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Soil Microbial Communities: Critical Roles in Control of Non-Native Invasive Species and Restoration of Ecosystem Functions

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Ecological restoration efforts can increase the diversity and function of degraded areas and inhibit establishment of non-native invasive plant species. However, current restoration practices cannot typically re-establish the full diversity and plant species composition of intact remnant plant communities. This project focuses on the role of soil microbes in improving the establishment of native plants and in ameliorating the negative effects of non-native invasive plant species in grasslands. The researchers particularly focus on the beneficial effects of a group of soil fungi called arbuscular mycorrhizal (AM) fungi, which form symbiotic associations with most plant species.

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ABSTRACT

Objective:

Ecological restoration efforts can increase the diversity and function of degraded areas and inhibit establishment of non-native invasive plant species. However, current restoration practices cannot typically re-establish the full diversity and plant species composition of intact remnant plant communities. Our project focusses on the role of soil microbes in improving the establishment of native plants and in ameliorating the negative effects of non-native invasive plant species in grasslands. We particularly focus on the beneficial effects of a group of soil fungi called arbuscular mycorrhizal (AM) fungi, which form symbiotic associations with most plant species. We evaluate the potential beneficial effects of AM fungi on native grassland restoration and management and the potential to suppress non-native invasive plant species.

Technical Approach:

We evaluate the potential beneficial effects of AM fungi on native grassland restoration and management through a series of field surveys, field inoculation experiments, and greenhouse experiments. To better understand how AM fungal community composition is affected by anthropogenic disturbance across US grasslands, we sampled plant roots from pairs of remnant and disturbed sites spanning from western Oklahoma to eastern Illinois and assessed AM fungal community composition using environmental sequencing. To evaluate the sensitivity of non-native invasive plant species and native plant species to AM fungi we tested plant growth response with and without native AM fungi in a series of greenhouse assays. These assays are combined within a meta-analysis with data from previously published assays to generate robust conclusions on the relative responsiveness of native and non-native plant species.

The value of reintroduction of the native microbiome was tested in a series of field inoculation experiments. Five large field inoculation experiments were conducted across three Department of Defense properties (Fort Riley Army Base in Kansas, Tinker Air Force Base in Oklahoma, and the decommissioned Chanute Air Force Base in Illinois). In our grassland restoration experiments, native soil microbes were introduced in association with native plant species that are known to be good hosts for AM fungi. In each experiment, the same four host plant species, which we call nurse plants, were planted into each plot. Individual plot treatments varied with whole plots having 16 nurse plants inoculated with AM fungi isolated from nearby unplowed remnant grasslands, soil microbes freshly derived from native unplowed remnant grasslands, or the control non-inoculated plots. The generality of these field experiments was supplemented by three additional field inoculation assays featuring variations of the basic design. The context dependence in which inoculation of AM fungi would benefit native plant establishment was investigated in a series of greenhouse assays that manipulated different aspects of the environment.

Results:

We find that AM fungi are sensitive to anthropogenic disturbance, with weedy AM fungi dominating following mechanical disturbance and dominance of non-native invasive plant species. This degradation of the mycorrhizal community may facilitate the continued dominance of non-native invasive plant species because we find that non-native invasive and weedy native

plant species are generally not responsive to mycorrhizal fungi and are not sensitive to different AM fungal taxa. In contrast, desirable, late successional native plant species are very responsive to mycorrhizal fungi and very sensitive to differences between AM fungal species, suggesting that the degradation of the soil community that occurs with non-native plant species invasion can inhibit native grassland restoration.

We find strong evidence that non-native invasive plant species can be inhibited and the quality of restorations can be improved by reintroducing native AM fungi into disturbed areas. Individual studies show that reintroduction of the native microbiome and native mycorrhizal fungi can improve plant diversity, accelerate succession, and increase the establishment of plants that are often missing from restored communities. We do not find significant differences between the beneficial effects of inoculation with the local native soil microbiome compared to inoculation with cultured native AM fungal community, which suggests that the AM fungi are the major components of the soil microbiome that benefit native plant establishment and growth. We also find that the benefits of inoculation can extend to non-inoculated neighboring native plants. Re-establishment of late successional plant species within a diverse native grassland can inhibit the dominance of non-native invasive plant species.

