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ARTICLE

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Legume life history interacts with land use degradation of rhizobia: Implications for restoration success

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Abstract

Restoration of soil microbial communities, and microbial mutualists in particular, is increasingly recognized as critical for the successful restoration of grassland plant communities. Although the positive effects of restoring arbuscular mycorrhizal fungi during the restoration of these systems have been well documented, less is known about the potential importance of nitrogen-fixing rhizobium bacteria, which associate with legume plant species that comprise an essential part of grassland plant communities, to restoration outcomes. In a series of greenhouse and field experiments, we examined the effects of disturbance on rhizobium communities, how plant interactions with these mutualists changed with disturbance, and whether rhizobia can be used to enhance the establishment of desirable native legume species in degraded grasslands. We found that agricultural disturbance alters rhizobium communities in ways that affect the growth and survival of legume species. Native legume species derived more benefit from interacting with rhizobia than did non-native species, regardless of rhizobia disturbance history. Additionally, slow-growing, long-lived legume species received more benefits from associating with rhizobia from undisturbed native grasslands than from associating with rhizobia from more disturbed sites. Together, this suggests that native rhizobia may be key to enhancing the restoration success of legumes in disturbed habitats.

KEYWORDS
arbuscular mycorrhizal fungi, legume life history, mutualism, restoration ecology, rhizobia, soil microbes

INTRODUCTION

Legumes (plants in the family Fabaceae) are integral parts of grassland communities that enhance biodiversity, provide resources for pollinators, provide high-quality forage, and can improve soil quality through their interactions with nitrogen-fixing bacteria (Koricheva et al., 2000; Potts et al., 2009; Tilman et al., 2001). Despite their importance, legumes (particularly long-lived, slow-growing late-successional species) are underrepresented in grasslands restored via seed broadcasting relative to undisturbed remnants (Kindscher & Tieszen, 1998; Urban, 2020).

Given that microbial mutualists play strong roles in plant establishment (Delavaux et al., 2021, 2022) and can influence restoration success (Koziol et al., 2018), it is possible that native late-successional legume establishment is limited in restorations by microbial mutualists. That is, communities of microbial mutualists may be degraded by anthropogenic disturbance of soils, which then affect the establishment success of these species.

Strong evidence has accumulated that the establishment of high-quality late-successional prairie plant species can be limited by native arbuscular mycorrhizal (AM) fungi. AM fungi associate with most prairie plants, including most prairie legumes, and provide plants with many benefits, including increased access to phosphorus (Smith & Read, 2008). Late-successional native prairie species generally benefit more from AM fungi (Bauer et al., 2018; Bryant & Bever, 2024; Koziol & Bever, 2015) and are more sensitive to AM fungal composition (Cheeke et al., 2019; Koziol & Bever, 2016a) than early-successional native or non-native species. AM fungal composition has been shown to be degraded by disruption of prairie soil such as tillage (House & Bever, 2018; Jansa et al., 2002; Kabir, 2005), and late-successional native prairie species are particularly responsive to AM fungi from undisturbed habitats (Koziol et al., 2022). Inoculation with AM fungi from remnant prairies enhances the establishment and growth of late-successional plant species, including legumes, in disturbed sites (Koziol et al., 2022; Koziol & Bever, 2016b; Middleton et al., 2015). Overall, this indicates that inoculation with native AM fungi can be used to enhance the restoration success of late-successional, difficult-to-establish legume species.

Less is known about the potential importance of nitrogen-fixing rhizobium bacteria, a second group of microbial mutualists associated with legumes. Rhizobia can greatly enhance plant fitness by providing plants with fixed atmospheric nitrogen in exchange for carbon, which suggests that rhizobia have the potential to influence the establishment of legumes in restorations. There is some evidence that, like for AM fungi, land-use change may degrade rhizobium communities. Long-term nitrogen addition can lead rhizobia to evolve to provide fewer benefits to their hosts (Weese et al., 2015), which can lead to degradation of rhizobium communities in former agricultural fields. Grman et al. (2020) found that plants inoculated with microbial communities (including, but not limited to, rhizobia) from remnant prairies produce more root nodules (structures to house rhizobia) than plants inoculated with soil from site disturbed by agriculture, suggesting that disturbance may potentially decrease rhizobium quantity/quality. Similarly, inoculation with certain rhizobium strains improved legume establishment in a restored prairie (Beyhaut et al., 2014), indicating that high-quality strains may be missing from disturbed sites. Whether potential degradation of the rhizobium community differentially inhibits late-successional legumes compared to early-successional species is unclear. In Grman et al.'s (2020) study, inoculation increased plant growth regardless of plant successional status. By contrast, Herzberger et al. (2015) found that growth of a late-successional legume was greater when inoculated with microbial communities from recently restored (i.e., recently anthropogenically disturbed) sites than those from remnant prairies. Better understanding of legume interactions with rhizobium communities in degraded grasslands may be essential for improving legume establishment in these systems.

In this study, we examine the responses of grassland legume species varying in life history (late-successional native, early-successional native, and non-native species) to microbial and rhizobium communities from grasslands varying in land-use history (remnant prairies, post-agricultural grasslands, and agricultural fields) to better determine when and where rhizobia may be most useful in restoration efforts. Specifically, in a series of greenhouse and field experiments, we test the following hypotheses: (1) growth of late-successional legume species will respond more strongly to rhizobia than that of early-successional or invasive species; (2) legumes (particularly late-successional legumes) will benefit most from rhizobia from undisturbed, remnant prairies; and (3) inoculation with rhizobia from remnant prairies will increase legume establishment in a post-agricultural grassland restoration. By identifying patterns in legume responses to rhizobia, we can determine how rhizobia may best be used to enhance the establishment of legumes in degraded grassland systems.

METHODS

To assess the outcomes of legume–rhizobium interactions across plant life history and land-use history, we conducted an observational study of rhizobial community composition across sites varying in land-use history and a series of greenhouse and field experiments. In the greenhouse, we first assessed growth responses of legume species from three life-history categories (native late-successional, native early-successional, and non-native) to whole soil microbial communities collected from remnant prairies, post-agricultural grasslands, and agricultural fields from several sites in western Kansas. We then inoculated legumes with rhizobium strains isolated from these soil microbial communities to assess plant response to rhizobia specifically. Finally, we inoculated a set of focal legumes with our isolated rhizobia strains and planted

them in a post-agricultural grassland to assess the effect of rhizobia on plant growth and survival in the field.

