


RESEARCH ARTICLE

Inoculation with remnant prairie soils increased the growth of three native prairie legumes but not necessarily their associations with beneficial soil microbes

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Restoring the diversity of plant species found in remnant communities is a challenge for restoration practitioners, in part because many reintroduced plant species fail to establish in restored sites. Legumes establish particularly poorly, perhaps because they depend on two guilds of soil microbial mutualists, rhizobial bacteria and arbuscular mycorrhizal (AM) fungi, that may be absent from restored sites. We tested the effect of soil microorganisms from remnant and restored prairies on legume growth by inoculating seedlings of *Lespedeza capitata*, *Amorpha canescens*, and *Dalea purpurea* with soil from 10 restored prairies and 6 remnant (untilled) prairies from southwest Michigan. We generally found support for the hypothesis that restored prairie soils lack microbes that enhance prairie plant growth, although there was variation across species and mutualist guilds. All three legumes grew larger and two legumes (*Lespedeza* and *Amorpha*) produced more nodules when inoculated with soil from remnant prairies, suggesting that low quantity and/or quality of rhizobial partners may limit the establishment of those species in restored prairies. In contrast, no legume experienced greater root colonization by AM fungi in remnant prairie soils, suggesting equivalent quantity (but not necessarily quality) of fungal partners in remnant and restored prairie soils. We detected no evidence of spontaneous recovery of the community of beneficial soil microbes in restorations. These results suggest that the absence of rhizobia, a largely overlooked component of prairie soils, could play a strong role in limiting restored prairie diversity by hindering legume establishment. Active reintroduction of appropriate rhizobial strains could enhance prairie restoration outcomes.

Key words: *Amorpha canescens*, *Dalea purpurea*, *Lespedeza capitata*, mycorrhizal fungi, rhizobia

Implications for Practice

- Establishment of prairie legumes could benefit from inoculation with remnant prairie microbes. Inoculations increased the growth of three species, including two difficult-to-establish species (*Dalea purpurea* and *Amorpha canescens*), and nodulation of *Amorpha* and *Lespedeza capitata*.
- Soil from older restored prairies was no better for legumes than younger restored prairies, suggesting that active reintroduction of rhizobia and other beneficial soil microorganisms may be required to create suitable condition for the establishment of some legume species.
- Several recent studies have supported the use of only native, locally adapted soil microorganisms in restoration efforts. As locally adapted rhizobia are not widely available, we suggest conservation practitioners collaborate with soil microbiology research laboratories to produce strains adapted to their sites and plant species of concern.

Introduction

Positive species interactions are a critical component of restored ecosystems, and establishing target species within damaged ecosystems may require restoring mutualisms (Gomez-Aparicio 2009; van der Heijden & Horton 2009). Soil microbial mutualists enable plants to persist in harsh environments and may be

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particularly important for plant resource uptake in degraded soils. However, sites intended for restoration across several ecosystem types commonly host degraded soil microbial communities (Helgason et al. 1998; Fierer et al. 2013; Vályi et al. 2015). This widespread disruption of soil microbial communities with land use change presents a broad problem with critical practical significance: How essential are missing mutualists for recovery of plant communities in restored ecosystems?

Inoculation with soil microbial communities can radically reshape plant community composition and increase the establishment of late-successional plants (Wubs et al. 2016), indicating that some microbes do not spontaneously recolonize disturbed sites and that their addition could enhance restoration outcomes. For example, inoculation with arbuscular mycorrhizal (AM) fungi in restorations, especially taxa from local native habitats, typically increases desired plant growth in restored ecosystems (Maltz & Treseder 2015). However, often we do not know the identity of the beneficial microbes causing these dramatic effects, limiting our ability to apply soil inoculations to restorations on the large scales required for biodiversity conservation.

Here, we investigate plant–microbial interactions in the tallgrass prairie, where restoration attempts have become widespread following the destruction of more than 96% (in some areas 99.9%) of our tallgrass prairies (White et al. 2000). These intentional re-creations of complex ecosystems provide an ideal testing ground for exploring the basic mechanisms underlying community assembly. Despite the importance and widespread nature of this practice, restored prairies often fall short of practitioners' goals. Plant diversity in restorations is often lower than in undisturbed, remnant ecosystems (Rey Benayas et al. 2009) including prairies (Sluis 2002; Martin et al. 2005; Polley et al. 2005), highlighting our inadequate understanding of processes that enable species establishment and coexistence. Many desirable sown species, particularly late-successional species, fail to establish for unknown reasons (Grman et al. 2015).

