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RESEARCH ARTICLE

Rare legumes are missing mutualists, but herbivory and environmental filtering are more important determinants of reintroduction success

Emily Galloway[1,2], Paul A. Price[3], Emily Grman[3], Jonathan T. Bauer[1,4]

Soil microbial mutualists like rhizobia bacteria can promote the establishment of rare, late-successional legumes. Despite restoration efforts, these mutualists are often absent in the microbiome. Therefore, restoring this mutualism by directly inoculating rare legumes with rhizobia mutualists may increase plant establishment. We inoculated seedlings of *Amorpha canescens*, *Dalea purpurea*, and *Lespedeza capitata* with three strains of species-specific rhizobia each to investigate how this mutualism would promote growth in the field and in the greenhouse. Because many herbaceous plants are vulnerable to herbivory, we used exclosures for half of our field transplantations to prevent mammalian herbivory. We did not find that rhizobia bacteria directly promoted the growth of our legumes in the field but rather that herbivory and environmental conditions overwhelmed the effects of the rhizobia. Of the plants transplanted, only 17.78% of 180 survived to the end of the growing season, all of which were protected from herbivory. Survival at the end of the growing season was also greater in the northern, drier end of the field site. In the second growing season, plants were more likely to survive in the exclosure treatment, while only four recovered in the open treatment. In the greenhouse, we found increased nodulation with inoculations, supporting the hypothesis that species-specific mutualists are absent from restoration sites. Though several recent studies have shown that restoring mutualistic interactions has the potential to dramatically improve the outcomes of ecological restoration, our results show that protecting rare species from herbivory after transplantation might achieve greater gains in establishment.

Key words: establishment limitation, inoculation, microbiome, prairie restoration, rhizobia

Implications for Practice

- Soil microbial mutualists are missing in restored sites, and inoculating leguminous plant species with species-specific rhizobia can increase nodule formation on legumes in restored environments, but other ecological interactions have the potential to overwhelm the benefits of microbial mutualists.
- Successful restoration of rare plant species may require installing exclosures to prevent herbivory.
- Our study suggests that herbivory and environmental context play a larger role in the success of rare legume establishment than missing microbial mutualists. These factors must be considered when developing restoration efforts to increase plant diversity.

Introduction

Mutualistic species interactions have clear potential to improve the outcomes of ecological restoration (Handel et al. 1994; Ribeiro da Silva et al. 2015; Derksen-Hooijberg et al. 2018). For example, mycorrhizal fungi can improve the establishment of rare species in prairie restorations (Koziol et al. 2022) and promoting frugivores can increase dispersal and diversity of tropical trees (Holl et al. 2020). However, despite these high-profile successes, many examples also exist where restoration of mutualism fails to contribute to restoration goals (Herzberger et al. 2015; Docherty & Gutknecht 2019; Holl et al. 2022). It is possible that this occurs because of the context dependency of mutualisms (Johnson et al. 1997). Environmental conditions at an ecological restoration site may alter the benefits provided by mutualistic interactions (Carrell et al. 2022), or other species interactions may overwhelm the potential positive effects of mutualisms (Reid & Holl 2013). We need a better understanding of how mutualists interact with other species interactions and environmental conditions to be able to predict when mutualisms will have the most benefits for ecological restoration.

Author contributions: EGa, PAP, EGr, JTB conceived and designed the research; EGa set up and measured the experiments; EGa, JTB analyzed the data and drafted the manuscript; EGa, PAP, EGr, JTB revised and edited the manuscript.

[1]Department of Biology, Miami University, Oxford, OH 45056, U.S.A.
[2]Address correspondence to E. Galloway, email gallowe@miamioh.edu
[3]Department of Biology, Eastern Michigan University, Ypsilanti, MI 48197, U.S.A.
[4]Institute for the Environment and Sustainability, Miami University, Oxford, OH 45056, U.S.A.

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We investigated legume-rhizobia mutualism to test if mutualistic microbial interactions can improve the establishment of rare legumes in restored prairies. Compared to weedy legumes that are not as particular about their rhizobia partners, rare legumes are an ideal system because of their strong dependence on rhizobia mutualists (Graham 2005; Van Der Heijden et al. 2006), and because many species of these legumes are difficult to restore in our study system (Grman et al. 2015). Previous research has shown that this dependency on rhizobia could cause establishment limitation when these bacteria are absent (Simonsen et al. 2017; Grman et al. 2020), and the presence of rhizobia in the soil plays a crucial role in the ability of legumes to coexist with competitive plant species (Tilman 1997; Trannin et al. 2000; Elias & Agrawal 2021). Further, the presence or absence of legume-rhizobia mutualisms have strong effects on community assembly (Bauer et al. 2012; Keller 2014), and restoring this mutualism is potentially important because of the essential role of legumes and rhizobia in nitrogen cycling (Xiao et al. 2019). Given the potential importance to rare species establishment, plant community assembly, and ecosystem function, the effectiveness of rhizobia mutualists when challenged by other ecosystem factors must be explored.

Pressures from herbivory may be an additional challenge in the reintroduction success of plants (Ritchie & Tilman 1995; Fletcher et al. 2001; Ruhren & Handel 2003). Frequent grazing has been found to reduce carbon allocation in root biomass (Holland & Detling 1990), which could reduce the survival of perennial plants (Knops et al. 2000; Buisson et al. 2015), decelerate N-cycling of legumes (Ritchie et al. 1998), limit seed recruitment (MacDougall & Wilson 2007), and decrease plant establishment (Garzón-Machado et al. 2010). The introduction of rhizobia, however, may positively or negatively interact with the effects of herbivores. The alleviation of nitrogen limitation by rhizobia mutualists has the potential to increase the palatability of legumes, subjecting them to a higher susceptibility to herbivory (Kempel et al. 2009; Winbourne & McCulloch 2022). On the other hand, the increase in nitrogen availability may promote the plant's defense mechanisms and compounds against herbivore pressure and perhaps improve a plant's ability to recover from damage (Thamer et al. 2011; Barker et al. 2022). To successfully reintroduce rare legumes to a restored ecosystem, it may be necessary to both enhance the plant via rhizobia inoculation and protect the plant via herbivore exclosures.