Bacterial community sampling across sites varying in land use

To examine patterns in how land use affects rhizobium communities, we took advantage of data from a large study examining soil microbial communities across the state of Kansas. Briefly, soils were sampled to a 5–15 cm depth in remnant prairies, post-agricultural grasslands, and agricultural sites at 12 locations spanning an east–west gradient in Kansas (Appendix S1: Table S3) in 2019 and 2021. DNA was extracted using the Qiagen DNeasy PowerSoil Pro kit. The V4 region of the 16S small subunit ribosomal gene was amplified using the modified Earth Microbiome Project (EMP) (Thompson et al., 2017) primers 515F-Y (Parada et al., 2016) and 806R (Apprill et al., 2015) and EMP PCR program. Amplicons incorporating Illumina Nextera (Illumina, Inc., San Diego, CA, USA) indices were sequenced on the Illumina MiSeq platform using Illumina 2 × 300 bp MiSeq v2 chemistry at the University of Kansas Genomic Sequencing Core (Lawrence, KS, USA). The sequences were clustered at 97% sequence identity and assigned taxonomy using the SILVA 132 database for the bacterial 16S rRNA gene (Quast et al., 2013). We then subset ASVs identified as genera/groups of genera that typically act as nitrogen fixers (*Bradyrhizobium*, *Ensifer*, *Mesorhizobium*, and those in the *Allorhizobium–Neorhizobium–Pararhizaobium–Rhizobium* and *Burkholderia–Caballeronia–Paraburkholderia* groups) and calculated the relative abundance/sample of rhizobia.

Plant and soil materials

We selected 15 legume species that are commonly found in remnant prairies and/or post-agricultural grasslands in eastern Kansas (Table 1). We specifically targeted native species that are commonly used in seed mixes for grassland restoration and invasive species that are commonly found, but not necessarily dominant, in restored prairies (with the exception of *Lespedeza cuneata*, which often forms dense stands in restored sites). We categorized the native legume species as either early- or late-successional based on their coefficient of conservatism (CC) scores (Haddock et al., 2015), with species with CC ≥6 considered late-successional and ≤5 early-successional. We purchased most native seed from a local supplier (Missouri Wildflowers Nursery, Jefferson City, MO, USA) and the rest from Prairie Moon Nursery (Winona, MN, USA). We collected non-native seed from plant populations in disturbed sites on the University of Kansas West Campus in fall of 2019 and 2020, making sure to harvest seeds from at least 20 individuals of each species across at least two populations >100 m apart.

TABLE 1 Legume species of each life-history category included in our experiments.

Life-history category and plant species	Seed source
Late-successional native	
Amorpha canescens[a]	Missouri Wildflowers Nursery
Baptisia australis	Missouri Wildflowers Nursery
Dalea candida	Missouri Wildflowers Nursery
Dalea purpurea	Missouri Wildflowers Nursery
Lespedeza capitata	Missouri Wildflowers Nursery
Early-successional native	
Chamaecrista fasciculata[a]	Missouri Wildflowers Nursery
Crotalaria sagittalis	Prairie Moon Nursery
Desmanthus illinoensis	Missouri Wildflowers Nursery
Desmodium illinoense	Prairie Moon Nursery
Non-native invasive	
Kummerowia stipulacea	Hand-collected
Lespedeza cuneata[a]	Hand-collected
Lotus corniculatus	Hand-collected
Medicago lupulina	Hand-collected
Melilotus alba	Hand-collected
Melilotus officinalis	Hand-collected

[a]Our three focal species.

We sampled soil microbial communities at three sites (an agricultural field, a post-agricultural field, and an undisturbed remnant prairie) in each of two locations in eastern Kansas (Clinton Lake and the KU Field Station; see Appendix S1: Table S1 for exact location details) in September 2020 and from Clinton Lake again in May 2021 for use in our greenhouse experiments. The agricultural site at Clinton Lake had grown corn in the previous season, while at the KU Field Station, the agricultural site had grown soybeans. Agriculture had ceased at the post-agricultural sites at both locations approximately 60–65 years prior, and neither post-agricultural site had been actively restored to prairie. At each site, we took 10 cm deep × 2.5 cm wide soil cores from 25 points evenly spaced across four 100-m transects. We homogenized the samples from a given site after collection. We stored these samples in plastic bags at 4°C to maintain microbial communities for approximately 1 week before the soils were used for inoculation in greenhouse experiments. We sampled microbial communities in post-agricultural and

remnant prairie sites at three other locations (Konza Prairie, Welda, and Leavenworth; Appendix S1: Table S1) using similar methods in order to isolate rhizobia for use in our field experiment.

Experimental design

Greenhouse experiment 1

In part (a) of this experiment, we tested the response of three focal legume species (*Amorpha canescens*, *Chamaecrista fasciculata*, *L. cuneata*) representing our three life-history categories (native late-successional, native early-successional, and non-native invasive) to whole soil microbial communities collected from our three different land-use types from both locations. In part (b), we tested the response of an additional four native late-successional, three native early-successional, and five non-native invasive species to microbial communities from the different land-use types at just the Clinton Lake location (we split this experiment into two parts in order to test responses to microbial communities both from several locations and with many plant species while keeping sample sizes manageable). We surface-sterilized seeds in 75% ethanol for 1 min, scarified them with sandpaper, placed them in moist paper towels in sealed plastic bags, kept them at 4°C for 1 week to promote germination, and then germinated them in sterilized potting soil in the greenhouse. When seedlings produced their first true leaves, we transplanted individual seedlings into 1-L Deepots filled with a 1:1 mix of Kansas topsoil and sand that we steam sterilized at 165–170°F twice for 4 h with a 1-day rest period. In inoculated treatments, pots also contained 30 cc of field soil placed under a cap of sterile soil near the top of the pot. Each plant species/land-use type combination was replicated 10 times [part (a): 3 plant species × 4 land-use treatments (ag, post-ag, remnant, uninoculated control) × 2 locations × 10 replicates = 240 total plants] and [part (b): 12 plant species × 4 land-use treatments × 10 replicates = 480 total plants].

We harvested aboveground and belowground biomass 12 weeks after initial transplanting. We counted root nodules and haphazardly selected 10 nodules from each plant and weighed them to estimate mean nodule biomass. We dried plant biomass at 60°C for 48 h and weighed it.

