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RESEARCH ARTICLE

Native plant abundance, diversity, and richness increases in prairie restoration with field inoculation density of native mycorrhizal amendments

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Ecological restoration efforts can increase the diversity and function of degraded areas. However, current restoration practices cannot typically reestablish the full diversity and species composition of remnant plant communities. Restoration quality can be improved by reintroducing key organisms from the native plant microbiome. In particular, root symbionts called arbuscular mycorrhizal (AM) fungi are critical in shaping grassland communities, but are sensitive to anthropogenic disturbance, which may pose a problem for grassland restoration. Studies of mycorrhizal amendments include inoculation densities of 2–10,000 kg of inocula per hectare. These studies report variable results that may depend on inocula volume, composition, or nativeness. Here we test eight different densities of native AM fungal amendment, ranging from 0 to 8,192 kg/ha in a newly installed prairie restoration. We found that native plant establishment benefited from native mycorrhizal inocula, resulting in improvements in native plant abundance, richness, and community diversity. Moreover, the application of very low densities of native mycorrhizal inocula, as suggested on commercial mycorrhizal products, were ineffective, and higher concentrations were required to benefit native plant abundance and community diversity. These data suggest that higher densities of mycorrhizal amendment or perhaps alternative distribution methods may be required to maximize benefits of native mycorrhizal amendments in restoration practices.

Key words: arbuscular mycorrhizal fungi, inoculation density, plant microbiome, prairie restoration

Implications for Practice

- The plant microbiome can be amended with native mycorrhizal fungi to alter restoration outcomes; the more inocula applied, the better the restoration outcome.
- There is a large discrepancy between the recommended application densities of commercial inoculum and what has been tested in scientific applications that needs further resolving.
- The low densities of mycorrhizal application suggested on commercial products were not enough to improve restoration outcomes. Land managers and restoration ecologists need to apply mycorrhizal amendments in greater densities or use alternative distribution methods to influence native plant establishment.

Introduction

Native plants are commonly established in grassland restoration, but outcomes are widely variable (Brudvig et al. 2017). Restorations commonly have lower plant community diversity relative to comparable nearby remnant grasslands (Kindscher & Tieszen 1998; Martin et al. 2005; Middleton et al. 2010), and plant species richness generally declines over time, especially for forb and legumes (Baer et al. 2002). Native plant community establishment, and particularly late successional prairie species, may be limited by arbuscular mycorrhizal (AM) fungal communities (Koziol & Bever 2017). Many of these prairie grassland species are strongly dependent on mycorrhizal fungi (Koziol & Bever 2015; Bauer et al. 2018) and highly sensitive to AM fungal composition (Koziol & Bever 2016; Cheeke et al. 2019; Koziol & Bever 2019). AM fungi are known to improve plant growth by acquiring soil nutrients which are difficult for plants to acquire, such as inorganic phosphorus. AM fungi can also provide non-nutritional benefits to their plant host through alleviation of environmental stressors such as drought (Davies et al. 1993; Koziol et al. 2012) as well as providing resistance to pathogens (Sikes et al. 2009) and herbivory (Bennett & Bever 2007). AM fungi contribute to other valuable ecosystem services, such as mitigating rising CO_2 levels by acting as carbon sinks (Leake et al. 2004) and decreasing erosion by producing soil-binding proteins that increase soil aggregate

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stability (Rillig 2004). AM fungal abundance is also tightly correlated with nitrogen and carbon sequestration (Wilson et al. 2009).

Although AM fungi are commonly present in soils, common agricultural practices are known to disrupt AM fungal communities (House & Bever 2018). For instance, processes such as tilling (Abbott & Robson 1991; Jasper et al. 1991), the use of soluble fertilizers and biocides (Ryan et al. 1994), and the planting of crop monocultures (Oehl et al. 2003) can lead to reduced AM fungal abundance, infectivity, and diversity. Given that many restorations are installed in highly disturbed landscapes, these changes in AM fungal communities can have implications to native plants that strongly depend on native mycorrhizal fungi. Reintroducing native AM fungal communities into sites with disturbed soil communities have been shown to benefit grassland plantings by improving native survival, growth, fecundity (Middleton et al. 2015; Koziol & Bever 2017), soil aggregate stability (Duchicela et al. 2012). and weed inhibition (Koziol & Bever 2017; Lubin et al. 2019). Increased native plant establishment has been found in other independent inoculation experiments both in North American grasslands. including tallgrass prairie, desert plains, and western coastal plains (Richter & Stutz 2002; Bever et al. 2003; Vogelsang & Bever 2010; Middleton & Bever 2012; Middleton et al. 2015; House & Bever 2019), and in Eurasian grasslands (Zhang et al. 2012; Wubs et al. 2016). Positive feedback between native plants and native AM fungi can amplify these benefits over time (Koziol & Bever 2019). Amending native AM fungi in restorations can improve native establishment, and greater native establishment can in turn improve habitat quality for wildlife (Debinski & Babbit 1997; Tonietto & Larkin 2018).