Plant establishment may be further limited by abiotic conditions within the landscape. Plants have clear niche differences along environmental gradients (Kobe 1999; Silvertown et al. 1999; Comita & Engelbrecht 2009), and environmental conditions such as water availability and soil nutrients can determine restoration outcomes (Sherry et al. 2012; Barak et al. 2017; Catano et al. 2022). Mutualistic interactions, such as the presence of N-fixers in low N environments, could broaden a species' niche, allowing persistence and enhancing restoration outcomes across a broader range of environmental conditions (Koffel et al. 2021). However, the benefit of mutualisms may depend on environmental conditions. For example, rhizobia may be most beneficial under dry conditions by increasing drought tolerance (Staudinger et al. 2016; Álvarez-Aragón et al. 2023). The complexity and context-dependent nature of mutualisms emphasizes the importance of considering environmental context when exploring how mutualisms influence the establishment of rare legumes.

In this study, we tested the hypothesis that missing mutualists can limit the establishment of rare plant species in restorations. We further explored the relative importance of legume-rhizobia mutualisms compared to other ecological interactions. We predicted that rhizobia inoculation in the field would increase the success of legumes, but that the effects of inoculation will only matter if our plants are able to withstand herbivory and acclimate to a novel environment. In the greenhouse, we predicted that rhizobia inoculation would increase nodulation and growth in our legumes in both sterile soil and live field soil.

Methods

Plant Selection and Preparation

We chose the three leguminous plant species *Lespedeza capitata*, *Amorpha canescens*, and *Dalea purpurea* as our three study plant species for both the greenhouse and field experiments. *Amorpha canescens* and *D. purpurea* are two late-successional plant species, meaning they tend to be slow-growing, long-lived, and sensitive to disturbance (Bauer et al. 2018). These species are frequently absent from restorations despite being included in seed mixes. *Lespedeza capitata* is a mid-successional plant that does not establish reliably. Our past research has shown that these plant species are more reliant on soil microbial mutualisms compared to those without microbial associations and that these essential microbes are often absent from restored prairie soils (Grman et al. 2020).

Rhizobia Selection and Inoculation Preparation

Due to the high degree of specificity these legumes express with their partners, we chose strains of rhizobia that can successfully associate with each study plant species (Table S1). These rhizobia were cultured from nodules on roots of our three study species grown in soils sourced from remnant prairies throughout Michigan and Indiana (full methods in Grman et al. 2020). Of these strains, we chose three strains for each plant species based on nodule quality and similarity to our study site's soil conditions based on soil texture. Plump pink nodules were assumed to be higher quality. Frozen glycerol stocks of each strain of rhizobia were individually cultured on sterile TYME media plates (Mohamed et al. 2021) for 5–7 days. Each cultured plate was repeatedly cultured three times to ensure we had established a pure culture.

Individual strains were measured to 0.1 at OD_{600} (Gourion et al. 2013) suspended in deionized (DI) water using a spectrophotometer to standardize the cell densities of each strain. We combined the three strains of rhizobia for a single plant species and measured spectrophotometry again. We inoculated our treatment plants with their respective rhizobia by

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pipetting 1 mL of the suspensions at the base of each plant; control plants received 1 mL of sterile DI water at their base.

Field Experiment

The field portion of this study was established in the prairie restoration at the Ecology Research Center (ERC) of Miami University (69 ha, 39.53253, −84.72331). This site was converted from agricultural land to restored prairie in 1974 by sowing with a mix of grass species, including *Andropogon gerardii*, *Schizachyrium scoparium*, *Sorghastrum nutans*, *Bouteloua curtipendula*, *Panicum virgatum*, and *Phalaris arundinacea* (Huffman et al. 1986). The site has retained its heavy stands of C4 prairie grass species, primarily *A. gerardii* and *S. nutans*, and additional non-sown species have colonized the site from the surrounding landscape, with the most common species being *Solidago canadensis*, *Dipsacus laciniatus*, and *Poa pratensis* (additional vegetation survey information in Cazzato et al. 2022). Vegetation surveys of this prairie show a gradient of plant community composition from north to south, with a dry-mesic community at the north end, where *Poa* and *Solidago* are most common, and a wet-mesic community at the south end, where *Andropogon* is more abundant. *Sorghastrum* and *Dipsacus* are common throughout the site.

We created a 2 × 2 factorial experiment to test the effects of rhizobia inoculation and herbivory on legume growth and survival. This experiment included 15 replicates of each plant species, rhizobia treatment, and herbivore exclosure treatment for a total of 180 plants. Three days after inoculation, we randomly transplanted seedlings 2 m apart along five 48 m north–south transects at the ERC and noted their position as a covariate. We chose this timing to allow nodules to begin to develop while avoiding large differences between treatments before planting in the field. We created individual exclosures to prevent herbivory from large ungulates, such as white-tailed deer, by rolling fencing material (stainless steel, 1 cm mesh, 60 cm height, 10 cm in diameter) into cylinders and placing them around our transplanted seedlings. We measured stem height bi-weekly from the transplantation date in July–October 2021 to measure the change in response to inoculation. Through this, we were also able to record the presence or absence of aboveground biomass throughout the growing season. We recorded “present aboveground” rather than survival because we could not reliably distinguish