To isolate rhizobia strains from microbial communities for greenhouse experiment 2, we used the nodules selected from each plant species for estimating nodule biomass. We surface-sterilized nodules by dipping them in 100% ethanol followed by 2 min in commercial bleach, rinsed them in sterilized water, then crushed them, and streaked them onto tryptone yeast (TY) agar plates (Somasegaran & Hoben, 1994). We transferred strains onto successive TY plates until we obtained single colonies. We picked a single colony from each final plate to inoculate into sterile TY broth, which we then incubated at 30°C and 120 rpm for 48 h. We archived a portion of each of these cultures in 50:50 culture:60% glycerol solution at −80°C and used another portion for DNA extraction. We isolated DNA with the MasterPure Complete DNA and RNA Purification Kit (Biosearch Technologies) following the kit protocol and then sent samples to GeneWiz (Azenta Life Sciences) for 16S sequencing (16S rRNA gene V1–V9 regions). We conducted a BLAST search (https://blast.ncbi.nlm.nih.gov) of the 16S rRNA sequences to verify identity as rhizobia (Appendix S1: Table S2).

Greenhouse experiment 2

In this experiment, we tested the response of legumes to the rhizobia isolated from the three different site types (from location 1 only—Clinton Lake). We used the same plant species and prepared, germinated, and planted seedlings in sterilized soil as described in experiment 1. In all pots (including controls), we included 30 cc of arbuscular mycorrhizal fungi (AMF) inoculum (a mixture of species isolated from midwestern prairies and maintained in live culture by the Bever Lab at the University of Kansas). We included AMF inoculum because prior studies have shown that these mutualists can be extremely important for the growth of some legumes (Bauer et al., 2018; Larimer et al., 2014). After transplanting seedlings, we inoculated with 1 mL of a mixture of rhizobium strains isolated from ag, post-ag, or remnant sites (*c.* 0.25×10^6 cells based on OD670), or sterile liquid media control. Rhizobium cultures were grown in Modified Arabinose Gluconate liquid culture (van Berkum, 1990) at 30°C for 48 h. Each rhizobium mixture comprised 30 strains, two isolated from each plant species—these were selected at random from the 5–10 strains successfully isolated from each species/land-use combination. We measured cell density for each strain with a spectrophotometer and standardized by diluting each individual strain culture with sterile media before combining into mixture. Each plant species/rhizobium type combination was replicated 10 times [15 plant species × 4 rhizobium treatments (ag, post-ag, remnant, control) × 10 replicates = 600 total plants]. We harvested plants after 12 weeks using the methods described in experiment 1.

Field experiment

To determine whether rhizobia inoculation affects legume establishment and survival in post-agricultural

grasslands, we planted seedlings of our three focal species inoculated with either rhizobia from remnant prairies or rhizobia from post-agricultural grasslands or with no rhizobia into plots in a post-agricultural grassland in eastern Kansas (39°00′03″ N, 95°19′01″ W). To isolate rhizobia strains for our field experiment, we harvested nodules from trap cultures (individually grown focal species grown in sterile soil in the greenhouse and inoculated with our soil samples), using the isolation methods described in greenhouse experiment 1. In spring 2020, we germinated seeds of our three species as described above and then transplanted them into Cone-tainers filled with sterilized 1:1 topsoil sand mix in the greenhouse. Half of the pots also contained 30 cc of native AMF inoculum (as described in greenhouse experiment 2) to test whether AMF influences the effects of rhizobium inoculation, as synergistic effects of these two symbionts have been shown in numerous studies (Magnoli & Bever, 2023; Primieri et al., 2022). Plants were inoculated with 1 mL of a mixture of rhizobium strains isolated from one of our three remnant or post-agricultural sites or a sterile liquid media control. Each rhizobium mixture contained nine strains (three isolated from each focal species) and was prepared as described above. We transplanted plants into the field in May 2020 when seedlings were 3 weeks old. In a 9 m × 6 m block, we established nine 0.5 m × 0.5 m plots, spaced 1.5 m apart. We planted nine plants (three of each species) into each plot. All plants in a plot came from a single rhizobium treatment, and each treatment was replicated three times within a block. All plants in a block came from a single AMF treatment. Each block was replicated five times [9 plants/plot × 3 rhizobium treatments (remnant, post-ag, control) × 3 plot replicates × 2 AMF treatments × 5 blocks = 810 total plants]. In the 2 weeks following transplanting, we watered plants as needed and replaced any plants that died.

In September 2020, we recorded plant survival and harvested *Chamaecrista* by clipping aboveground biomass at the base, as it is an annual and had reached then end of its lifecycle. We also measured the diameter of the base of the stem, as this is highly correlated with biomass in this species (S. M. Magnoli, unpublished data) and some plants experienced late-season browsing by deer and rodents that prevented us from harvesting all biomass. We also harvested *Lespedeza* in the same way as, although it is a perennial, we did not want this invasive species to establish at the site (we also dug up the roots to prevent establishment). All harvested biomass was dried at 60°C for 48 h and weighed. We did not harvest *Amorpha* because it is a slow-growing species and we wanted to track its growth over a longer period of time, but we did count leaves as an estimate of plant size. In June 2021 and 2022, we re-surveyed all plots to record *Amorpha* survival.

Statistical analyses

Bacterial community data

We used a linear mixed model in the *lme4* package (Bates et al., 2015) in R v 4.1.2 (R Core Team, 2022) to analyze N-fixer relative abundance, with land use, soil sample depth, and their interaction as fixed effects and sample location as a random effect. We conducted PERMANOVA that accounts for site effects using the adonis2 function in the *vegan* package (Oksanen et al., 2025) to determine whether N-fixer community composition differs between land uses. Site was included first in the model as adonis2 uses sequential sums of squares.

We tested the effects of microbial community/rhizobium type in our greenhouse and field experiments on plant growth, survival, and nodulation using mixed models. For plant biomass and estimated nodule biomass, we used linear mixed models in the *lme4* package; for nodule number, we used generalized linear mixed models with a negative binomial distribution in the *glmmTMB* package (Brooks et al., 2017); and for survival, we used generalized linear mixed models with a binomial distribution. We standardized plant biomass data prior to analysis in greenhouse experiments 1 and 2, as it spanned several orders of magnitude across species.

Greenhouse experiment 1

We combined biomass and nodule data from parts (a) and (b) for analysis. Models included microbial community type, plant life history and their interaction, as well as experiment (a or b), and microbial community type nested within site as fixed effects and plant species nested within life history and microbial community type nested within plant species and life history as random effects. We used a priori, orthogonal contrasts to decompose the effects of inoculation and plant life history. Specifically, we partitioned inoculation into the average effect of rhizobia (inoculation vs. uninoculated) and agricultural versus perennial grassland origin of inocula. We partitioned the effect of plant life history into native versus non-native legumes and late-successional versus early-successional native legumes. The inoculation × life history interaction was partitioned into the product of the individual inoculation and life-history contrasts.