Previous work has shown that high-quality soil mutualists may not spontaneously colonize restored prairies, limiting the establishment of dependent plant species (Bever et al. 2003; Koziol & Bever 2019; Neuenkamp et al. 2019). In particular, late-successional, highly conservative species rarely seen outside intact habitats benefit from whole soil inoculations that incorporate a diverse community of remnant prairie microbes (Middleton & Bever 2012; Herzberger et al. 2015). Subsequent follow-up studies with isolates of AM fungi from remnant prairie soils show that those highly beneficial mutualists dramatically improve plant performance in restored prairies (Middleton et al. 2015; Koziol & Bever 2017). Importantly, these positive effects can extend beyond the target seedlings to increase the growth and establishment of late-successional species in the surrounding plots (Middleton et al. 2015; Koziol & Bever 2017), increasing plant diversity of the restored prairie (Bever et al. 2003). In contrast, other work has suggested that some components of prairie soil microbial communities do recover spontaneously over decadal timescales. Barber et al. (2017) showed that soil bacterial communities in older restored prairies were more similar to remnant prairies than younger

restored sites. Yet, studies of inoculation chronosequences often show that the components of microbial communities that most affect plant growth do not consistently improve between young and old restorations (Anderson 2008; Herzberger et al. 2015). Thus, we do not yet fully understand whether active intervention is required for the reestablishment of diverse prairie soil microbial communities that increase plant diversity.

Legumes (Fabaceae) provide an interesting opportunity to investigate the degree to which mutualistic soil microbes recover in restored prairies. Legumes are particularly desirable components of prairie ecosystems because they contribute to plant species diversity, provide resources for rare specialist insects (Belth 2012; Fenner et al. 2018), increase soil health including carbon and nitrogen content (Fornara & Tilman 2008), and play an important role in biodiversity–ecosystem function relationships (Tilman et al. 1997). Most prairie legumes interact with two types of mutualistic soil microbes: rhizobia bacteria that fix atmospheric nitrogen in root nodules and AM fungi that colonize plant roots and extend into soil to scavenge nutrients. Furthermore, legumes, especially late-successional species, are particularly difficult to establish in restored prairies (Grman et al. 2015). We hypothesize that this dependence on two guilds of soil microbial mutualists exacerbates the consequences of depauperate soil microbial communities for legume establishment relative to other prairie plants. Existing evidence for our hypothesis is conflicting. Inoculations with the complete soil microbial community (whole-soil inoculations) from remnant prairies increase the growth of some desirable late-successional legumes (Middleton & Bever 2012), although they may not increase the number of root nodules. Other desirable late-successional legumes develop fewer nodules and obtain less growth benefit when inoculated with remnant prairie soil (Herzberger et al. 2015). Some legumes nodulate in restorations without inoculation, but others may be symbiont-limited (Larson & Siemann 1998; Tlustý et al. 2004) and inoculation with appropriate strains can increase their establishment (Beyhaut et al. 2014). Given the economic and conservation importance of prairie restoration, resolving the importance of soil mutualist limitation for the establishment of a diverse prairie legume community is a critical direction for research.

Although it is widely accepted that lack of appropriate AM fungal strains can limit the success of prairie restorations (Koziol et al. 2018) and that rhizobia limitation can limit the spread of dependent legumes (Simonsen et al. 2017), the importance of rhizobia limitation for the establishment of a diverse prairie legume community has not been fully explored. We tested the hypothesis that high-quality soil microbial mutualists are lacking in the soils of restored prairies. We predicted that prairie legumes inoculated with soil from remnant (untilled) prairies would grow larger, produce more nodules, and have increased root colonization by AM fungi than legumes inoculated with soil from restored (post-agricultural) prairies. We also investigated whether the benefit of the soil microbial community for legume growth in restored prairies would spontaneously increase over time. We predicted that soil from older restored prairies would support more legume growth, more nodules, and more AM fungal colonization than soil from younger

restored prairies. Support for this spontaneous recovery hypothesis would indicate that passive restoration can enhance the function of prairie soil microbial communities and that no intervention is necessary. In contrast, support for the missing mutualists hypothesis, but not the spontaneous recovery hypothesis, could point to critical new strategies for enhancing the establishment of rare prairie legumes in restored ecosystems.