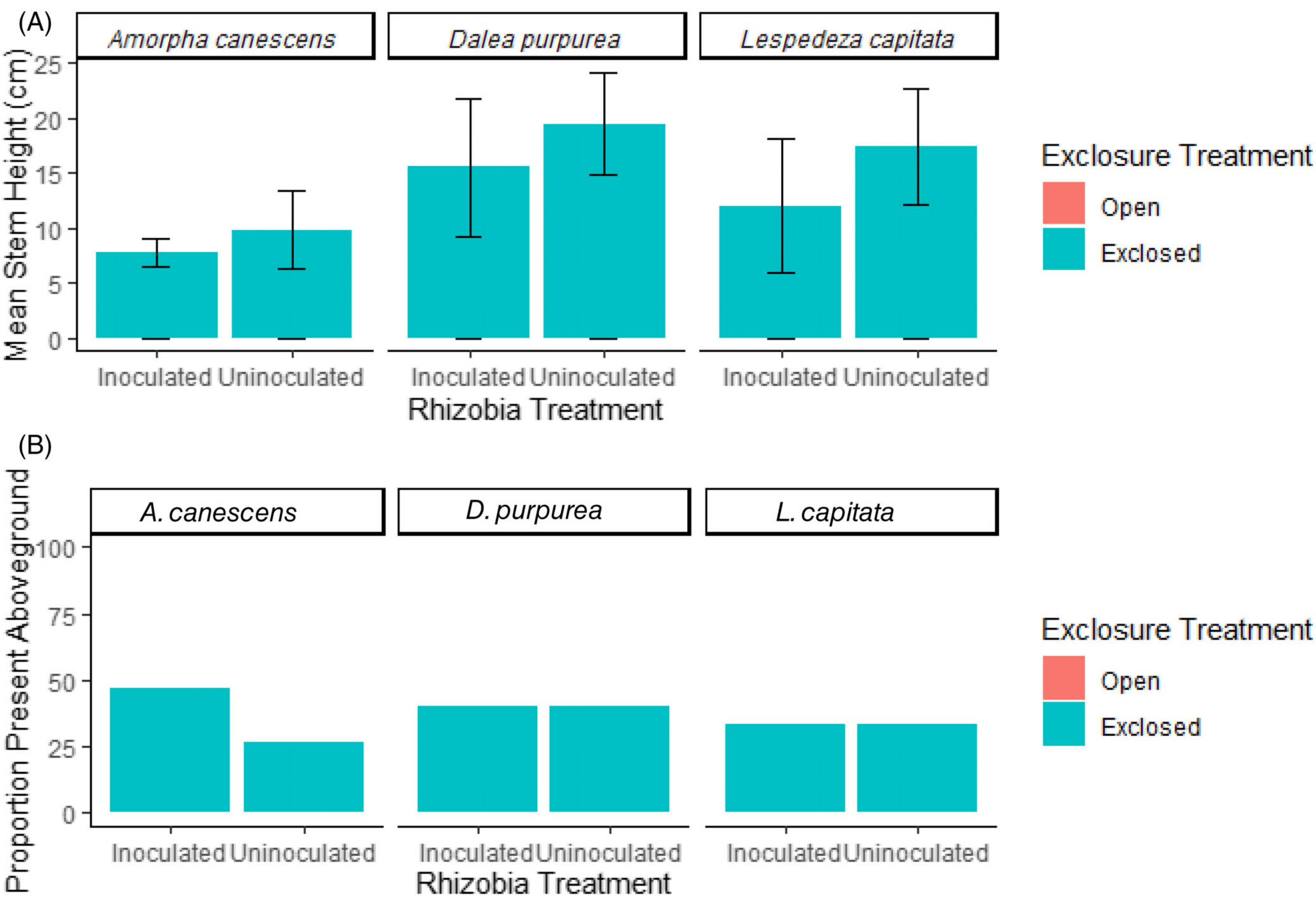

Figure 1. (A) Mean stem height (± SE) of each plant species and (B) proportion of plants that were present aboveground with or without rhizobia inoculation on the final day of measurements (day 107) in each exclosure treatment. There is no height data for plants in the open treatment because there were no plants present aboveground on the final day of measurements.

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between plants that had been browsed, senesced aboveground, or actually died. In early June 2022, we measured stem height and survival after one growing season.

Greenhouse Experiment

To test the effects of rhizobia under more controlled conditions, we tested the effects of rhizobia on plants in both sterile soil and live soil collected from the ERC. The inclusion of the live soil treatment gave us the ability to test whether our inoculation treatments performed better than any rhizobia already in the soil and if our isolates improved plant growth. We randomly collected and homogenized 10 soil cores throughout the same location as our field study using a 2-cm-wide soil probe to a depth of 15 cm.

Each replicate was planted in 656 mL D40H Deepots (Stuewe and Sons, INC, Tangent, OR, U.S.A.) that were filled 75% with 50:50 autoclaved sand:soil mix. Plants included in the live soil mixture received 15 mL of live soil followed by a cap of sterile sand: soil mix. Control soil treatments received only the 50:50 sterile sand:soil mix. We transplanted one germinated seedling of each plant species into each Deepot and measured its aboveground height, and then rhizobia inoculated each treatment as described above. In total, we had seven replicates of each of the three plant species, two soil treatments (sterile and live), and two rhizobia treatments (inoculated and control) for a total of 84 plants. After growing in the Deepots for 14 weeks, we measured stem height, dry weight of below- and aboveground biomass, and nodule count.

Statistical Analysis

We conducted all data analyses in R 4.3.1 (R Core Team 2023). For the field experiment, we conducted quasi-binomial generalized linear models, the function glm in base R, to analyze plant survival in 2021 and 2022. We used the variables of rhizobia treatment, enclosure treatment, transplantation position at the ERC, and the interactions between these variables in our analysis. To obtain test statistics, we conducted F tests on our quasi-binomial models.