Greenhouse experiment 2

Models analyzing biomass and nodule data included rhizobia type, plant life history and their interaction as

fixed effects, and plant species nested within life history and rhizobia type nested within plant species and life history as random effects. We used a priori, orthogonal contrasts to decompose the effects of inoculation and plant life history as described above.

Field experiment

We ran separate models for each plant species to analyze the effects of rhizobia and AMF on growth in the first year (*Chamaecrista* stem diameter, *Lespedeza* biomass, and *Amorpha* leaf number). Each model included rhizobia treatment, AMF treatment, and their interaction as fixed effects, with block and rhizobia site origin as random effects. We analyzed survival using generalized linear models with a binomial distribution and the same fixed and random factors.

For all mixed models, we validated model fit by inspection of simulated residuals using the *DHARMa* package (Hartig, 2019). We tested the significance of fixed effects using type III sums of squares in the ANOVA function in the *car* package (Fox & Weisberg, 2011) with sum contrasts, and we calculated estimated marginal means and conducted Tukey's post hoc multiple comparisons tests using the *emmeans* package (Lenth, 2018).

RESULTS

Bacterial communities

Relative abundance of N-fixing bacteria was significantly higher in remnant prairies than in agricultural sites ($\chi^2 = 7.38$, $p = 0.02$; Figure 1a). In addition, PERMANOVA revealed a significant effect of land use on rhizobium community composition ($F = 2.03$, $p = 0.031$), with remnant prairies and post-agricultural sites being distinctly different from agricultural sites (Figure 1b). We found that abundance changes in certain strains underlie the differences between rhizobium communities with different land-use histories. Specifically, although all genera were represented in each land-use type, differences in two *Bradyrhizobium* strains and a *Burkholderia*, *Mesorhizobium*, and *Rhizobium* strain drove differences in community composition.

Greenhouse experiment 1

Inoculation with microbial communities significantly increased plant biomass relative to controls for all plant groups, but the effects of microbial community type varied with life history (life history × community type $\chi^2 = 15.19$, $p = 0.02$; Figure 2a). Late-successional native species had significantly higher biomass when inoculated with remnant or post-agricultural microbes than agricultural microbes, while early-successional native and invasive species had no biomass differences between microbial types. We found similar patterns in nodule number production (life history × community type $\chi^2 = 10.6$, $p \leq 0.03$; Figure 2b), with late-successional native species producing significantly more nodules with remnant microbes than with agricultural microbes, early-successional natives with no differences between microbial treatments, and invasive species producing significantly more nodules with post-agricultural microbes than with remnant microbes. We found qualitatively similar patterns with nodule mass data (Appendix S1: Figure S1a). An a priori contrast showed that although all plant life-history groups benefitted from inoculation with microbial communities, native plant groups received greater fitness benefits from inoculation than the invasive group did ($p = 0.02$).

Greenhouse experiment 2

The effects of inoculation with only rhizobia varied with plant life history and rhizobium type (life history × rhizobium type $\chi^2 = 13.7$, $p = 0.03$; Figure 3a). Inoculation significantly increased the biomass of both native plant groups, but the magnitude of this effect did not vary with rhizobium type, with the exception of the effect of inoculation with agricultural rhizobia on late-successional native plant species, which did not significantly differ from uninoculated controls. By contrast, invasive plants had significantly higher biomass when grown with rhizobia from agricultural sites than controls, but they did not significantly benefit from inoculation with rhizobia from remnant or post-agricultural sites, again indicating that native plant groups generally benefit more from inoculation than invasive plants (a priori contrast of interaction of inoculation and native vs. non-native plant species: $p = 0.01$). Nodule numbers varied similarly with plant life history and rhizobium type (life history × rhizobium type $\chi^2 = 10.5$, $p = 0.03$; Figure 3b). There were no differences between rhizobium types for either native plant group, but invasive species made significantly more nodules with post-agricultural and agricultural rhizobia than with remnant rhizobia. Patterns in nodule mass were qualitatively similar to nodule number (Appendix S1: Figure S1b). We found evidence of rhizobia contamination in our control treatment, with many plants forming nodules (although control plants formed significantly fewer nodules than inoculated