Methods

We tested the effect of remnant and restored prairie soils on the growth and nodulation of three prairie legumes in a growth chamber experiment. We collected soil inocula from 10 restored prairies and 6 remnant prairies in southwestern Michigan. All restored sites were sown between 2004 and 2014 with a modified Truax seed drill into former row-crop agricultural land and maintained with periodic prescribed fire (see Grman et al. 2014 for more information). At each site, we collected 10 soil samples at 5-cm intervals along a 46-m transect with a 2-cm-wide stainless steel soil probe to a depth of 10 cm, and then composited and mixed the samples from a single site. We filled 2.5-cm-diameter, 12-cm-deep, 49-mL containers (Stuewe and Sons, Tangent, OR, U.S.A.) $\frac{3}{4}$ full with a 2:1 mixture of autoclaved turface and vermiculite, then added 5 mL of inoculum soil (approximately 10% of total soil volume), and then topped with another approximately 1 cm of the turface/vermiculite mixture.

We scarified seeds of *Amorpha canescens*, *Dalea purpurea*, and *Lespedeza capitata* by placing the seeds on a board lined with fine grit sand paper and rubbing with a fine grit sanding block. We then sterilized the outer coat of the seeds by placing them in a 5% bleach solution and shaking for 3 minutes and then washing with distilled water five times. To germinate them, we placed the seeds in sealed Petri dishes in the refrigerator with enough sterile 0.5mM KH_2PO_4 to just cover the seeds. We drew the liquid off the seeds after 3 days, and then resealed the plates and placed them upside down in a warm dark cabinet until germination when we transplanted them to the pots. Each species by soil inoculation treatment combination was replicated five times (16 collection sites \times 3 species \times 5 replicates = 240 pots total).

We arranged the pots randomly in racks; to minimize contamination, we surrounded pots with pots containing only turface/vermiculite mixture and lacking plants. We placed the racks in a growth chamber set to 26°C and 26% humidity on the campus of Eastern Michigan University (MI, U.S.A.). Racks were elevated on a Plexiglas sheet for improved air flow, positioned approximately 100 cm from the chamber lights, and covered with Plexiglas lids to reduce desiccation. We watered plants with approximately 3 mL of sterile standard nodulation medium (1mM KH_2PO_4 , 0.5mM MgSO_4 , 0.5mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 2 mL/L of a minor salts solution [minor salts solution/L = 9.5 g $\text{Na}_2\text{-EDTA} \cdot 2\text{H}_2\text{O}$, 7 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g H_3BO_3 , 250 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 50 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 50 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 50 mg CuSO_4 , and 10 mg CoCl_2]; Price et al. 2015) daily for the first week and three times a week thereafter.

After 10 weeks of growth, we harvested the plants by washing the roots, counting and removing root nodules, drying plant roots and shoots separately at 60°C for 48 hours, and then weighing roots and shoots. We assessed root colonization by first clearing roots in boiling 2.5% KOH for 5–8 minutes, staining by boiling in 5% solution of Schaeffer black ink and white vinegar for 5–8 minutes (Vierheilig et al. 1998), and then examining each sample at 40 \times magnification at approximately 90 locations for the presence of fungal hyphae, arbuscules, or vesicles (McGonigle et al. 1990).