The benefits of inoculation with AM fungi will likely depend upon environmental context and on the source of the inocula. We find that warming and drying environments may increase the competitive ability of the non-native invasive plant species, like old world bluestem, for example, by decreasing the importance of the interactions of soil microbes with native plants. We also find that while using locally-adapted native AM fungal inocula is beneficial in general, the specific effects depend upon the ecological context. In particular, at early stages of succession, non-target native plant species may benefit most from well matched mycorrhizal fungal inocula, particularly during the first year of a restoration.

Benefits:

Our work identifies AM fungi as keystone components of the plant microbiome and illustrates the potential value of reintroduction of native AM fungi as a native grassland management strategy to facilitate recovery and control of non-native invasive plant species. We show that degradation of the AM fungal community is a major problem resulting from anthropogenic disturbance, such as due to military training, and dominance of non-native invasive plant species. Reintroduction of native AM fungi can accelerate grassland recovery by improving establishment and growth of highly desirable native plant species, and suppressing undesirable plant species including non-native invasive plant species. As the reintroduction of native AM fungi increases competitive ability of late successional long-lived native plant species, the implementation of this management approach could offer long-term solutions to recovery of grasslands from a history of disturbance and dominance by non-native invasive plant species.

The implementation of the inoculation approach on a large scale would require the development of native AM fungal cultures from native grasslands across the US. In addition, further research is required to test the conditions in which inoculation of native AM fungi is most advantageous and to document the long-term benefits of reintroduction of native AM fungi.

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I. OBJECTIVES

Biological invasion by non-native plants is a major cause of native ecosystem loss. This is particularly true for grasslands in North America, where non-native plants can dominate grasslands following soil disturbance and become persistent problems for land managers. Disturbance may facilitate dominance by non-native plants because it destroys the native soil community on which native plant species depend, including symbiotic fungi. Arbuscular mycorrhizal (AM) fungi form symbiotic associations with plant roots and aid in plant uptake of limiting soil resources such as phosphorus, nitrogen, and water. In exchange, plants deliver carbon to their obligate symbionts in the form of sugars. Disruption of the association of native plants with AM fungi can reduce rates of carbon sequestration and reduce soil aggregate stability, thereby potentially contributing to soil erosion. Therefore, restoration of native AM fungi can help restore native plants and ecosystem function of grasslands.

We evaluated the potential benefits of native soil fungal inoculation to control non-native plant invasions and enhance restoration of ecosystem function at U.S. military bases. We specifically tested whether disturbance of soil and dominance by non-native invasive plant species results in reductions in diversity and changes in composition of AM fungi and reductions in soil aggregate stability. We also tested whether inoculation with native AM fungi at disturbed sites can improve the establishment of native plant species and suppress non-native invasive plant species. Finally, we evaluated whether changes in soil communities due warmer temperatures or increased droughts alter plant-AM fungal interactions.

We combined field surveys, field inoculation studies and laboratory mesocosm tests to accomplish our objectives. We inoculated with native AM fungal communities in association with native nurse plants and assayed their effectiveness in improving establishment and survival of native plant species and suppression of introduced species. We also measured establishment and spread of the inoculated fungi using assays of environmental DNA sequences.

We conducted field surveys and field inoculations in parallel across disturbed and invaded grasslands within three military bases in the Midwestern US: Chanute Air Force Base, IL; Fort Riley, KS; and Tinker Air Force Base, OK. Problem non-native invasive plant species on these bases vary, with a C_3 NIS being dominant at the northern-most facility, (Chanute Air Force Base), a C_4 NIS being extensively invasive at our southern-most facility (Tinker Air Force Base), while both the C_3 and C_4 species are problems at our centralized location (Fort Riley Army Base).

Our research goal was to improve understanding of the ecological causes and consequences of invasion by non-native invasive plant species. Our research directly addressed key natural resource program goals of Department of Defense (DoD) facilities by testing novel approaches to restore disturbed grasslands of US military bases. The general principles tested through our research are relevant to land management of DoD properties across the U.S.

BACKGROUND

AM fungal mediation of non-native invasive plants

Biological invasion by non-native plants is one of the major causes of native ecosystem loss (Watkinson & Ormerod 2001) and global change (Vitousek et al. 1997). Most research on the factors influencing non-native plant invasions has focused on propagule availability or aboveground plant traits of the invading species (e.g. Tilman 1988). As a result, we know considerably less about invasibility as an emergent property of the comprehensive plant-soil interactions or the factors influencing it (Levine et al. 2004). However, aboveground and belowground communities are inextricably linked, and it is well documented that soil organisms play important roles in regulating ecosystem-level processes in native systems. Additionally, plants can alter soil characteristics in ways that feed back to affect the performance of that species or other plant species (Bever et al. 1997, Bever 2003). These soil feedbacks can alter the success of invasive species, with individual studies showing both positive and negative feedbacks during invasion (Reinhart et al. 2003; Bever et al. 2010).