While the above research highlights improvements in restoration response to native mycorrhizae, restoration outcomes have been shown to benefit less from commercial mycorrhizal inoculum products. Late successional plants have been shown to be inhibited by commercial fungi (Middleton et al. 2015; Emam 2016) and commercial fungi have been shown to have little effect on reducing soil erosion (Vogelsang & Bever 2010), native plant richness (Perkins & Bennett 2017), or cover (White et al. 2008; Ohsowski et al. 2017). Commercial inoculum products may fail because many AM fungal species that are commercially available are likely highly abundant in disturbed, post-agricultural, and early successional soils (Koziol et al. 2018). Alternatively, commercial inocula may be applied at too low of a density to be effective (but see [Middleton et al. 2015] for a study comparing native and commercial inocula at similar densities).

While there is evidence to suggest that AM fungal amendments, and in particular native AM fungal amendments, may benefit restoration establishment, the recommended application rates and methods provided by commercial producers of AM fungi are highly variable and there are no standards for native inocula application method or densities. Inoculation methods commonly include planting pre-inoculated seedlings or broadcasting inocula (Koziol et al. 2018). Generally, the inoculation densities recommended by commercial producers of inocula range from around 2–120 kg of inocula per hectare. This volume tends to be much lower than what has been reported to be effective application densities from within the scientific literature, which have ranged from an estimated 700–75,000 kg per hectare and have utilized native inocula (Bever et al. 2003; Middleton & Bever 2012; Emam 2016; Wubs et al. 2016; Koziol & Bever 2017). It remains to be seen whether the ineffectiveness of commercial mycorrhizal products stems from too low of recommended application densities, or from being nonbeneficial for other reasons, such as non-native isolates of commercial fungi being maladapted to specific soils, plant communities, or water regimes in which they are applied.

In this study, we test whether the benefits of native mycorrhizal inoculation on prairie community success depends on inoculum density. Specifically, we ask how inoculation density impacts richness, abundance, and diversity of native and non-native plant communities. We apply a native mycorrhizal inoculum previously shown to benefit native prairie plant establishment and growth (Koziol et al. 2019; Wang et al. 2019). Using this beneficial native inoculum, we designed an inoculation density gradient that covers the range of application densities suggested by commercial producers of mycorrhizal inocula as well as several higher densities corresponding to effective inoculation densities reported in the scientific literature. Eight inoculation densities were established by broadcasting and tilling. We followed the establishment and growth in the resulting prairie communities.

Methods

Field Site Preparation

This field inoculation study was initiated in the spring of 2017 at The Land Institute's Perennial Agriculture Project Field Station located in Lawrence, Kansas, U.S.A. (39.001311°, −95.320337°). The site was dominated by *Bromus inermis* (smooth brome) that was planted at least 20 years prior to this experiment. In the fall of 2016 and spring of 2017, the land was disked and tilled prior to initiating field plots. Soil nutrient status after tilling was total N 0.19%, total C 1.92%, 2.67 P-M ppm via Melich, 9.5 ppm NO3-N, 53.69 ppm NH4-N via KCL extraction. Replicate 2×4 m plots were created with 3 or more meter aisles.