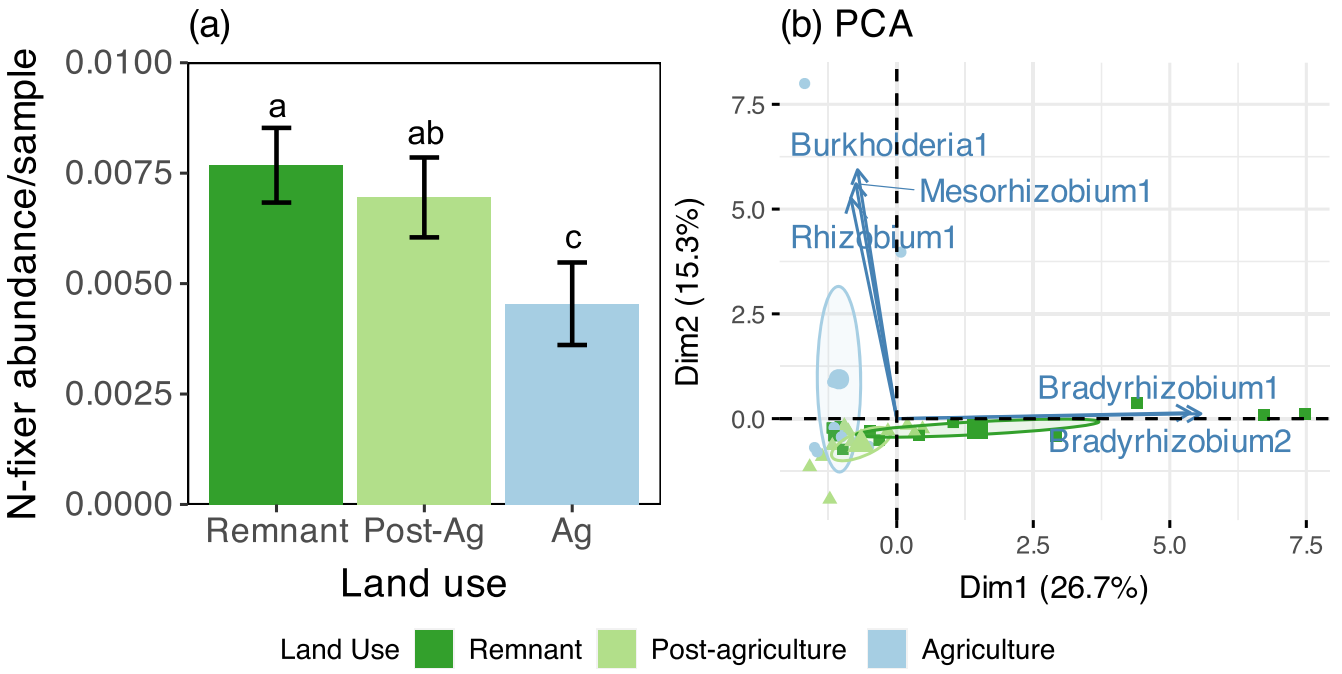

FIGURE 1 (a) Relative abundance of rhizobia in bacterial soil communities from sites varying in land-use history. Bars represent estimated marginal means (±SE). Different letters represent statistically significant differences between sites with different land-use histories. (b) Principal components analysis biplot of rhizobium communities from sites varying in land-use history. Ellipses show confidence estimates around centroids for each group, while arrows indicate the genera of the five rhizobium strains (out of 19) with the strongest contributions to dimensions 1 and 2.

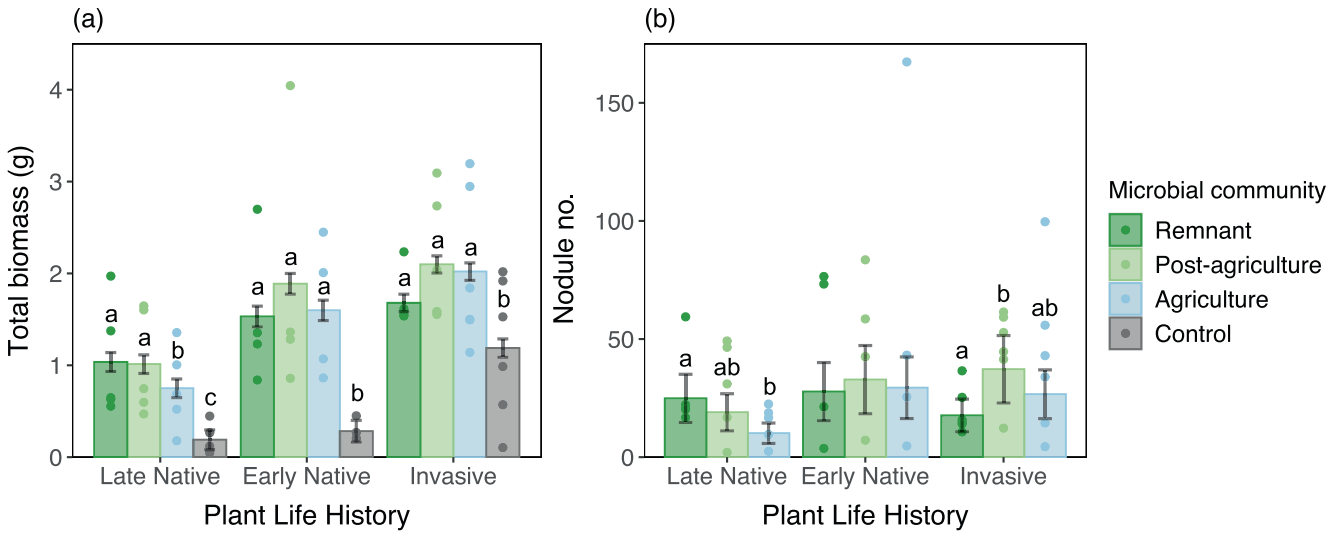

FIGURE 2 (a) Total plant biomass and (b) number of nodules produced by plants from different life-history groups inoculated with microbial communities from sites with different land-use histories. Bars represent estimated marginal means (±SE), and points represent individual species means from fixed-effects models including microbial community, plant life history, their interaction, and species interaction with life history as predictor variables (note that we used these models to generate values for plotting purposes only; all statistics and significance testing were done with the mixed-effects models described in *Methods*). Letters show the results of Tukey's post hoc multiple comparisons tests between microbial community types within a plant life-history group (different letters indicate significant differences).

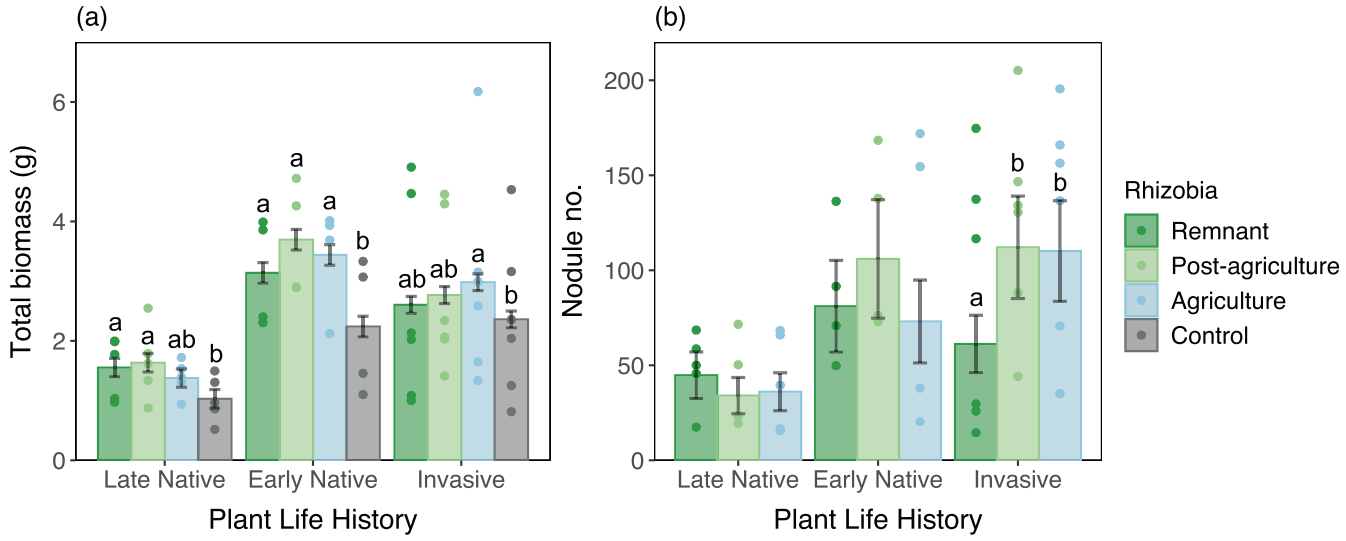

FIGURE 3 (a) Total plant biomass and (b) number of nodules produced by plants from different life-history groups inoculated with rhizobium communities from sites with different land-use histories. Bars represent estimated marginal means (±SE), and dots represent individual species means from fixed-effects models including rhizobia, plant life history, their interaction, and species interaction with life history as predictor variables (note that we used these models to generate values for plotting purposes only; all statistics and significance testing were done with the mixed-effects models described in *Methods*). Letters show the results of Tukey's post hoc multiple comparisons tests between microbial community types within a plant life-history group (different letters indicate significant differences).