AM fungi can play an important role in plant invasions (Pringle et al. 2009). AM fungi can contribute to plant soil feedback, as AM fungal taxa can exhibit host-specific growth responses (Bever 2002), and the benefits a given plant receives can depend on the identity of its AM fungal associates (e.g. Johnson et al. 2010; Hoeksema et al. 2010). Non-native invasive plant species (NIS), in particular, have been shown to alter the density and/or composition of AM fungal communities, which may feedback on the subsequent spread of the introduced plant species (Bever 2002, 2003; Reinhart & Calloway 2006). For example, Vogelsang & Bever (2009) found that NIS in California grasslands were poorer hosts for AM fungi than native plant species, and dominance by NISs reduced the density of AM fungi and thereby inhibited the growth rate of the native plant species which are highly dependent on AM fungi. A similar story has been demonstrated with invasive garlic mustard (Stinson et al. 2006, Wolfe et al. 2008), which suggests that degradation of the mycorrhizal mutualism may be common for NISs in North America. In fact, some NISs have evolved reduced dependence on AM fungi during the invasion of North America (Seifert et al. 2009), suggesting that there is a selective advantage to this strategy.

In contrast, the growth and fitness of other grassland NISs, such as old world bluestems (*Bothriochloa* spp.) which have invaded the Central and Southern Great Plains, are highly dependent on AM fungal associations and AM fungi may promote their invasion into native grasslands (Wilson & Hartnett 1997; Wilson et al. 2011). Nevertheless, even in these systems, the AM fungal community may be changed in a way that inhibits highly dependent native grassland species (Wilson et al. 2011). This inhibition likely results from host-specific changes in AM fungal community composition. Host plants shape distinctive AM fungal communities even when inoculated with the same AM fungal species (Bever et al. 1996; Uibopuu et al. 2009) and these altered communities have been shown to differentially impact growth of native and NIS plants (Bever 2002). As plants can allocate preferentially to the most beneficial fungal partner (Bever et al. 2009; Kiers et al. 2011; Zheng et al. 2015; Ji and Bever 2016), it is possible that invasive plants may alter AM fungal communities to promote their own success. Moora et al. (2011) found an NIS associated with non-host specific AM fungi, while the native plant-host species associated with a more diverse community of AM fungi, a change that could influence future success of NISs. More work is required on the Old World bluestem invasion to test

whether such AM fungal community changes are contributing to the inhibition of native grassland species.

In both of these scenarios, the change in the AM fungal communities by NISs can generate a positive feedback that can stabilize communities dominated by NISs and prevent the re-establishment of native plant species (Bever et al. 2010, 2012).

Ecosystem consequences of invasion by non-native invasive plants

The invasion of non-native plant species can have cascading effects through the terrestrial ecosystem. We have found, for example, evidence of a negative effect of non-native invasive species on an important metric of ecosystem function, soil aggregate stability. Soil aggregation is the physical structure of the soil and it influences virtually all nutrient-cycling processes and soil biota (Jastrow & Miller 1998; Diaz-Zorita et al. 2002). Carbon sequestered within soil aggregates can be protected from decomposition, thereby contributing to the stabilization of the soil carbon pool (Jastrow & Miller 1998; Six et al. 1998; Miller & Jastrow 2000). The stability of soil aggregates to exposure of the disruptive force of wetting reflects positively on the permeability of the soil, the resistance of the soil to erosion, and the potential for the soil to sequester carbon (Rillig et al. 2010). In field sampling across North American grasslands, we found soil aggregate stability was negatively correlated with the diversity of NISs (Duchicela et al. 2012). Moreover, in California grasslands, we found that mesocosms dominated by NISs had reduced soil aggregate stability compared to mesocosms dominated by native plant species (Duchicela et al. 2012). In this study, the mesocosms dominated by NISs also had reduced densities of AM fungi (Vogelsang & Bever 2009), suggesting that the negative effect of NISs on soil aggregate stability was mediated by the degradation of the AM fungal community. This is consistent with the evidence that soil aggregate stability is disrupted by reductions in AM hyphae (Wilson et al. 2009). Given these negative effects, we expect negative effects from the dominance of NISs on other ecosystem properties including rates of carbon sequestration.