We used R 3.6.0 (R Core Team 2019) to analyze the data. We excluded all plants that died before the end of the experiment; sample sizes were insufficient to allow analysis of treatment effects on mortality. First, we summed root and shoot biomass for a plant to obtain total plant biomass (excluding nodule biomass). To assess whether restored and remnant prairie soils affected plant biomass, nodule number, or root colonization, we analyzed each legume separately. We tested for an effect of our treatment (inoculation) on log-transformed plant biomass in a linear mixed-effects model (library lme4; Bates et al. 2015) with soil origin (site identity) as a random effect and using type II Wald F tests with Kenward–Roger degrees of freedom (function Anova in library car; Fox & Weisberg 2019). Analogous generalized linear mixed-effects models with negative binomial distributions (for nodule number) and binomial distributions (for root colonization) did not converge, so we averaged the number of nodules and root colonization across all growth chamber replicates for a site and performed general linear models using the number of sites as the sample size and F tests with type II sums of squares (function Anova).

Results

All three legume species produced more plant biomass when inoculated with soil from remnants than restored prairies (Fig. 1–1). *Amorpha* grew 74% larger ($F_{[1,12.66]} = 5.8$, $p = 0.03$), *Dalea* grew 31% larger ($F_{[1,13.18]} = 9.0$, $p = 0.01$), and *Lespedeza* grew 367% larger with remnant prairie soil microbes ($F_{[1,10.23]} = 38.5$, $p < 0.001$). In soil from restored prairies, nodulation was very low: 38% of *Amorpha*, 58% of *Dalea*, and 57% of *Lespedeza* individuals did not form any nodules. *Amorpha* and *Lespedeza* produced 179 and 647% more nodules in remnant soil, respectively (Fig. 1 & 1; *Amorpha* $F_{[1,14]} = 6.66$, $p = 0.02$; *Lespedeza* $F_{[1,14]} = 21.96$, $p < 0.001$). However, *Dalea* plants in remnant prairie soil also failed to nodulate in 80% of cases and on average did not produce more nodules than plants in restored prairie soil (Fig. 1; $F_{[1,14]} = 1.61$, $p = 0.2$). None of the legumes experienced higher root colonization by AM fungi in remnant prairie soils, and in fact *Amorpha* and *Lespedeza* had nonsignificantly less root colonization in remnant prairie soils (Fig. 1 & 1; *Amorpha* $F_{[1,14]} = 1.95$, $p = 0.2$; *Dalea* $F_{[1,14]} = 0.13$, $p = 0.7$; *Lespedeza* $F_{[1,13]} = 3.02$, $p = 0.11$).

Soil from older restored prairies failed to produce more plant biomass (*Amorpha* $F_{[1,3.98]} = 0.36$, $p = 0.6$; *Dalea* $F_{[1,8.84]} = 0.50$, $p = 0.5$; *Lespedeza* $F_{[1,8.69]} = 1.52$, $p = 0.2$), nodules (*Amorpha* $F_{[1,8]} = 0.42$, $p = 0.5$; *Dalea* $F_{[1,8]} = 0.47$,