plants [$\chi^2 = 100.09$, $p < 0.0001$] and there were no significant differences in nodule production in control treatments across plant life-history groups [$\chi^2 = 5.10$, $p = 0.08$]). We suspect that the contamination came from the native AMF inoculum that every plant received and not pot-to-pot contamination, as we had no contamination issues in experiment 1 controls and followed the same soil sterilization, watering, and spacing protocols in both experiments. As all plants received the AM fungal inoculation, we expect that these contamination issues would diminish the effects of rhizobium inoculation treatments and therefore view responses to inoculation as conservative estimates of potential benefits of rhizobia.

Field experiment

We found variable effects of inoculation with rhizobia and/or AM fungi on plant survival and growth in the field. In the first year of the experiment, the late-successional native legume (*A. canescens*) tended to have higher survival when inoculated with rhizobia, regardless of rhizobia type ($\chi^2 = 5.81$, $p = 0.055$; Figure 4a). In years 2 and 3, we found significant effects of rhizobia inoculation on *Amorpha* survival, with plants inoculated with rhizobia from remnant prairies having higher survival than uninoculated plants (year 2: $\chi^2 = 8.75$, $p = 0.01$; year 3: $\chi^2 = 9.08$, $p = 0.01$; Figure 4a). In the third year, AM fungi inoculation also increased *Amorpha* survival ($\chi^2 = 5.56$, $p = 0.02$). *Amorpha* also had more leaves when inoculated with AM fungi ($\chi^2 = 17.58$, $p < 0.0001$; Figure 4b) in the first year of the experiment (the only year we estimated growth) and tended to have more leaves when inoculated with rhizobia in the absence of AM fungi (rhizobia × AM fungi: $\chi^2 = 4.96$, $p = 0.08$; Figure 4b). There was no indication in the survival or growth of *Amorpha* of synergistic responses to co-inoculation with rhizobia and AM fungi (i.e., no significant rhizobia × AM fungi effects). Our early-successional native species (*C. fasciculata*) had both significantly higher survival and growth when inoculated with AM fungi (survival: $\chi^2 = 17.14$, $p < 0.0001$; growth: $\chi^2 = 40.62$, $p < 0.0001$; Figure 5a) and tended to have lower survival when associating with remnant rhizobia and lower growth when associating with both types of rhizobia when also inoculated with AM fungi (survival rhizobia × AM fungi: $\chi^2 = 5.36$, $p = 0.07$; growth: $\chi^2 = 5.32$, $p = 0.07$; Figure 5b). Neither symbiont affected the survival of the invasive legume (*L. cuneata*) (Figure 5c) but inoculation with AM fungi increased biomass ($\chi^2 = 136.41$, $p < 0.0001$), and plants inoculated with rhizobia from post-agricultural sites grew larger than those inoculated with remnant prairie rhizobia ($\chi^2 = 7.94$, $p = 0.02$; Figure 5d). Again, we found no evidence of synergistic effects of rhizobia and AM fungi on growth or survival of either of these plant species.