AM fungal inoculation and the restoration of native plant biodiversity

Restoration outcomes vary widely, and it is often not clear why (Brudvig et al. 2017). Generally, restorations have lower plant species diversity compared to that of nearby remnant grasslands (Kindscher and Tieszen 1998, Martin et al. 2005, Middleton et al. 2010), and plant species richness, especially forb species, can decline over time (Baer et al. 2002). In addition, some of the plant species seeded into restorations are not well represented in the resulting plant community (Grman et al. 2015). Although management strategies and site histories can explain some variation in restored plant community composition (Grman et al. 2013), much of the variation in restoration outcomes remains unexplained. This remaining variation in restoration outcomes is likely due to restoration protocols that focus primarily on the plant community rather than establishment of other important ecological components of grassland ecology. Here, we will argue that the focus on reintroduction of plants without re-establishment of native plant microbiomes may be limiting restoration success.

Accumulating evidence identifies the plant microbiome as an important driver of plant community composition. Experiments and field studies have identified that microbes play important roles in plant local adaptation (Schultz et al. 2001, Johnson et al. 2010), coexistence (Bever et al. 2015), relative abundance (Klironomos 2002, Mangan et al. 2010), succession

(Kardol et al. 2007, Bauer et al. 2015, Koziol and Bever 2016b), and invasions (Callaway et al. 2004, Pringle et al. 2009, Vogelsang and Bever 2009). Given this growing realization that microbiomes can structure plant communities, it is logical that successful restoration of native plant communities may require re-establishment of native microbiomes. Although many microbiome components might be important to plant and ecosystem function, plant mutualists such as mycorrhizal fungi are obvious first candidates to aid restoration.

It has long been known that reintroduction of mycorrhizal fungi can be critical to the establishment of plant species in artificial and severely degraded landscapes such as reclamation of mine spoils. Prairie restoration practitioners have inoculated plants with mycorrhiza and seed companies offer mycorrhiza, *Rhizobia*, and other soil microbial amendments for purchase (Prairie Moon Nursery 2017). Across studies the response to inoculation can be highly variable, with a recent review expressing skepticism in the value of inoculation to restoration quality (Hart et al. 2017). We suggest that some of this variation in response reflects the lack of attention to the source of the AM fungal inocula and the ecological target of the restoration.

While tremendous attention is paid to the origin and native status of the seeds put into grassland restorations, current restoration practice fails to consider the origin of mycorrhizal inoculants. Commercial inocula tends to be dominated by the weediest of AM fungal species that has been shown to have deleterious effects on native plant establishment (Middleton et al 2015). However native soil microbiomes, and native mycorrhizal fungi in particular, can confer strongly positive effects on restoration quality (Middleton et al. 2015, Koziol and Bever 2016). Based on the last fifteen years of experiments, we suggest that the value of reintroduction of native AM fungi to restorations will likely depend upon the context in which they are being used, including the land-use history of the site, the plant species planted, and the type of AM fungi chosen for inoculation.

In the sections that follow, we present tests of (1) whether AM fungal communities degrade with disturbance and dominance of non-native invasive plant species, (2) whether native plant species, particularly desirable native plant species, are more responsive to AM fungi than non-native invasive plant species, (3) whether reintroduction of native AM fungi in areas previously dominated by non-native invasive plant species can improve establishment, (4) whether changes in climate will alter the relationship of non-native invasive plant species with AM fungi, and (5) whether the source of native AM fungi influences the success of native plant species.

METHODS

METHODS—SURVEY OF AM FUNGAL COMMUNITY COMPOSITION

To better understand how AM fungal community composition in prairie remnants may vary across a large precipitation gradient and the effects of disturbance on these communities, we sampled plant roots from pairs of remnant and disturbed sites spanning western Oklahoma to eastern Illinois. We assessed AM fungal community composition by sequencing a portion of the nuclear large subunit ribosomal RNA (rRNA) gene from the sampled roots and then clustered similar sequences into operational taxonomic units (OTUs) that formed the basis for all community analyses. Using these data, we sought to address two main questions:

1) How are AM fungal communities in remnant prairies and nearby disturbed sites structured, and does community composition change across the precipitation gradient?
2) Are particular AM fungal OTUs consistently over-represented in remnant sites compared to disturbed sites, and therefore sensitive to loss by disturbance?