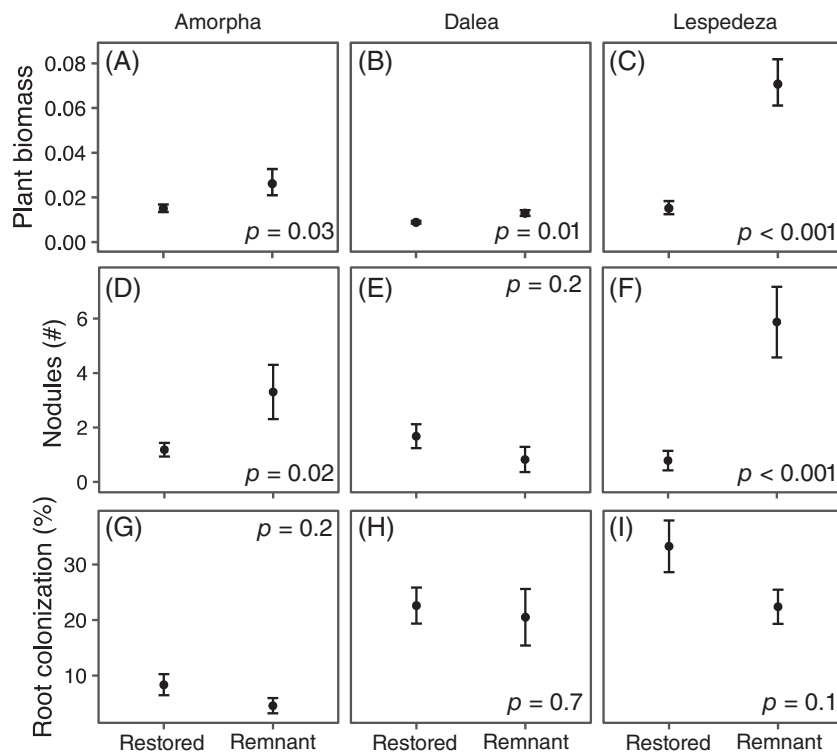


Figure 1. Effect of inoculation with remnant and restored prairie soil on plant biomass (A–C), number of nodules (D–F), and root colonization by AM fungi (G–I) in *Amorpha canescens* (A, D, G), *Dalea purpurea* (B, E, H), and *Lespedeza capitata* (C, F, I). Means shown of 10 restored sites and 6 remnant sites; error bars represent \pm SE.

$p = 0.5$; *Lespedeza* $F_{[1,8]} = 1.01$, $p = 0.3$), or root colonization (*Amorpha* $F_{[1,8]} = 0.11$, $p = 0.8$; *Dalea* $F_{[1,8]} = 0.81$, $p = 0.4$; *Lespedeza* $F_{[1,7]} = 0.05$, $p = 0.8$) compared to soil from younger restored prairies in any species (Fig. 2).

Discussion

This study contributes to a growing body of knowledge indicating that beneficial soil microbes may be lacking from restored ecosystems and suggesting that inoculation with appropriate microbes could enhance restoration outcomes by boosting growth of sensitive plant species. In particular, we showed that the abundance of rhizobia bacteria capable of nodulating focal legume species was lower in restored prairie soil, highlighting a previously overlooked component of soil microbial communities.

Our results support our first hypothesis that high-quality microbial mutualists are missing from the soils of restored prairies. All three legumes grew significantly larger with access to remnant prairie soil microbes. Although isolation of mutualistic rhizobia and mycorrhizal fungi from remnant and restored ecosystems is necessary to experimentally determine their effects on legumes independently of the suite of soil microbes that could affect plant growth responses, these results are consistent with several previous studies demonstrating positive effects of soil microorganisms from remnant communities (Middleton & Bever 2012; Maltz & Treseder 2015; Neuenkamp et al. 2019).

However, both in our data and in previously published studies, species vary in the degree to which they benefit from remnant soil microbes. We suggest two alternative hypotheses that could allow better prediction of which legume species are most likely to benefit from reintroduction of microbial mutualists.

One possibility is that late-successional legumes may be more dependent on their rhizobia mutualists, much like late-successional plant species that tend to be more reliant on AM fungi (Kozioł & Bever 2015; Bauer et al. 2018). However, data from this growth chamber experiment do not support this hypothesis. The mid-successional legume that establishes fairly well in restorations, *Lespedeza*, had the strongest positive growth response to remnant prairie soil, increasing 367% relative to restored prairie soil. Our two late-successional species, *Amorpha* and *Dalea*, are highly conservative (coefficient of conservatism values of 8 and 10, respectively; michiganflora.net), almost never appear in transects in restored prairies (Bauer et al. unpublished data; Grman et al. 2015), and increased biomass by only 74 and 31% when inoculated with remnant prairie soils.

An alternative possibility is that plant specificity as hosts for mutualistic rhizobia determine legume establishment limitation in restored ecosystems. Data from the literature support this prediction. Species that have been shown to nodulate without inoculation of rhizobia or remnant prairie soil (*Chamaecrista fasciculata*, *Desmodium canadense*, *Lespedeza capitata* [Tlustý et al. 2004], and *Baptisia lactea* [Herzberger et al. 2015]) are