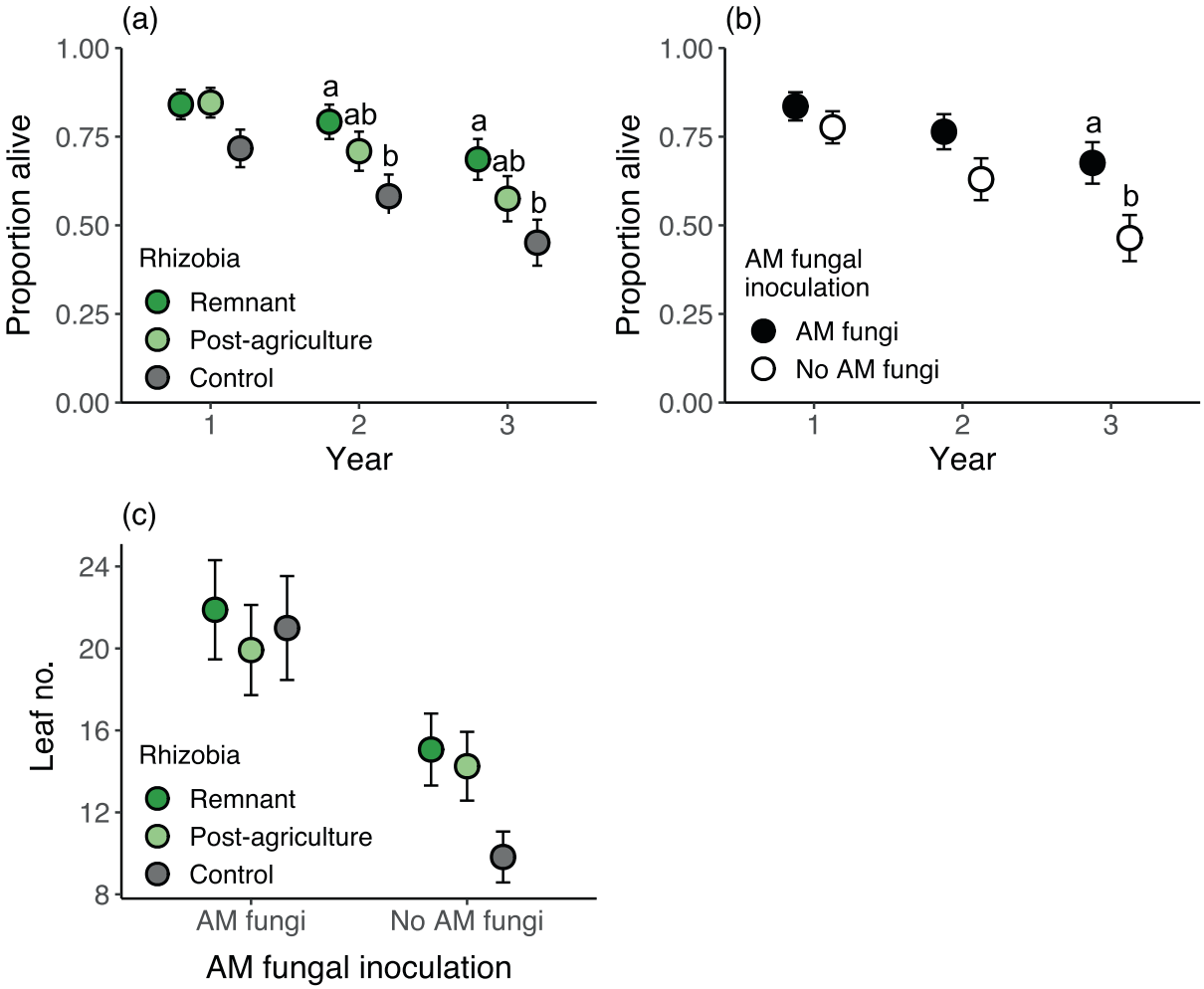

FIGURE 4 Survival of the late-successional native species (*Amorpha canescens*) over the 3-year experiment when inoculated with (a) rhizobia and (b) arbuscular mycorrhizal (AM) fungi, and (c) the number of leaves produced by each plant in the first year of the experiment. Colors represent different rhizobia inoculation/AM fungi treatments, and points show estimated marginal means ± SE. In (a) and (b), different letters indicate significant differences between rhizobia and AM fungi treatments in a given year as estimated with Tukey's post hoc multiple comparisons tests.

DISCUSSION

Rhizobia can have large effects on legume fitness, with consequences for plant productivity and distributions. Here we find that land use, specifically agricultural practices, affects native rhizobium communities by altering nitrogen-fixer relative abundance and community composition. These effects remain even after agricultural practices are abandoned, and affect legume survival and growth. These results suggest that native rhizobium communities do not recover on their own following disturbance and add to the growing body of evidence that rhizobium distribution may be limited by dispersal or co-limited by the difficulty of simultaneous colonization of compatible host plants. Analyses of global patterns of legume distribution show that legumes that associate with rhizobia are less likely to establish as invaders in novel habitats than legumes that do not associate with rhizobia (Delavaux et al., 2022; Simonsen et al., 2017), likely due to the absence of compatible rhizobia outside their native ranges. Native rhizobia may similarly limit the establishment of native legumes in restoration, and inoculation with native legumes may be critical for enhancing the restoration success of legume species in disturbed habitats.

Native rhizobia may be especially important for restoration given our findings that native legumes are more dependent on rhizobia than non-native invasive legumes. In our greenhouse experiment, both late- and early-successional native legume species that are commonly included in restoration seed mixes generally benefited more from association with rhizobia than did invasive species that are commonly found in post-agricultural grasslands and restored prairies. This mirrors broad

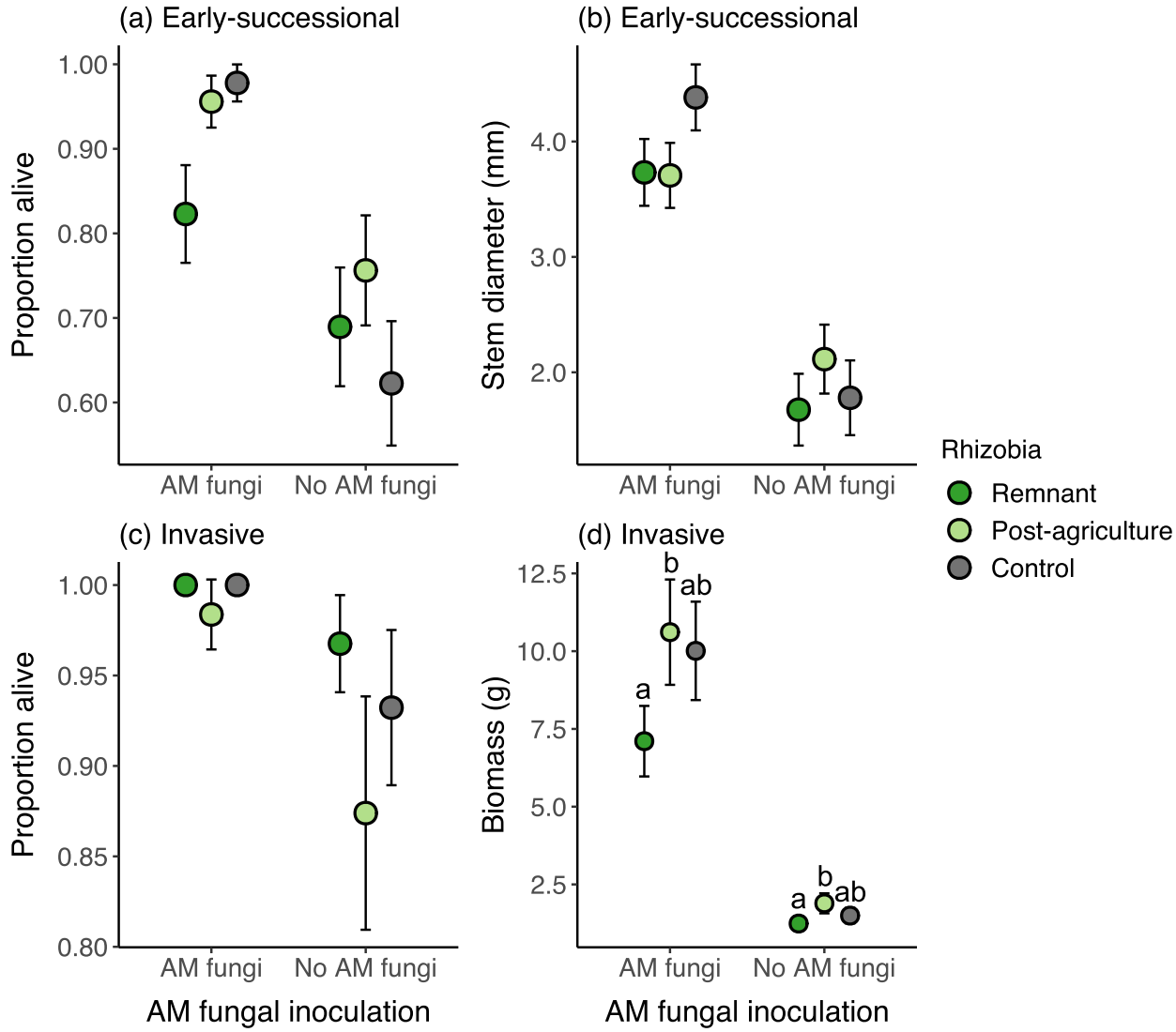

FIGURE 5 Survival (a) and stem diameter (b) of the early-successional native legume (*Chamaecrista fasciculata*), and survival (c) and biomass (d) of the invasive legume (*Lespedeza cuneata*) inoculated with or without arbuscular mycorrhizal (AM) fungi in the first year of our field experiment. Points represent estimated marginal means (±SE), and colors indicate rhizobia treatment. Different letters represent statistically significant differences between rhizobia treatments within an AM fungi treatment as estimated with Tukey's post hoc multiple comparisons tests.