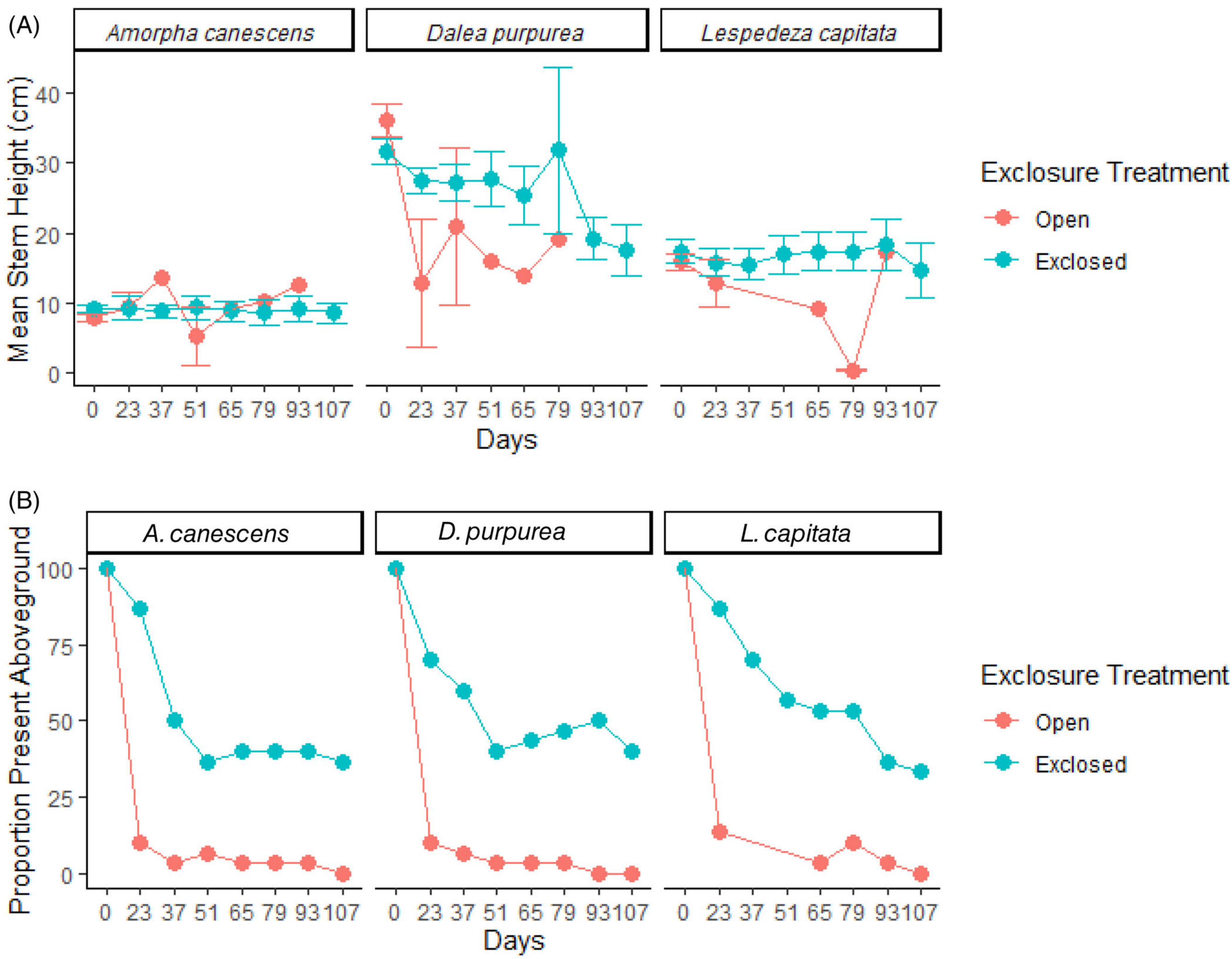

Figure 2. (A) Mean stem height (± SE) and (B) proportion of plants that had aboveground biomass present of all three plant species summarized at each biweekly measurement day in both exclosure treatments.

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For our analyses of stem height in 2021 and 2022, we removed plants that were not present aboveground from the dataset. For our 2021 analysis, we conducted a repeated measures ANOVA, a function in base R, on stem height with the variables: rhizobia treatment, exclosure treatment, plant species, transplantation position at the ERC, and the interactions between these variables as fixed effects. We used these same variables and the interactions between the variables in our ANOVA model for analysis of stem height in 2022, once again excluding plants that were not present aboveground from the dataset.

For the greenhouse experiment, we first summed the dried above- and belowground biomass of each plant (library doBy; Højsgaard & Halekoh 2023). We used a linear model to analyze the biomass data with rhizobia treatment, soil treatment, plant species, and all interactions between these variables. Each species was divided into subsets for our nodule analysis and then fit a quasi-Poisson generalized linear model with distribution on nodule counts with the variables rhizobia treatment, soil treatment, and the interactions between the two.

Results

Field Experiment

Rhizobia inoculation did not increase stem height; instead, inoculated plants tended to be shorter (Table S2; Fig. 1A; $F_{[1,92]} = 2.859$, $p = 0.094$). The effects of inoculation did not interact with exclosure treatment (Table S2; $F_{[1,92]} = 0.169$, $p = 0.682$). Similarly, we did not find evidence of inoculation improving the presence of aboveground biomass of our legumes in the field (Table S3; Fig. 1B; $F_{[1,1,437]} = 0.033$, $p = 0.857$).

However, exclosures substantially improved seedling survival throughout the 2021 growing season. Exclosure treatment did not appear to affect the size of our transplanted seedlings