Site selection and sampling

We sampled a total of 19 remnant and 15 disturbed sites across the Midwestern United States (Fig. 1), including Fort Riley (KS), Tinker Air Force Base (OK), and the retired Chanute Airforce Base (IL). We defined remnant sites to be locations that have not been tilled, but may be grazed or hayed and are still dominated by prairie plant species. Near each remnant site or group of remnant sites we selected disturbed sites that were dominated by non-native plant species, principally *Bothriochloa ischaemum* (L.) Keng (yellow bluestem), *Bromus inermis* Leyss. (smooth brome), or *Schedonorus arundinaceus* (Schreb.) Dumort. (tall fescue).

Each sample comprised four soil cores (2 cm × 15 cm) collected within a $1m^2$ plot. After collection, samples were kept on ice until processing, generally within 12 hours. At least 50 mg (wet mass) of fine roots were removed from each sample, rinsed and blotted dry; samples were then stored frozen or lyophilized (samples from Fort Riley, KS) until DNA extraction. Soil chemical analyses (pH, C/N, cation exchange capacity (CEC), Bray 1 phosphorus, Bray 2 phosphorus, bicarbonate phosphorus, magnesium, calcium, and potassium) were conducted for most soil samples (A&L Great Lakes Labs, Fort Wayne, Indiana). DNA was extracted, sequenced, and processed as described in Appendix A1.

Effect of site history and environmental conditions on AM fungal communities

The average annual aridity index (Allen et al. 1998) that incorporates measures of precipitation, temperature, wind speed, and dew point was highly correlated with average precipitation near each site for the five years preceding sampling (2011-2015) and analyses from the two metrics gave nearly identical results; therefore for simplicity we report average precipitation. Some analysis methods we used required further simplification of the precipitation-based analyses by creating two groups of samples: one group representing western samples from sites with < 800 mm annual precipitation (n = 58), and one group representing eastern samples from sites with > 800 mm (n = 50). The effects of precipitation on AM fungal community composition as measured by PERMANOVA (Anderson 2001) were qualitatively comparable regardless of whether we used precipitation as a continuous or a categorical predictor. We used

PERMANOVA to test whether site history (remnant/disturbed) and location along the precipitation gradient (West/East), added as marginal effects in the model, explained differences in AM fungal community composition across all sites, and visualized these differences at a community level using principal coordinates analysis. We also used PERMANOVA to determine the effects of soil factors on AM fungal communities, as described below.

Correlations among site history, precipitation, and soil variables were calculated using Spearman's rank correlation. To determine how soil variables may correlate with differences in AM fungal community composition between remnant and disturbed sites, we used partial constrained principal coordinates analysis (Anderson and Willis 2003) controlling for geographic location. All samples from Kansas, Missouri, and Illinois with soil nutrient results (72 of 141) were used, and the following soil variables were $\log_{10}$ transformed before analysis: CEC, Bray 1 phosphorus, Bray 2 phosphorus, bicarbonate phosphorus, magnesium, calcium, and potassium.

Differential abundance of OTUs

We used the *DESeq2* package in R (Love et al. 2014) to determine the differential abundance of each OTU in pairwise comparisons of different groups of sites while correcting for both variation in sequence number across samples and variance in sequence number for each OTU (McMurdie and Holmes 2014). We used this pairwise contrast to determine: 1) differences in OTU abundance between remnant and disturbed sites considered together, and 2) differences in OTU abundance with location along the precipitation gradient (West/East) separately for only remnant sites and for only disturbed sites. Using these differential abundance results, we calculated the net relatedness index (NRI) (Webb et al. 2002) to test for phylogenetic clustering among OTUs that were more abundant in either remnant or disturbed sites when considering all samples, or were more abundant in either western or eastern sites when considering only remnant samples or only disturbed samples separately. We also used the OTU table after *DESeq2* correction to conduct linear discriminant analysis (LDA), an alternative to a random forest classifier in grouping samples by site history or location on the precipitation gradient using the AM fungal community composition. Experimental details are reported in House and Bever (2018).

METHODS—EFFECT OF OLD WORLD BLUE INVASION NEAR FT. RILEY

A large area at the Konza Prairie Biological Station near Fort Riley Army Base has been invaded by the warm-season non-native grass, *Bothriochloa bladhii*. Eight years of glyphosate applications have removed much of the invasive grass, but there has been little to no success in re-establishing native plant species. We established 6 replicate 2 x 2 m permanent plots. In each replicate plot, we assessed soil microbial biomass (including AM fungal abundance) using phospholipid and neutral lipid fatty acid (PLFA/NLFA) (as described in Appendix A1).