Our approach was to apply a common native inocula across a density gradient. Inoculation density treatments were randomized within each of eight replicate blocks. Each block included all seven inoculation density treatments and two non-inoculated control plots, totaling 72 restoration plots (Fig. 1). Inoculation densities increased on a log progression from no inocula to more than 8,000 kg/ha. Our four lowest densities of native inocula amendment were inspired by those listed on commercial mycorrhizal products and are henceforth referred to as "low densities" of native inoculum (2–128 kg/ha, Table 1). Our three highest densities of native inocula were intended to reflect those used in past scientific studies and are henceforth called "high densities" of native inoculum (512–8,192 kg/ha, Table 1). Inocula for each plot was evenly scattered onto the soil surface by hand broadcasting and then tilled in to the top 4 inches of the plot with a rototiller. No effort was made to remove the existing mycorrhizal community in the old field.

North

0	2,048	8,192	2,048	512	512	2	0
32	0	32	0	2	128	128	2,048
0	8,192	2,048	8,192	0	2	0	8
2	8	0	8	8192	0	32	128
512	0	128	128	128	32	2,048	8,192
128	512	0	0	0	8	512	2
2,048	2	2	32	32	2,048	8	0
8	128	512	512	2,048	8,192	0	32
8,192	32	8	2	8	0	8,192	512

South

Figure 1. Experimental randomization of our inoculation density plots. Eight blocks were arranged from east to west and included each of the seven densities of a common mycorrhizal inoculum, ranging from 2 to 8,192 kg/ha, and two non-inoculated controls. Replicate 2 × 4 m plots were randomized from north to south and 3 or more meter aisles were established between plots.

Native Mycorrhizal Propagation

A native mycorrhizal inoculum was created as described in a previous study (Koziol & Bever 2017). Briefly, single-species fungal cultures were created based on spore morphology from spores isolated from an unploughed native prairie in Lawrence, Kansas, 22 km from the restoration location. Cultures were grown for 1 year in a sterilized sand:soil mixture (10.15 P ppm via Melich extraction, 7.375 NO3-N ppm, 22.2 NH3-N ppm via KCl extractions) prior to being used as inocula. A native fungal community mixture of these cultures was homogenized prior to being applied in the field. The mixture contained the fungal species *Scutellospora dipurpurescens*, *Gigaspora gigantea*, *Funneliformis mosseae*, *Funneliformis geosporum*, *Glomus mortonii*, *Rhizophagus diaphanous*, and *Claroideoglomus claroideum*. Of these native mycorrhizae species, *S. dipurpurescens*, *F. geosporum*, *G. mortonii*, and *R. diaphanous* are not found in

Table 1. Eight different inoculation densities of a native mycorrhizal inoculum were chosen for this study. No mycorrhizal application reflects contemporary conventional restoration practices. "Low density" represents various application densities listed on commercial mycorrhizal products. "High density" represents a few of the inocula application densities utilized in past successful research using native inocula.

kg/ha of Native Inocula	*Native Inocula Density Applied*	*Products/Scientific Studies Using Similar Densities*
0	0	
2	Low density	MycoApply (Mycorrhizal Applications) Endo ~2 kg/ha
8	Low density	Sustainable Agricultural Technologies, Inc. ~11 kg/ha
32	Low density	Root Naturally Granular EndoMycorrhize ~24 kg/ha
128	Low density	MycoBloom Mycorrhizae ~168 kg/ha
512	High density	Emam 2016 (772 kg/ha whole soil)
2,048	High density	Koziol and Bever 2017 (1,790 kg/ha mycorrhizae)
8,192	High density	Bever et al. 2003 (10,000+ kg/ha whole soil)