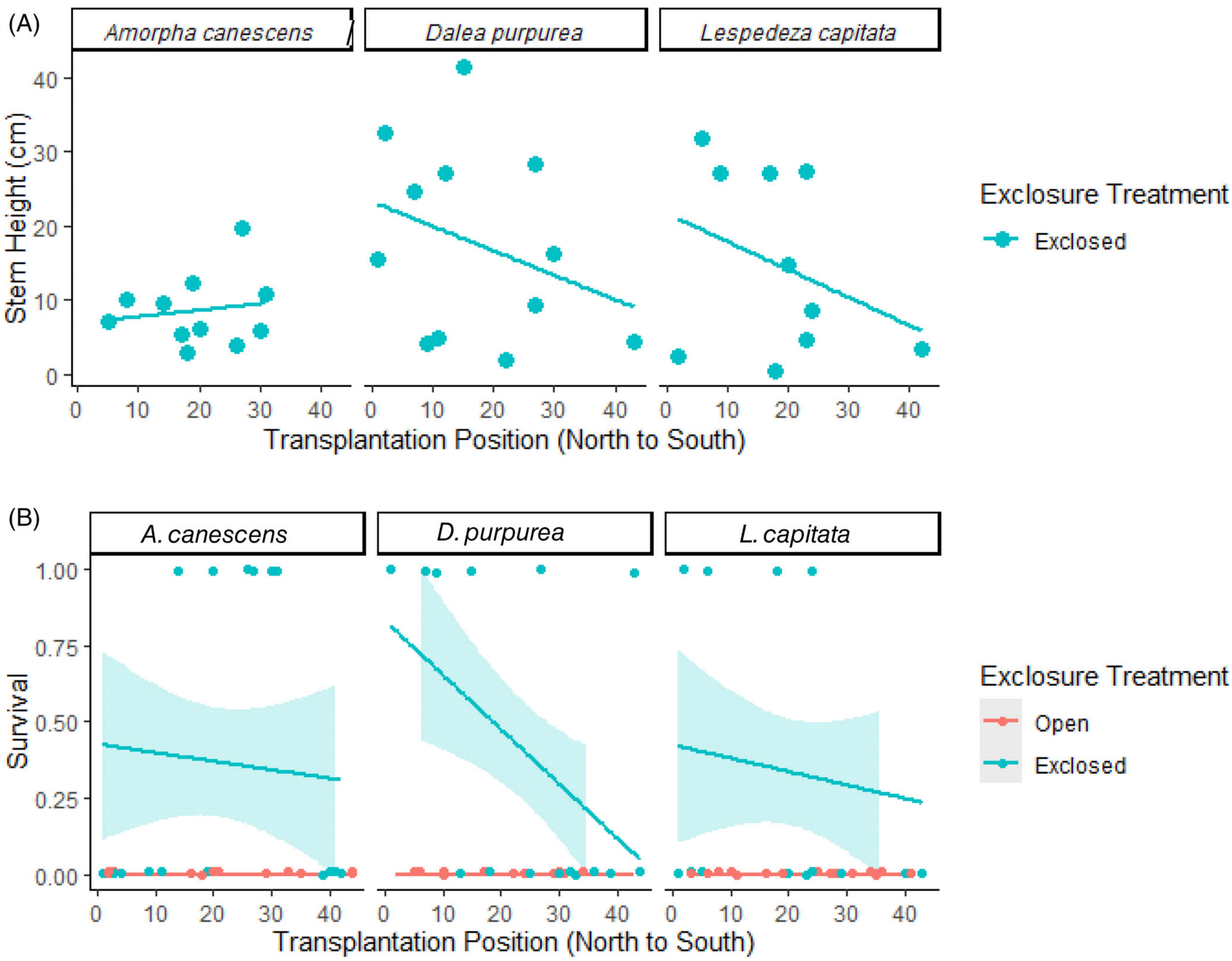

Figure 3. (A) Mean stem height and (B) survival at each transplantation position (north to south). The transplantation position numbers follow the environmental shift in the prairie restoration where the community composition changes from dry-mesic in the north end to wet prairie in the south end of the site. Data is missing for (A) mean stem height of each plant species in the open treatment because there were no plants present aboveground on day 107.

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(Table S2; Fig. 2A; $F_{[1,92]} = 0.187$, $p = 0.667$), but legumes in exclosures had greater survival at each time point (Table S3; Fig. 2B; $F_{[1,1438]} = 270.479$, $p < 0.0001$). At the end of the first growing season, 35.56% of the exclosed plants were present aboveground, but no plants were present aboveground when herbivores had access.

Along our transects, mean stem height (Fig. 3A) and presence (Fig. 3B) were greater toward the northern, drier end of the prairie (represented by lower position numbers) on the final day of measurements (Table S4; $F_{[1,21]} = 2.363$, $p = 0.139$; Table S5; $F_{[1,176]} = 7.201$, $p = 0.008$). The effects of environmental conditions on seedling size were only apparent when herbivores were exclosed since no plants were present aboveground when exposed to herbivores. We additionally did not find an interaction between rhizobia inoculation and transplantation position within the prairie on stem height (Table S4; Fig. 4A; $F_{[1,21]} = 0.315$, $p = 0.581$) or survival at the end of the growing season (Table S5; Fig. 4B; $F_{[1,173]} = 1.462$, $p = 0.228$).

In the second year of our experiment, 87.5% of the plants that were remaining aboveground at the end of the previous year resprouted. Of the seedlings that were not recorded aboveground at the end of the previous year, only 13.51% resprouted. Overall, rhizobia inoculation did not increase the stem height of all seedlings (Table S6; Fig. 5A; $F_{[1,33]} = 0.924$, $p = 0.343$). In the exclosures, 47.78% of plants were present aboveground in the second year, but only 4 of 90 plants (4.4%) were present when herbivores were not exclosed (Table S7; Fig. 5B; $F_{[1,178]} = 70.522$, $p < 0.0001$). Notably, all four of the plants that survived to the second year in the open treatment were individuals that were inoculated with rhizobia. However, few enough survived in the open treatment that differences in survival between inoculated and control plants were not statistically significant (Table S7; $F_{[1,177]} = 2.474$, $p = 0.118$).