METHODS—GREENHOUSE ASSAY OF PLANT RESPONSIVENESS TO AMF

Plant species mycorrhizal responsiveness were collected from published reports (Wilson and Hartnett 1998, Koziol and Bever 2015, Bauer et al. 2017) and from a greenhouse experiment which followed the methods of (Koziol and Bever 2015). Generally, we grew plants in 500 cm^3 pots filled with sterilized 1:1 soil sand mixtures. The five mycorrhizal and non-mycorrhizal

replicates for each plant species were the same with the exception being that a community of AM fungi inoculated at 10% by volume in the mycorrhizal pots whereas sterile background soil was added to the non-mycorrhizal pots. AM fungal species were collected from mid and late successional prairies at the Kankakee Sands Prairie and Beaver Lake Prairie Chicken Refuge near Morocco, Indiana. Species included *E. infrequens, C. lamellosum, C. claroideum, F. mosseae, C. pellucida, S. fulgida,* and *A. spinosa.* Increases (or decreases) in proportion of biomass was analyzed by constructing the mycorrhizal response ratio for each plant/fungal combination was determined using the following equation:

$$\frac{\text{Total plant biomass with fungal inoculation}}{\text{Total plant biomass without fungal inoculation}}$$

(Hoeksema et al. 2010). Mycorrhizal responsiveness was analyzed using a simple linear regression models with plant native status or plant successional category. Successional stages were assigned based on coefficient of conservatism scores (CC) (Swink and Wilhelm 1994, Freeman 2014), which have been shown to be strongly correlated with field observations of plant species abundances across prairie succession (Koziol and Bever 2015).

METHODS—GREENHOUSE ASSAY OF SPECIFICITY OF PLANT RESPONSIVENESS TO AMF

In two greenhouse experiments, we tested for differences in plant-AM fungal specificity (i.e. variation in plant growth response to individual fungal isolates) using 17 grassland plant species from different successional stages, plant families, and native and non-native status. Individuals of each plant species were grown in a full factorial design with a single AM fungal species, a mixture of AM fungal species, or no inocula for four months (as in Koziol and Bever 2016a).

The average mycorrhizal responsiveness was calculated as above, and the variance was then calculated for each plant species. The coefficient of variation (a measure of plant-fungal specificity) was determined for each plant species using the following equation:

$$\frac{\text{variance in mycorrhizal responsiveness}}{\text{average mycorrhizal responsiveness}}$$

Data from our two greenhouse studies showed that 1) non-native invasive plant species were less responsive to mycorrhizal fungi that native plants. 2) Both non-native invasive plant species and weedy, early successional native plant species do not benefit from mycorrhizal fungi, while late successional native plant species are very responsive to mycorrhizal fungi. 3) Late successional native plant species are also sensitive to AM fungal species identity, significantly more so than non-native invasive plant species or weedy, early successional native plant species. To confirm that these results are general, we combined these data with 11 other studies examining variation in growth response to different AM fungal species in grassland plants of North America. We report the results of the meta-analysis of these papers within results section.

METHODS—FIELD INOCULATION OF AM FUNGI-CORE EXPERIMENT

We evaluate the potential beneficial effects of AM fungi on native grassland restoration and management through a series of field inoculation experiments. The field inoculation experiments test the fundamental importance of native soil microbes to native plant communities. These experiments also evaluate the pragmatic costs and benefits of one approach to field inoculation by collecting data on the rate of spread of the native soil microbes in the restored landscape and the spread of benefits to ecosystem function associated with the spread of these microbes. In these experiments, we manipulate the introduction of soil microbial inocula into the restoration by either inoculating with whole native soil communities, AM fungal species isolated from nearby remnants, or inoculation with sterile soil (control: no addition of microbes). The experiments are distributed across three Department of Defense properties (Fort Riley in Kansas, Tinker Air Force Base in Oklahoma, and the retired Chanute Air Force Base in Illinois). In these experiments, we manipulated soil microbes within replicated grassland restoration experiments.

In our grassland restoration experiments, native soil microbes are introduced in association with native plant species that are known to be good hosts to AM fungi. In each experiment, the same four host plant species, which we call nurse plants (Middleton and Bever 2013 Middleton et al 2015), are planted into each plot. Individual treatments vary with whole plots having 16 nurse plants inoculated with AM fungi isolated from nearby unplowed remnant grasslands, soil microbes freshly derived from native unplowed remnant grasslands, or inoculated with sterile soil (no addition of microbes). We expect these root symbionts to thrive with the nurse plants.