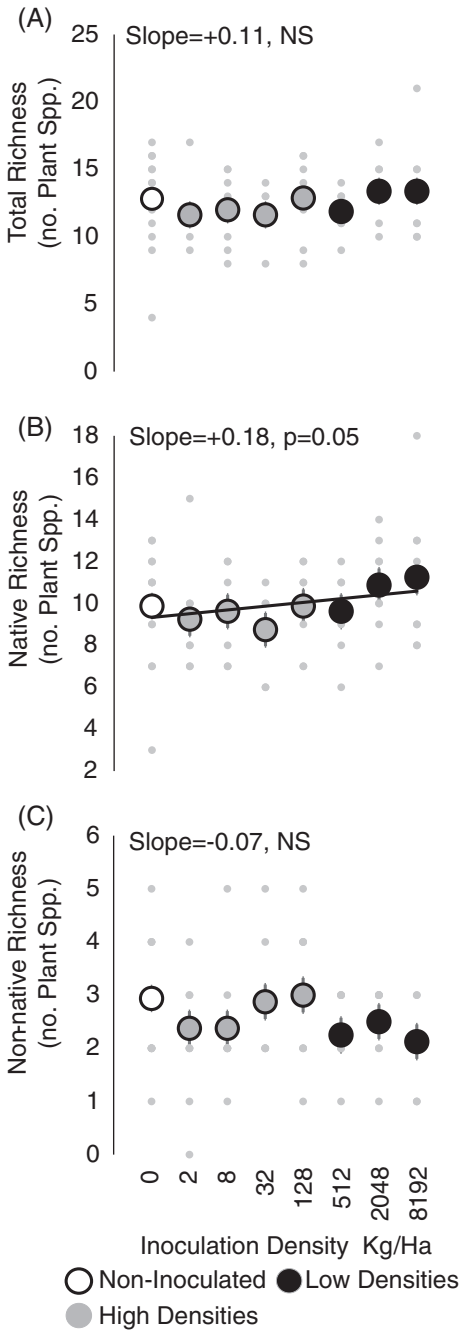

Figure 2. Total (A), native (B), and non-native (C) plant richness in the field. Lines and slopes represent the best-fit lines for the linear response relationship to inoculation density for the individual plots (small gray circles ●). Large circles represent the average richness for a given inoculation density (non-inoculated represented by white, open circles (○), low densities of mycorrhizal amendment recommended by commercial producers if inoculum represented by large gray circles (●), and higher densities of mycorrhizal amendment are represented by large black circles (●)). Error bars on the large circles are ± SE.

any commercial mycorrhizal products we are aware of, while *G. gigantea*, *F. mosseae*, and *C. claroideum* are widely available in commercial mycorrhizal products. Past work has shown that a community of these native mycorrhizal species benefit native prairie plants from this region (Koziol et al. 2019; Wang et al. 2019). Mycorrhizae for the field experiment was grown during the 2016 growing season. The concentration of the homogenized native inocula was around 30 spores/cm^3 or 25,132 spores/kg. The number of fungal propagules does not include hyphae or infected root fragments because not all fungal species can germinate via fungal propagules such as hyphae and infected root fragments (Klironomos & Hart 2002).

Seeds were obtained from Missouri Wildflowers (Jefferson City, Missouri, U.S.A.) (Table S1). For each of the 46 plant species, ½ of the seed weight was put aside for distribution into isles and borders. The other ½ was evenly distributed into 72 bags, one for each plot. All seeds were then cold moist stratified for 2 months. In April of 2017, the seed mixture was hand broadcast after inoculation prior to tilling. Final seed density was 12.5 kg/ha (11 lb./acre).