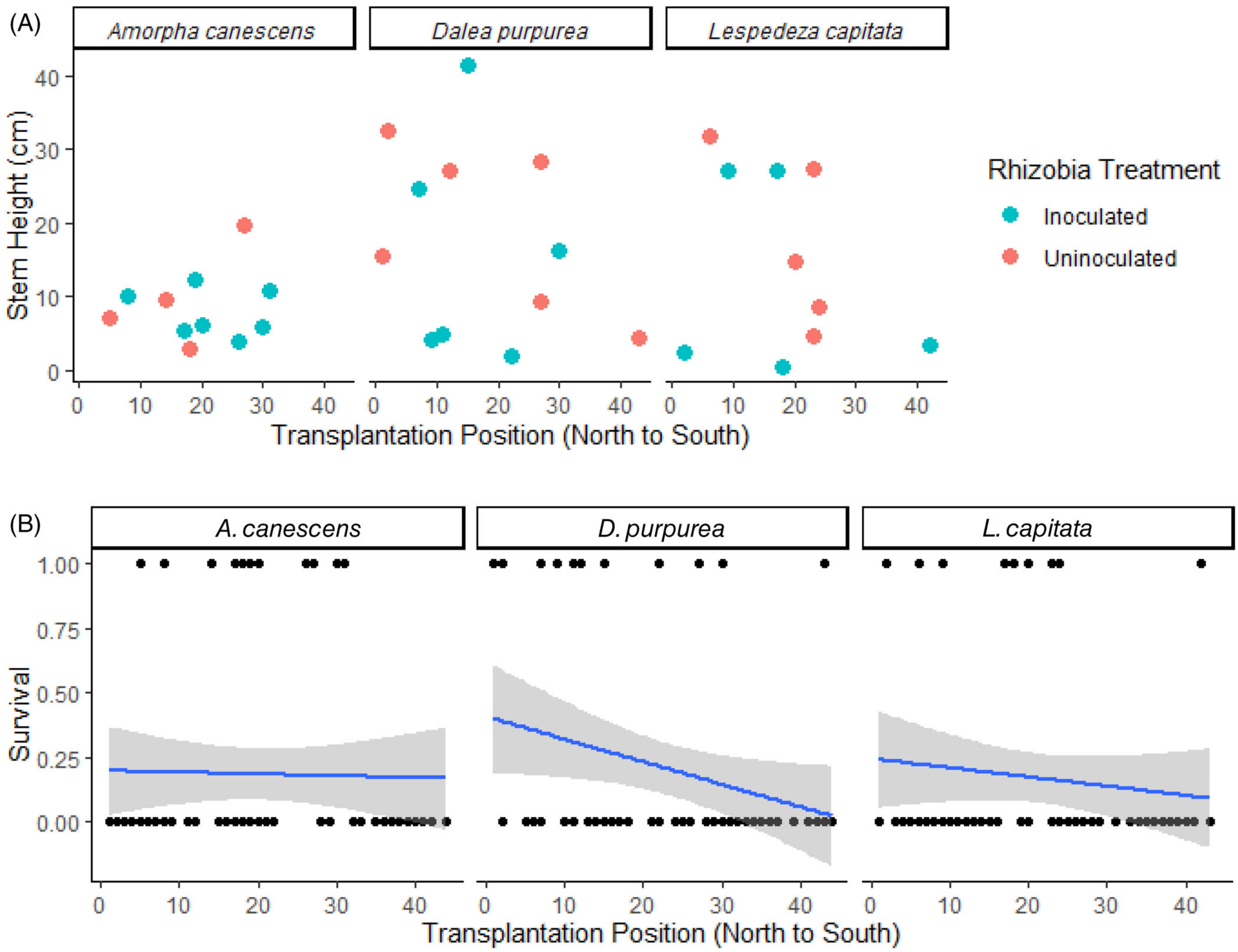

Figure 4. (A) Mean stem height and (B) survival at each transplantation position. The transplantation position numbers follow the environmental shift in the prairie restoration where the community composition changes north to south from dry-mesic to wet prairie. Rhizobia inoculation treatments for survival (B) were summarized because there was not a significant difference in survival for each plant species whether they were inoculated with rhizobia bacteria or not.

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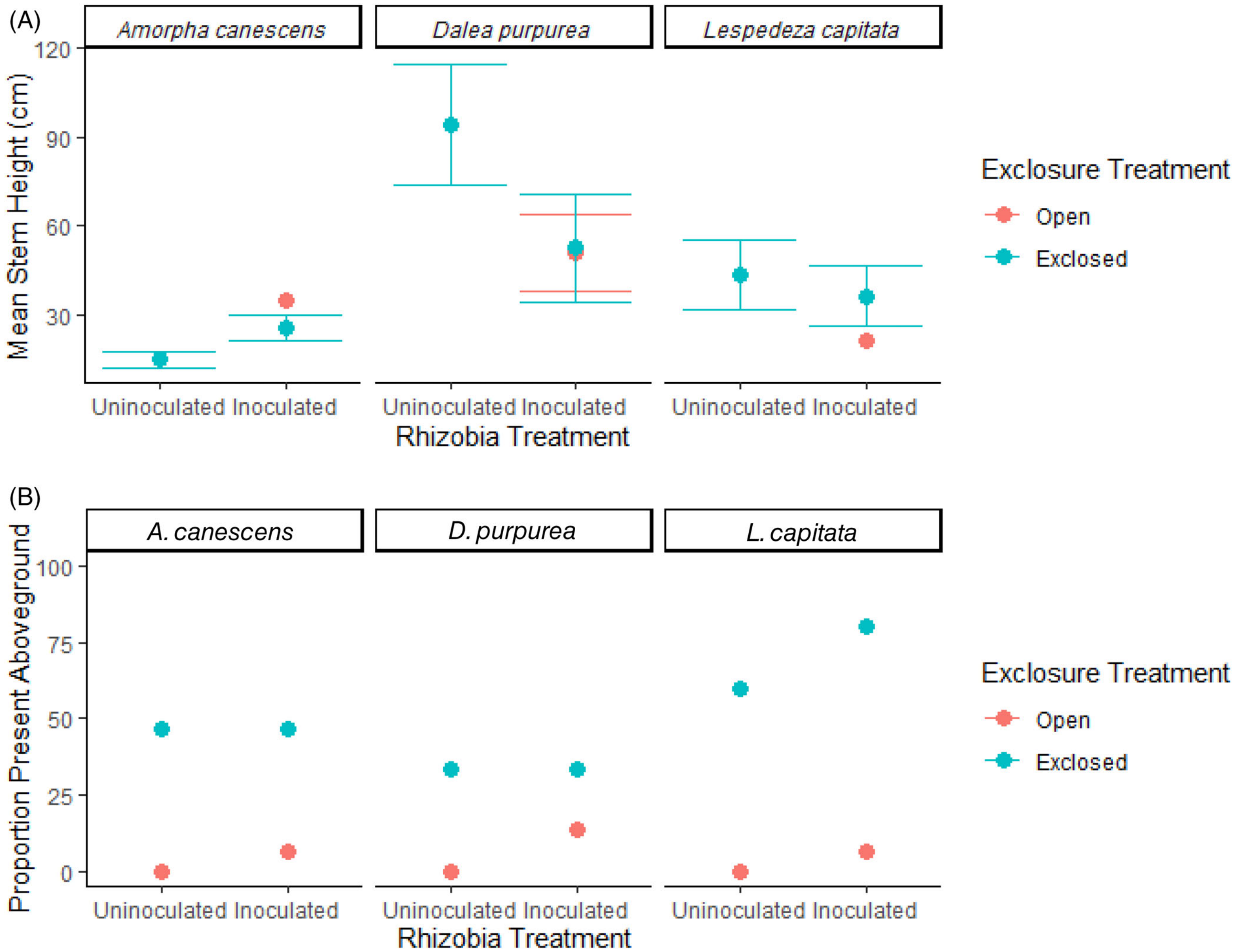