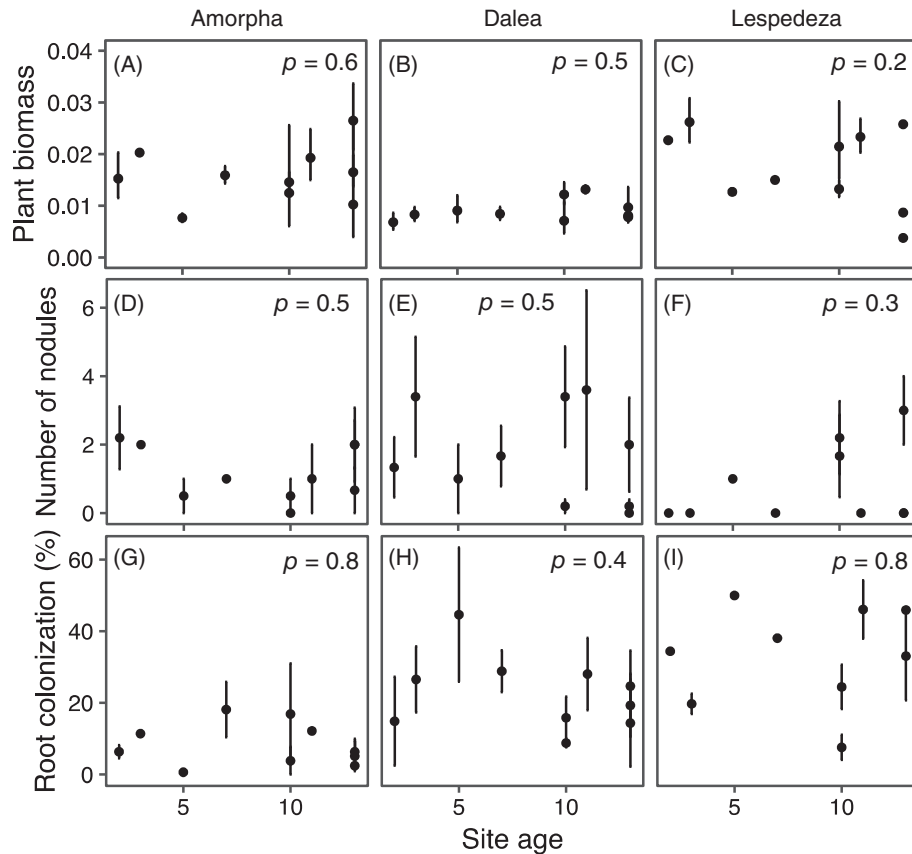


Figure 2. Variation in plant biomass (A–C), number of nodules (D–F), and root colonization by AM fungi (G–I) in *Amorpha canescens* (A, D, G), *Dalea purpurea* (B, E, H), and *Lespedeza capitata* (C, F, I) when inoculated with soils from restored prairies of different ages. Error bars represent \pm SE from growth chamber replicates (maximum 5 per site).

those that established better in Michigan restored prairies (in 23, 94, 69, and 69% of sites, respectively; Grman et al. 2015). In contrast, species that have been shown to benefit from inoculation (*Amorpha canescens* and *Dalea purpurea*; Middleton & Bever 2012) tend to establish more poorly in Michigan restorations (in 0 and 8% of sites, respectively; Grman et al. 2015). Therefore, poor establishers may be more sensitive to the identity of potential rhizobial partners than better establishers. However, data from more species are necessary to support or refute both of these hypotheses.

Our nodulation results roughly support our missing mutualist hypothesis that remnant prairies contain more mutualistic rhizobia than restored prairies. Both *Lespedeza* and *Amorpha* produced considerably more nodules when inoculated with remnant prairie soil, indicating that the abundance of rhizobia capable of nodulating these legumes is low in restored prairie soils and higher in remnant soils. Although higher nodule numbers do not necessarily indicate better mutualistic functioning (Heath 2010), in this case plants with more nodules grew more, indicating higher availability of quality rhizobia from remnant prairie sites. In *D. purpurea*, nodulation was low in both soil types, perhaps suggesting that propagules of appropriate rhizobial partners are absent from all the sites we sampled. Low *Dalea* regional abundance and its absence from the remnant

prairie sites we sampled could lead to low prevalence of their rhizobial partners. However, *Amorpha* is also very regionally rare and absent from most of the remnant sites we sampled (Bauer et al. unpublished data; Grman et al. 2015), and yet this species benefited substantially from remnant prairie soil microbes. Another possible explanation is that the growth stage reached by *Dalea* was unsuitable for nodulation. Although *Dalea* grew for the same amount of time as the species that nodulated well, it achieved far less biomass (mean of 0.01 g total biomass, compared to 0.03 and 0.05 g for *Amorpha* and *Lespedeza*, respectively). Although we cannot explain why we did not see the expected increase in *Dalea* nodulation in remnant prairie soil, it is clear that nodulation for all three species was very low in restored prairie soil. This result suggests that inoculation with suitable rhizobia could enhance nodulation of legumes in restored prairies, possibly enhancing their growth, reproduction, and population viability, increasing overall plant diversity in restored prairies.