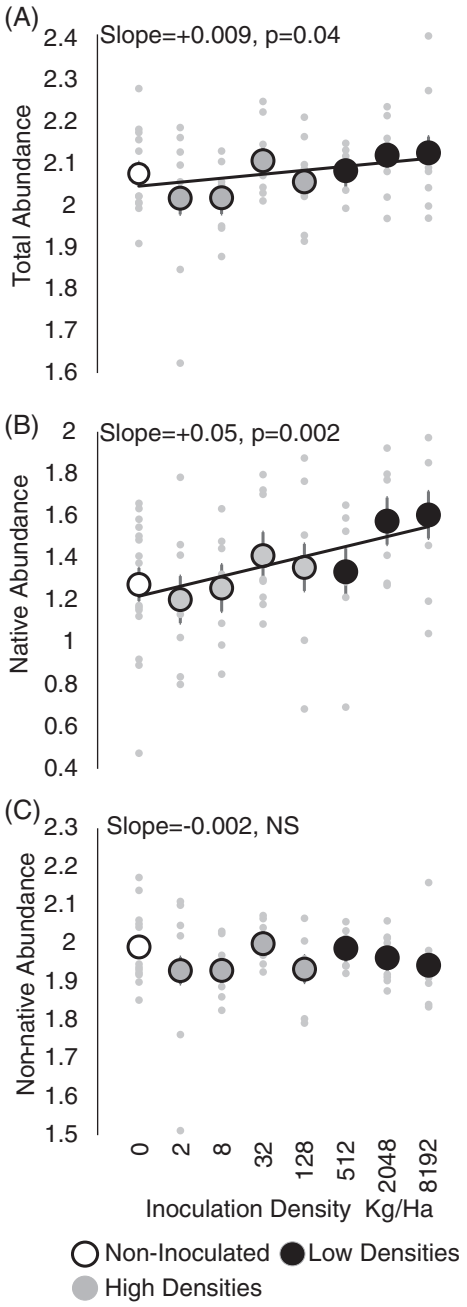

Figure 3. Total (A), native (B), and non-native (C) plant abundance in the field. Lines and slopes represent the best-fit lines for the linear response relationship to inoculation density for the individual plots (small gray circles ●). Large circles represent the average plot abundance for a given inoculation density (non-inoculated represented by white, open circles (○), low densities of mycorrhizal amendment recommended by commercial producers if inoculum represented by large gray circles (●), and higher densities of mycorrhizal amendment are represented by large black circles (●)). Error bars on the large circles are ± SE.

Data Collection and Statistical Analyses

Establishment year biomass was collected in late August 2017 by clipping plant mass in two 15 cm × 1 m strips in each plot. Plants were sorted by species before being dried at 70°C and weighed. We analyzed the abundance, richness, and diversity of establishing plants, including separate analyses of total, native, and non-native native plant species. Biomass data were transformed as log (1+ biomass). We used the *vegan* package in R to calculate the inverse Simpson's index for each plot as a metric of plant community diversity using the aboveground biomass (g) of each plant species in plot (Oksanen et al. 2007). We analyzed richness, abundance, and diversity data using Proc Mixed in SAS (SAS 2015) using inoculation density as a categorical predictor and block as a random effect. We designed a linear contrast to test if plant response increased with rank of inoculation density. As this contrast was planned, it was tested with full power. Reported r^2 for the linear contrast was calculated sorting by inoculation density using Proc Corr in SAS (SAS 2015). We also tested whether low densities, consistent with commercial recommendations, or high densities were beneficial compared to the non-inoculated control. While these tests were also planned, they were not orthogonal to the linear contrast with rank of density and we therefore controlled for multiple comparisons using the Dunn-Sidák method of adjustment (Sokal & Rohlf, 1995).

Results

Total plot richness during the year of establishment was not affected by inoculation density (Table S2, Fig. 2A, Linear contrast slope = +0.11, $F_{1,57} = 1.59$, $p = 0.2$, $r^2 = 0.28$) nor any of the a priori contrasts we designed (Table S2). Across all richness metrics, low densities of inocula were not found to be different from the controls, suggesting that these densities of AM inocula are not great enough to produce an effect on plant community richness using the tested methods (Table S2, Low Density vs. Non-inoculated contrasts, all $p > 0.3$). However, this effect differed depending on whether plants were native or non-native. Linear contrasts indicated that native richness increased with

inoculation density (Linear contrast slope = +0.18, Table S2, Fig. 2B, $F_{1,57} = 3.94$, $p = 0.05$, $r^2 = 0.43$), where the plots with the highest density of native inocula amendment had 14% more native species than the non-inoculated controls. Non-native richness was similar across inoculation density (Linear contrast slope = −0.07, Table S2, Fig. 2C, $F_{1,57} = 1.8$, $p = 0.2$, $r^2 = 0.21$). We found a marginally significant effect for non-native richness to be reduced with high inoculation density (High Density vs. Non-inoculated contrast, Table S2, Fig. 2C, $F_{1,57} = 4.8$, $p = 0.08$), where plots had an average of 0.65 fewer non-native species with high density of native inoculation (Fig. 2C).

After harvesting the aboveground biomass in the plots, we found that total abundance increased with increasing density of native inocula amendment (Linear contrast slope = +0.009, Table S2, Fig. 3A, $F_{1,57} = 4.68$, $p = 0.04$, $r^2 = 0.49$). This effect was driven by increases in native abundance with increasing inocula density (Linear contrast slope = +0.05, Table S2, Fig. 3B, $F_{1,57} = 10.6$, $p = 0.002$, $r^2 = 0.75$) but not non-native abundance (Table S2, Fig. 3C). We found a marginally significant effect where native abundance was 18% greater on average in high-density inoculation treatments relative to the control (High Density vs. Non-inoculated contrast, Table S2, Fig. 2B, $F_{1,57} = 5.11$, $p = 0.08$). As found with plant richness metrics, total and native abundance with low-density application of inocula were not found to be different from the controls, suggesting that these densities of AM inocula are not great enough to produce an effect on native plant abundance using the tested methods (Table S2, Low Density vs. Non-inoculated contrasts).

