f) against legume successional status (coefficient of conservatism) for AMF (a,d), rhizobia (b,e), and synergistic multi-mutualists (c,f). Mutualistic effects significantly different from zero are marked with a red dot and non-significant mutualistic relationships are marked with a black dot.

The effect of AMF on legume height or biomass did not increase with increasing coefficient of conservatism (Fig. 12a, p=0.35; Fig 13d, p=0.84). However, legume biomass responses to rhizobia did tend to strengthen non-significantly in later successional species (Fig. 12e, p=0.18). The synergistic effect of both mutualists increased slightly but non-significantly with increasing successional status (Fig. 12c, p=0.72; Fig. 12f, p=0.88). There was no trend of increasing synergistic effects with successional status for biomass (Fig. 12e) or height (Fig. 12c).

DISCUSSION

Synergistic and additive effects on legumes with varying life histories

Our results found that dual inoculation with both rhizobia and AMF synergistically increased seedling health and above ground biomass across species. Four of the eight species experienced synergistic effects for above ground biomass with dual inoculation: *L. capitata*, *L. virginica, D. illinoense* (marginally significant)*,* and *A. canescens*. Similarly, three species experienced synergistic effects for height with dual inoculation: *L. capitata*, *L. virginica* (marginally significant)*,* and *A. canescens*. This supports our hypothesis that dual inoculation promotes synergistic growth in later successional species. However, despite mid and late successional species experiencing synergistic effects from dual inoculation, we did not find evidence for increasing magnitude of synergistic effects with increasing coefficient of conservatism (i.e., successional status). These results suggest that late successional species do not see greater returns for these multi-mutualistic relationships, but dual inoculation does synergistically increase mid and late successional species biomass and height.

Our findings that late successional species experience synergistic effects is supported by other studies. One study found synergistic effects for late successional legume *A. canescens* (Larimer et al. 2010), while a meta-analysis found that perennial legume species experienced synergistic effects from dual inoculation while annuals did not (Primieri et al. 2022). While these studies are consistent with our findings, it is important to note that we were unable to collect data on below ground biomass. This may be important because all eight species were perennials who invest in below ground biomass during their first growing season, therefore synergistic effects may be even stronger than our data can demonstrate.

Further research is needed in order to understand the full extent of the reliance of late successional legumes on multi-mutualists. To expand upon these findings, a similar field experiment with a greater number of replicates and annual native prairie species could confirm whether the impact of multi-mutualisms depends on successional status. Additionally, studying these relationships over a longer time frame rather than a single growing season would give further insight into multi-mutualistic impacts on mid and late successional species as they mature. This would help determine whether synergistic effects grow stronger or weaker over time.

Dual inoculation effects on legumes of varying life histories

We found that dual inoculation with both rhizobia and AMF increased seedling survivorship, health, above ground biomass, and height across species. Dual inoculation across species increased seedling survival from 41% to 66%, and surviving seedlings were more likely to have a greater above ground biomass and height. Among individual species, mid to late successional legumes inoculated with both AMF and rhizobia tended to have greater above ground biomass than uninoculated treatments, while early to mid-successional species tended to show no significant effect of dual inoculation. Height only increased for two species: one early and one late successional species. This partially contradicts our hypothesis that early successional species would experience additive effects on above ground biomass and height from dual inoculation.

We found that dual inoculation increased above ground biomass for mid and late successional species *L. virginica* and *A. canescens*. Other studies support our findings that above ground biomass increases with dual inoculation for late successional legume *A. canescens* (Larimer et al. 2010) and perennial legumes in general (Primieri et al. 2022). Though our results support rhizobia and AMF dual inoculation having positive impacts on mid and late successional legume growth, this is likely dependent upon nutrient availability (Larimer et al. 2010) and specific strains of AMF (Tsikou et al. 2023), as this can impact mutualistic interactions. Additionally, only two of the eight legume species saw significant positive effects for height and above ground biomass from dual inoculation, though this is likely due to high variation in seedling size. Our results along with supporting literature suggest that rhizobial and AMF communities used in tandem can increase the survival and growth rates of legumes, allowing seedlings to have an increased chance of establishment.

Independent inoculant effects on legumes of varying life histories

Our study found single inoculation with rhizobia or AMF did not increase seedling survivorship, health, above ground biomass, or height across species. Several legume species experienced significant effects (mutualistic or parasitic) with single inoculation of rhizobia or AMF for above ground biomass and height, however we did not find evidence for increasing or decreasing magnitude of single inoculation effects with increasing successional status. While we cannot say inoculation of rhizobia or AMF improved seedling survival, across species survivorship increased from 41% to 47% and 52% for rhizobia and AMF inoculations, respectively. Some species did appear to survive more often with single inoculation, but without more replication we cannot draw meaningful statistical conclusions regarding those relationships.

Our findings that single inoculation of rhizobia and AMF does not increase seedling survival and biomass conflicts with our hypothesis that regardless of successional status, inoculation with rhizobia or AMF would support increased above ground biomass in legumes. However, other studies have found that single inoculation of rhizobia and AMF can increase legume and other native prairie plant survivorship (van der Hiejden et al. 2016; Koziol and Bever 2017). These differences may be in part because the rhizobia strains used for inoculation in our experiment had been pulled from the respective legume species in restored and remnant prairies but had not yet been tested for effectiveness; species-specific relationships can be critical to mutualism success (Middleton et al. 2015; Maltz & Tresder 2015; Tsikou et al. 2023). Another explanation may be that legacy effects caused by agricultural practices that continue to impact restored prairie nutrient levels and microbial communities (MacDonald et al. 2012; Jangid et al. 2010; Vazquez et al. 2022). We suggest further studies be done regarding the impact of single inoculations, specifically comparing isolated strains of rhizobia and AMF to specific legume species with varying life histories.

Conclusions

Our results support the idea that multi-mutualistic communities are important for the establishment (seedling survival and health) and growth (above ground biomass and height) of mid and late successional legume species. Furthermore, our findings suggest that rhizobia and AMF alone are not enough to provide significant effects on legume growth and that dual inoculation may be necessary for initial legume success in restored prairies. Our findings and a growing body of evidence suggest that soil microbial community health should be a focus of prairie restoration and are important for restoration success. However, more research is necessary to understand (1) how synergistic and additive effects may shift over time for mid and late successional species, (2) how multi-mutualistic relationships respond to varying environmental conditions (i.e., nutrient availability, soil composition, etc.), and (3) how the introduction of soil mutualists may impact restored prairie community composition and dynamics. By understanding the complexities behind prairie community interactions, we may increase the chance of prairie restoration success, preserving these ecosystems for generations to come.

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