Study System

We target three DoD facilities spanning tallgrass prairies in Central North America: Chanute Air Force Base, IL: Fort Riley, KS; and Tinker Air Force Base, OK.

Chanute Air Force Base, located in Central Illinois in Champaign County is on the southern edge of Rantoul. The site was used to provide military and technical training for Airman and the Department of Defense personal. Military operations ceased in 1993 and the site is undergoing remediation that is expected to be complete by 2016. There are no remnant tallgrass prairies on the base, but we sampled several tallgrass prairie remnants nearby. Much of this site is currently invaded by Tall Fescue. Soils are loamy to silty clay loam. Average monthly temperatures range from -0.5° C in January to a high of 30.5° C in July. This region receives on average 99 cm of precipitation annually.

Fort Riley Army Base, located in the Flint Hills of northeast Kansas, is a large expanse of tallgrass prairie containing primarily silt loam to silty clay loam soils dominated by perennial C4 grasses with numerous subdominant grasses, forbs, and woody species. Our study sites are located within areas of the Base periodically burned by prescribed or wildfire and disturbed through military activities. Several disturbed sites have been invaded or are threatened by invasion by Old World Bluestems and Smooth Brome. Average monthly temperatures range from a minimum of -2.7° C in January to a high of 26.6° C in July. Annual precipitation

(averages 86 cm) is similar to the OK site, but this is offset by lower evapo-transpiration and a shorter growing season.

Tinker Air Force Base is located in central Oklahoma and is situated within the Cross Timbers, an ecoregion containing tallgrass prairie (silty loam soil) interspersed with oak dominated woodlands. Less than 2% of the presettlement prairie ecosystem currently remains on Tinker AFB and the majority of native prairie that remains has been disturbed through military activities and subsequently invaded or threatened by invasion by Old World Bluestem. Tinker AFB has attempted restoration of these sites with limited success. Long-term average monthly temperatures range from a low of 2.6° C in January to a high of 27.8° C in July and can experience more than 65 days per year of > 32 C temperatures. The region receives on average 90 cm of precipitation annually and can experience up to 280 growing season days per year.

Experimental Design and Details

Inoculating every plant in acres of restoration is not feasible. Our approach is to inoculate nurse plants and allow the native AM fungi to benefit adjacent noninoculated plants. We have found that neighboring plants do benefit from proximity to the inocuated plants, or nurse plants (Middleton & Bever 2012). The scale over which beneficial effects of inoculation spreads determines optimal inoculation designs. We evaluated the rate of spread of inoculated AM fungi in this project. We compared inoculation with fresh soil collected from nearby prairie remnants, AM fungi isolated from a nearby prairie, and a noninoculated sterile control. Seven replicate plots of each inoculation treatment were planted into areas previously dominated by NISs and previous, partially successful attempts at native grassland restoration. During 2014, we established four experiments of this general design, distributed across three DoD properties: 1 site history at Tinker (a partial restoration of native grassland for a total of 3 inoculation treatments x 7 replicates = 21 plots), 1 site history at Chanute (cool-season NIS for a total of 3 inoculation treatments x 9 replicates = 27 plots), and 2 site histories at Fort Riley (cool-season NIS and warm-season NIS, for a total of 2 x 3 inoculation treatments x 7 replicates = 42 plots). In the spring of 2015, we planted a second experiment at Tinker Air Force base in an area dominated by Old World Blue grasses. Seedlings were inoculated with 1 of 3 inoculation treatments: whole soil (fresh soil collected from nearby prairie remnants), AM fungi isolated from soil collected from a nearby prairie, or whole soil autoclaved for 2 hrs (sterile inoculum or 'noninoculated' control). Seedlings were started in sterile germination mix and transplanted into root trainers with sterile background soil from each of the military installations. Inoculum (whole prairie soil, native AM fungi, or sterile soil inoculum [control]) were mixed into the soil prior to seedling planting. The experimental treatments received 9% inoculum by soil volume.

Whole soil inocula

We collected whole soil inocula at remnant prairies ranging from 0-25 km from each field site. At remnant prairies, we collected five randomized samples of field soil, each 0.5 L. Soil was sieved with an 8mm sieve and stored at 4°C prior to being used as nurse-plant inoculum. Both Ft. Riley sites received the same whole soil inocula. We took sub-samples from each whole soil inocula and stored them at -20°C for molecular identification.