As species diversity considers both species richness and relative abundances, both of which we found to be affected by native AM fungal density treatments, it follows that total plot diversity also responded to inoculation density (Table S3, Linear contrast slope = +0.06, Fig. 4A, $F_{1,57} = 7.95$, $p = 0.007$, $r^2 = 0.59$). Specifically, total diversity was 31% greater than controls with the highest inoculation density on average (Fig. 4A). As observed with both plant community richness and abundance, the low densities of native inocula amendment had no effect on total, native, and non-native diversity compared to the non-inoculated controls (Table S3, Low Density vs. Non-inoculated contrasts, all not significant), indicating that greater densities of inocula may be required to affect plant community diversity. Native (Linear contrast slope = +0.02, Table S3, Fig. 4B, $F_{1,57} = 0.9$, $p = 0.3$, $r^2 = 0.06$) and non-native (Linear contrast slope = −0.006, Table S3, Fig. 4C, $F_{1,57} = 0.55$, $p = 0.46$, $r^2 = 0.14$) were not affected by inoculation density.

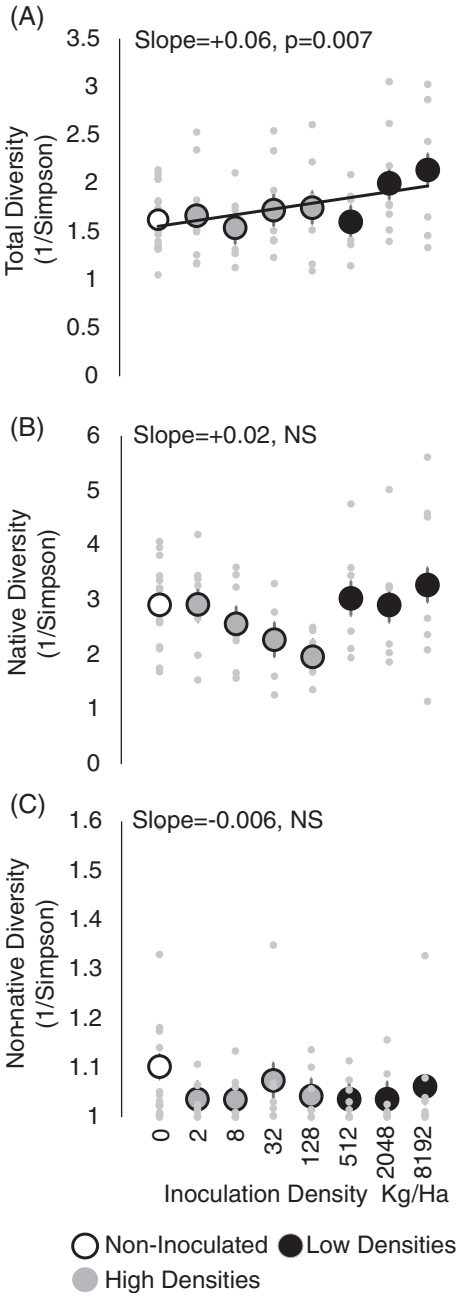

Figure 4. Total (A), native (B), and non-native (C) plant community diversity in the field. Lines and slopes represent the best fit lines for the linear response relationship to inoculation density for the individual plots (small gray circles ●). Large circles represent the average plot plant community diversity for a given inoculation density (non-inoculated represented by white, open circles (○), low densities of mycorrhizal amendment recommended by commercial producers if inoculum represented by large gray circles (●), and higher densities of mycorrhizal amendment are represented by large black circles (●)). Error bars on the large circles are ± SE.