patterns in plant responses to AM fungi, where native plants generally respond more strongly than invasives (Koziol et al., 2022, 2023), but we did not find response differences between late- and early-successional native species, which have been shown for AM fungi (Bauer et al., 2018; Bryant & Bever, 2024; Cheeke et al., 2019; Koziol & Bever, 2015, 2016a, 2016b). However, we also found that late-successional native legumes, which are often prioritized in grassland restorations, are more sensitive to rhizobia origin than early-successional or invasive species. In the greenhouse, late-successional natives received fewer growth benefits and made fewer root nodules when associating with whole microbial communities from agricultural sites, and they tended to also grow less with rhizobia-only inoculation from these sites, suggesting that rhizobia that are particularly beneficial to late-successional native legumes may be less abundant in these very recently disturbed sites. We found similar sensitivity differences in the field, where inoculation with native rhizobia from undisturbed sites, but not rhizobia from post-agricultural sites, increased late-successional legume survival but had no effects on the other legume species. Overall, this suggests that native rhizobia can provide benefits to late-successional native legumes that do not extend to weedier, easy-to-establish legumes.

Although our greenhouse and field experiments consistently show that native late-successional legumes benefit from interacting with rhizobia, we found greater plant

sensitivity to native rhizobia than post-agricultural rhizobia in the field but not in the greenhouse. This could potentially be due to the short length of our greenhouse experiments (4 months), which may not have been enough time to observe differential effects on slow-growing late-successional species like we observed in the field, where significant effects of native rhizobia inoculation were not observed until the second year of the experiment. However, differential effects of remnant and post-agricultural microbial communities were found in another greenhouse study of similar length with late- and mid-successional legumes (Grman et al., 2020), indicating that growth responses can be observed in this timeframe. Alternatively, the lack of difference between the effects of microbial communities from remnant and post-agricultural sites in our greenhouse studies may stem from the locations where we collected those communities. At each of the two locations we sampled (each with a remnant, post-agricultural, and agricultural site), the remnant and post-agricultural sites were in close proximity to each other (<50 m apart), and agricultural practices had been abandoned >60 years prior to sampling. Although the plant communities in the post-agricultural sites differed from those in the remnant sites (higher cover of non-native weedy species and fewer native species, S. M. Magnoli, personal observation), their microbial communities could be similar if microbes dispersed over the short distance from the remnant sites over time. By contrast, the remnant and post-agricultural sites where we collected the rhizobia used in our field experiment came from locations where these sites were separated by larger distances (>150 m) and agricultural practices had ceased more recently in the post-agricultural sites (ranging from <10–25 years ago). If microbial dispersal/recovery was less likely to occur in these post-agricultural sites due to distance and time, it may explain why we observed differential effects of rhizobia from these sites in our field experiment. This suggests that when degraded sites are not directly adjacent to undisturbed remnant sites, inoculation with native rhizobia will likely be beneficial for native legume establishment. Differences between the results of our field and greenhouse experiments could also stem from the simplified greenhouse environment, where plants interact with mutualists in the absence of other biotic interactions such as competition and herbivory that are present in the field. This underscores the importance of considering context dependence when evaluating plant–mutualist interactions.

Although our study focuses mainly on legume interactions with rhizobia, the fact that legumes are simultaneously interacting with other soil microbes can alter the importance of rhizobia. We observed, for example, differences in the magnitude of plant response to whole soil versus rhizobia only inoculation, which is consistent with rhizobia effects being at least partially influenced by the background soil microbial community. AM fungi are an obvious component of the soil microbial community that could alter rhizobium impacts on plant growth, as legumes, particularly perennial legumes, can receive synergistic benefits from associating with both mutualists at once (i.e., plants grow much larger than expected with both mutualists based on growth with individual mutualists) (Magnoli & Bever, 2023; Primieri et al., 2022). Inoculation with both native rhizobia and AM fungi could potentially have synergistic benefits to late-successional legumes, thereby enhancing restoration success. In our field experiment, which had both rhizobia and AM fungi inoculation treatments, we observed strong growth responses of all legumes to native AM fungal inoculation, whereas rhizobia only benefitted late-successional species survival and even had some negative effects on invasive species growth. These differences in mutualist effects could be due to environmental context dependence (e.g., soil fertility or water availability). Our observation of positive growth responses to native AM fungi of *C. fasciculata* in particular is consistent with previous tests (Reynolds et al., 2020). The high dependence of native and non-native legumes on AM fungi is also consistent with previous field inoculation results in which both native and non-native legumes benefit from these symbionts (Koziol et al., 2022). However, we found no evidence of synergistic effects of dual-inoculation with both AM fungi and rhizobia. This was somewhat surprising, given that the late-successional species in our experiment (*A. canescens*) had been shown to experience strong synergism when associating with both mutualists in a previous greenhouse experiment (Larimer et al., 2014). Annual legumes are less likely to experience synergism than perennial legumes (Primieri et al., 2022), which could explain why the early-successional species in our field experiment tended to have lower growth when inoculated with both mutualists. Exploring under what conditions and for which types of legumes synergism may occur in grassland systems would improve our understanding of how to use both these important mutualists to increase restoration success.

Restoration is essential to improving and enhancing grassland biodiversity and the ecosystem functions that grasslands provide. Legumes are integral components of grassland floras, and here we show patterns in how they interact with mutualistic rhizobia and demonstrate that rhizobia inoculations can be used to enhance the restoration success of a desirable legume species in a degraded habitat. While native AM fungi inoculants are increasingly used by restoration practitioners to enhance the establishment success of native plants in prairie restorations, our work demonstrates that including native rhizobia inoculants is also beneficial, particularly when

restoring native late-successional legumes. Future work should focus on long-term field experiments that include more legume species in order to better generalize these findings and determine the best ways to use rhizobium inoculations as a tool to improve restoration success.

AUTHOR CONTRIBUTIONS

Susan M. Magnoli and James D. Bever planned and designed the research. Susan M. Magnoli conducted all experiments and performed data analyses. Susan M. Magnoli and James D. Bever wrote the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data (Magnoli & Bever, 2025) are available in Dryad at https://doi.org/10.5061/dryad.s4mw6m9hp.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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