Figure 5. (A) Each plant species' response of mean stem height (± SE) and (B) proportion of plants that had aboveground biomass present in 2022 in both rhizobia inoculation treatments and exclosure treatments. Data points missing due to no survivors.

Greenhouse Experiment

Rhizobia inoculation did not increase plant dry weight (Table S8; Fig. 6A; $F_{[1,80]} = 1.199$, $p = 0.277$) in either soil treatment (Table S8; $F_{[1,80]} = 0.005$, $p = 0.943$). However, rhizobia inoculation increased nodulation of *Amorpha canescens* (Table S9; Fig. 6B; $F_{[1,26]} = 16.467$. $p = 0.0004$) in both soil treatments (Table S8; $F_{[1,26]} = 3.726$, $p = 0.082$). In sterile soil treatments, *Dalea purpurea* and *Lespedeza capitata* nodulated more with inoculation than in controls, but in the live soil treatments, these species were not as responsive to rhizobia inoculation (Fig. 6B; Table S10; $F_{[1,30]} = 2.062$, $p = 0.161$; Table S11; $F_{[1,25]} = 1.383$, $p = 0.251$).

Discussion

Rhizobia inoculation did not promote the growth or establishment of rare legumes in our study. Instead, legume establishment in a restored prairie was more strongly limited by herbivory and environmental context. Few transplanted seedlings survived when herbivores had access, and survival was much higher in the drier portion of our restoration site. Though mutualistic interactions have clear potential to improve restoration outcomes in some circumstances, our study shows that the potential benefits of mutualism can be overwhelmed by other ecological interactions.

We found some support for species-specific mutualists being absent from restoration sites: inoculation increased *Amorpha canescens* nodulation in live field soil in our greenhouse experiment. In contrast to the findings of Becknell et al. (2021), where nodule numbers were positively related to biomass, increased nodulation in our experiment's seedlings did not correlate to larger biomass, suggesting that something other than N-limitation hindered the growth of our seedlings in controlled greenhouse conditions. Additionally, because nodule formation is biologically expensive for the legume host, the effects of increased nodulation in our study plant species may be best seen if our study plants were grown for a longer time period or even maintained in a second year of growth. In the field, inoculating our transplanted seedlings did not

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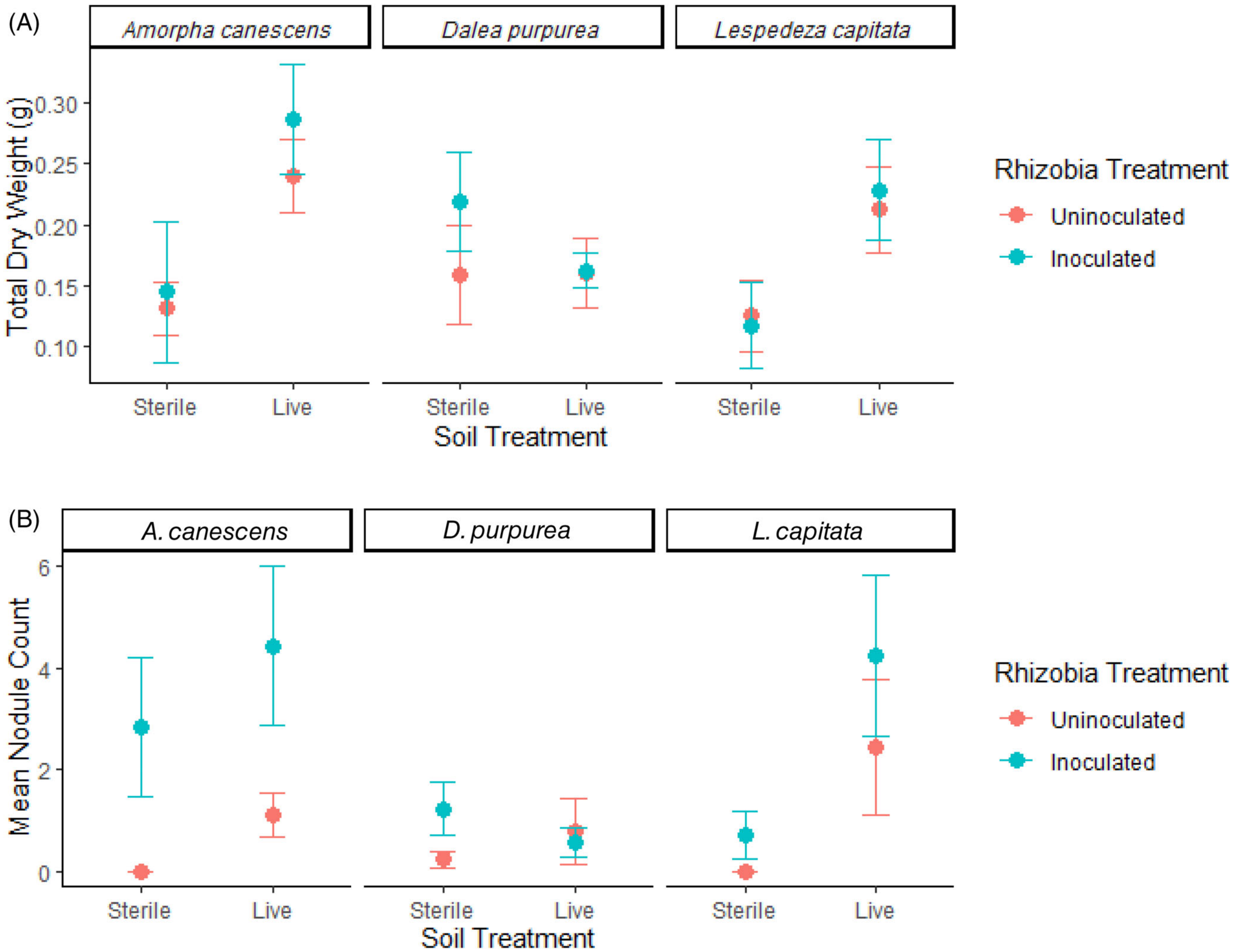