Discussion

Because soil microbial communities are sensitive to soil disturbances and other anthropogenic changes such as agrochemical inputs, crop monocultures, and tillage (Abbott & Robson 1991; Jasper et al. 1991; Ryan et al. 1994; Oehl et al. 2003), amending new plantings with beneficial microbiome components has the potential to improve plant productivity, particularly for prairie grassland species that are often strongly responsive to mycorrhizal fungal (Koziol & Bever 2015; Bauer et al. 2018). This body of work demonstrates the value of a holistic approach to restoration by highlighting the importance of the plant microbiome in plant community establishment. Many perennial plant species are strongly dependent on their

soil microbes including *Rhizobia* (Larimer et al. 2013), arbuscular mycorrhizal fungi (Koziol & Bever 2015; Bauer et al. 2018), and larger biota such as worms (Agarwal et al. 2010). We have shown conclusively that native plant abundance and plant community diversity in restorations can be improved by high-density applications of native AM fungi. Our findings support previous work which demonstrated that mycorrhizal amendment can improve native plant establishment and lead to decreased nonseeded abundance in establishment years (Koziol & Bever 2017).

Previous field trials have indicated that commercial mycorrhizal fungi are less effective than native, locally adapted mycorrhizal amendments (Maltz & Treseder 2015; Middleton et al. 2015; Emam 2016). Given that commercial AM fungi are likely non-native and that AM fungi can also be adapted to the specific nutrient and water conditions of their soil (Johnson et al. 2010), it is possible that the ineffectiveness of commercial fungi is the result of a mismatch to the soil in which they are being applied. However, commercial fungi application densities are also typically much lower than the density of native mycorrhizal amendments used in scientific studies (Bever et al. 2003; Emam 2016; Wubs et al. 2016) and inoculation density is rarely controlled in comparing inoculation types (but see [Middleton et al. 2015]). For this study, we applied a common native inocula across a density gradient that covered both the low densities recommended by commercial producers of mycorrhizae and the high densities of native inocula included in past restoration science. Across all plant community response metrics (richness, abundance, or diversity), we were unable to detect an inoculation effect at the low-density inoculation densities recommended by commercial mycorrhizal producers. We found that plant community richness and native abundance responded only to the highest inoculation densities. In fact, we did not observe a plateau in the plant community response to inoculation, which suggests application of native AM fungi at even higher densities than we investigated could yield additional benefits. In short, while our native inoculum was beneficial, our results suggest that the low densities of mycorrhizal amendment recommended on commercial products may be too low to be effective and that more propagules are needed to increase native plant establishment and productivity. However, it should be noted that our field trial tested inoculation density on small patches (0.3 m^2 subsamples per 2 × 4 m plot). Additionally, it is also possible that commercial inoculum that is non-native may not be effective even at high application densities due to nonlocal adaption. To resolve these issues, future work should assess the effects of native versus non-native mycorrhizal amendment (commercial or otherwise) at similar inoculation densities on a larger scale.

Applying mycorrhizal amendments at the high densities found to be successful in this study may be cost prohibitive. This study and others that found mycorrhizal amendment is successful when inocula is applied via broadcasting and tilling it into the top few inches of soil (Bever et al. 2003; Emam 2016; Wubs et al. 2016), a practice that may be wasteful of inocula given that (1) inocula may land on the soil surface and be exposed to harmful solarization and (2) inocula is placed into/on soil before seeds have broken dormancy and have developed the necessary fine roots that spores require to feed and support them. Given that AM fungal spores can germinate and cease growing within 5 days (Kokkoris et al. 2019), it is likely that many propagules of inocula die when applied via broadcasting had they not located a suitable plant host root by then. Alternative methods of inoculation introduction have proved useful, including introducing inocula via planting inoculated plant seedlings using much less inocula (Koziol & Bever 2017). Applications via seed drill have also been found to be effective at lower application densities of 25 kg/ha at the time of planting for corn, but only in conjunction with inorganic fertilizers (Cozzolino et al. 2013). More studies are needed on a wider range of application techniques and densities. Future work should investigate application methods that more precisely colocate inoculum with plant roots or seeds, such as drilling via seed drill or distribution on a seed coating. Additionally, more work is needed to understand the long-term effects of inocula in grassland restoration, as recent evidence from meta-analysis suggests that the benefits of inoculation may improve over time in the restoration of several plant community systems (Neuenkamp et al. 2019). Furthermore, future work should assess whether benefits of dilute native inoculations can be realized over longer periods of time.

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Supporting Information

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

Table S1. The name of applied volume of each of our prairie plant species.
Table S2. Plant total, native, and non-native richness and abundance in the field.
Table S3. Plant total, native, and non-native diversity in the field.

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