In opposition to our missing mutualist hypothesis, there was no difference in root colonization by AM fungi between restored and remnant sites. We had expected that root colonization would be higher in remnant prairie soils because a history of tillage can reduce population sizes of AM fungi (Helgason et al. 1998), and because past inoculation studies have showed increased root

colonization with remnant soils (Maltz & Treseder 2015). Although this finding suggests that the abundance of mutualistic fungi may not differ between our restored and remnant prairies, it cannot indicate whether the quality of mutualistic fungi differs. The identity of AM fungal partners can strongly determine the degree of benefit for late-successional plants such as *Dalea* and *Amorpha* (Koziol & Bever 2016), and other work implies that the quality of fungal partners is generally lower in restored compared to remnant prairies (Bever et al. 2003; Middleton & Bever 2012; Koziol et al. 2018). However, this pattern is not universal, and for many plant species, restored grassland soil microbes are superior or equivalent to remnant soil microbes (Carbajo et al. 2011; Herzberger et al. 2015; Grman & Hodges in preparation). Therefore, we suggest that differences in AM fungal community composition may have contributed to the growth differences of legumes in our restored versus remnant prairie soils, but further investigation is required.

We found no support for our spontaneous recovery hypothesis, that microbial communities would reassemble without inoculation in older restorations and provide more benefit to legumes than microbes from younger communities. This result agrees with previous inoculation studies from prairie systems, which often show that soil microbes from older restorations may have equal or inferior effects on plant growth compared to those from younger restorations (Larson & Siemann 1998; Anderson 2008; Herzberger et al. 2015; Grman & Hodges in preparation). However, this result is in contrast to studies using environmental sampling to monitor reassembly of soil microbial communities, where partial recovery is seen over decadal timescales (28-year-old restorations in Jangid et al. 2010, 27-year-old restorations in Barber et al. 2017) or even as quickly as 2–4 years after prairie planting (Herzberger et al. 2014). It is possible that microbes with the largest positive effects on plant growth—that is, plant mutualists—recolonize more slowly than the non-plant-associated microbes that are more abundant in soils and more easily detected with environmental sampling. It is also likely that recovery of specialist plant mutualist populations depends on the presence of appropriate host plants. Because restored prairies often lack target plant species (Martin et al. 2005; Grman et al. 2015) and the soils may lack the beneficial microbes that associate with those target plant species (Koziol et al. 2018), restoration practitioners may find themselves caught in a positive feedback loop. Indeed, recent work has suggested that positive feedbacks between late-successional plants and remnant-associated soil microbes can accelerate succession of late-successional plant communities when appropriate AM fungi are present (Koziol & Bever 2019). Although these positive feedbacks can enhance restoration outcomes, the possibility of these feedbacks also implies that late-successional microbes and late-successional plants must be reestablished concurrently.

This study indicates that appropriate rhizobial partners for three target legumes may be scarce or effectively absent from the soils of restored prairies, even after approximately 13 years of prairie ecosystem development. Furthermore, it suggests that targeted inoculations may help—may even be required—to increase the establishment and growth of these species. However, inoculation is not without risk or cost (Hart et al. 2017;

Koziol et al. 2018). For example, the establishment and spread of inoculated microbes is poorly understood (Beyhaut et al. 2014; Hart et al. 2017); if too low, inoculation may be ineffective and a poor use of practitioner time and funds, while if too high, inoculation can potentially introduce invasive species or strains (Hart et al. 2017; Koziol et al. 2018). Further work is required to (1) isolate effective specialist microbial strains, (2) evaluate their efficacy in the field, (3) understand the community and ecosystem consequences of inoculation, and (4) develop the infrastructure and knowledge base for practical inoculum application in a restoration setting.

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