Figure 6. (A) Mean dry weight (g) of each plant species (± SE) and (B) mean number of nodules on each plant species planted into sterile or live field soil (± SE).

improve their performance, suggesting that alleviating nutrient limitation by restoring plant–microbe mutualisms does not directly improve success and other interactions prohibit legume success. One possibility for these results is that the specificity that legumes form mutualistic relationships with rhizobia may be both site- and species-specific. We have chosen species-specific rhizobia strains that were cultured from prairie soils that were compositionally similar to that of our site's soil, but it may be that choosing rhizobia strains of the same local genotype may be a more compatible match for the rhizobia and legume due to the complexity of this relationship (Walker et al. 2020). Additionally, other species-specific strains of rhizobia strains may fix nitrogen more efficiently and increase nutrient uptake for their host (Allito et al. 2020).

In support of our second prediction, we found that pressures of herbivory disrupted plant establishment regardless of rhizobia inoculation. Previous studies have shown that overly abundant populations of herbivores are a limiting factor in the success of rare plant species (Fletcher et al. 2001; MacDougall & Wilson 2007), but plant–microbe mutualisms can increase resistance to this (Basu et al. 2022). Our study indicates that there is potential for herbivory to overwhelm the effects of mutualistic interaction, especially if a plant species is particularly vulnerable to herbivory. In our case, all three of our legumes were preyed upon without preference in the open treatments regardless of rhizobia inoculation. A possibility for this increased vulnerability may be because we transplanted plugs of greenhouse grown plants that are more nutritive and palatable to grazers. Additionally, white-tailed deer populations were already overly abundant on our site. To increase the chance of survival post-transplantation, it would be effective to protect the transplanted seedlings with herbivore exclosures (Albrecht & Long 2019). Depending on a species' vulnerability to herbivory, installing protective measures may be the most vital in the first growing season post-transplantation (Garzón-Machado et al. 2010; Buisson et al. 2015). In our assessment of the next year's survival, we found that some plants that were completely

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grazed in the prior year were able to recover. This result was found only in legumes that were inoculated with rhizobia bacteria. However, because recovery after herbivory was so rare (only four), we cannot attribute this to rhizobia inoculation.

We further expected the legume-rhizobia mutualism to interact with environmental conditions; however, our results show that, similar to herbivory, the environment overwhelms the possible benefits of the mutualism. In our study, legume growth and persistence in the first growing season were more strongly correlated with their planted location within the site, irrespective of their inoculation treatment. This was the case for our three study legumes, which are historically adapted to dry prairie (Gleason & Cronquist 1991). While we did not specifically measure soil moisture content throughout our study site, there was a clear shift in composition from having vegetation characteristic of dry-mesic environments in the northern end to becoming more similar to wet prairie in the southern end. This result emphasizes that environmental filtering is another limiting factor in plant establishment (Barber et al. 2019; Rodriguez-Barrera et al. 2022). More specifically, abiotic factors such as soil moisture and soil composition may be the driving factors in why rare plant species are often absent in restorations, despite being sown or transplanted. Identifying appropriate abiotic conditions may be more important when planning restoration efforts, rather than focusing solely on restoring missing soil mutualists.

Grman et al. (2020) showed that high-quality, host-specific rhizobia were missing in restorations for three leguminous plant species, *Dalea purpurea*, *Lespedeza capitata*, and *A. canescens*. We expanded this research by exploring if the inoculation of species-specific rhizobia bacteria from high-quality remnant prairies would promote the growth of these three legumes in a restoration. Additionally, we paired a greenhouse component to investigate whether the effects of rhizobia inoculation are generalizable to the field. Despite prior research that emphasizes the importance of restoring the rhizobia–legume mutualistic relationship to increase growth, we found that herbivory and environmental context are stronger predictors of plant establishment at our site. This research did nonetheless find support that these legumes are sensitive to rhizobia partners and that higher quality soil mutualists are sometimes absent in restored sites. Future research should explore the effects of mutualisms in a broader geographical range and different plant species. Expanding this understanding to other ecosystems may encourage the development of more effective restoration strategies to increase populations of rare plant species.

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Supporting Information

The following information may be found in the online version of this article:

Table S1. Rhizobia strains and respective location used as inoculum for study legumes.
Table S2. Repeated measures ANOVA of stem height of 2021 growing season.
Table S3. Survival analysis of plants from the whole growing season.
Table S4. ANOVA of stem height on final day of measurements of 2021.
Table S5. Survival analysis of plants from the final day of measurements of 2021.
Table S6. ANOVA of stem height measurements from second growing season.
Table S7. Survival of plants in second growing season.
Table S8. ANOVA of biomass of all plant species in greenhouse experiment.
Table S9. Quasi-Poisson nodules for *Amorpha canadensis*.
Table S10. Quasi-Poisson nodules for *Dalea purpurea*.
Table S11. Quasi-Poisson for *Lespedeza capitata*.

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