AMF Inocula

In 2012, we extracted spores from field collected remnant prairie soil from the same locations used in the whole soil treatments using the methods of Bever et al. 1997. AMF species were separated microscopically. We created single spore cultures using the methods of Koziol and Bever (2016). This entailed filling pots with an autoclaved sterilized 1:1 sand and soil mixture and then inoculating with spores from a single AMF isolate. *Sorghum bicolor* was planted in each pot and used as the host plant for all cultures. After a nine month growing season, spore-cultures were harvested by removing above-ground biomass and storing sand:soil mixture and belowground *S. bicolor* biomass at 4°C. We then extracted spores from each pot to confirm spore production and identity, and then homogenized soil and roots to inoculate new cultures grown on mixture of native prairie plants the following growing season. Prior to field inoculation, multiple spore cultures from each site were mixed to create three site-specific cultures for each of the restorations sites (Ft. Riley, Tinker, and Chanute). Each site-specific mixed culture was stored at -20°C for molecular identification.

Plot set-up

In May 2014, 16-m^2 plots were established at each site and assigned plot treatments using a randomized block design. Chanute contained 9 blocks and 27 plots, while all other sites contained 7 blocks and 21 plots total (69 plots total across all four sites). Each of the three plots in each spatially stratified block was randomly assigned one of the three inoculation treatments created by planting seedlings that were inoculated with either whole remnant prairie soil inocula, AM fungi inocula, or sterilized inocula. Henceforth we refer to inoculated plants as "nurse plants" as in Middleton et al (2009, 2015). Nurse plants included two forbs, one grass, and one legume at each restoration site. Each plot received four replicates of each nurse plant species. We planted sixteen nurse plants in a row down the center of each of the 16-m^2 plots. All nurse plants within a plot had the same inoculation treatment. Replicates were repeated in the same order across each plot in each site. Nurse plants species were inoculated with microbial treatments at 15% by volume in 150 cm3 conetainers™ with the remaining volume being sterilized soil:sand mixtures.

Nurse plant monitoring and analysis

Nurse plant size was measured at the time of planting into inoculation treatments and then annually each growing season. We used Proc Mixed in SAS to analyze plant growth with site, inoculation treatment, plant functional group (grass, legume or forb [non-legume forbs henceforth called "forbs"]), block X site interactions, functional group X treatment interactions, and site X treatment interactions as predictors. To compare the effects of different soil microbes on nurse plant success, we designed contrasts comparing (1) plant growth with living inocula (whole soil and AM fungal cultures) verses non-inoculated, (2) growth when inoculated with AM fungi verses whole soil inocula, and (3) the interaction of these contrasts by plant functional group. For these analyses, we identified plot*inoculation treatment*site and that interaction with plant functional group as random effects. We used presence absence data of each nurse plant to analyze plant survival using proc Glimmix in SAS. To calculate plant growth or survival relative to the controls, we used the LS Means outputs from our mixed model to make a ratio of plant growth or survival with inoculation relative to the controls.

Plant community composition

Because plant community composition was measured across distance from nurse plant row for all three treatments, we were able to analyze plant community across treatment and distance for sterile, whole soil, and AM fungi inocula plots in one analysis. Because analyses like PERMANOVA do not account for pseudoreplication within plots, we first analyzed community composition using NMDS in the vegan package in R for all sites and all years. We then extracted the values for axis 1 (NMDS1) and axis 2 (NMDS2) from this analysis. We also used the vegan package to calculate richness, Shannon diversity, and evenness. Then, mixed effect models were used to assess how treatment, distance from nurse plant row, and the interaction impacted NMDS1, NMDS2, NMDS1 X NMDS2, richness, Shannon diversity, and evenness. All mixed effect models were conducted in SAS.

Soil and root sampling for AMF spread

Each growing season, we collected soil and root samples for molecular and phospholipid and neutral lipid fatty acid (PLFA/NLFA) analysis. We collected at the nurse plant row, 0.5-m away from the nurse plant row, and 1.5-m away from the nurse plant row. We collected four 2-cm diameter samples approximately 10 cm deep using a soil core along each sampling row. Sampling was concentrated away from the plot edges. Soil corers were cleaned with 80% ethanol between each sampling row. Soil cores were mixed and split into a molecular and PLFA/NLFA sub-samples. Molecular and PLFA was analyzed as described in Appendix A1.

Spread of inoculated OTUs

For each site, we first determined which AMF OTUs were present in the AMF and whole soil inocula used in each experiment. We then used separate Multivariate Analysis of Variance (MANOVA) tests to determine first, whether inoculated OTUs were more abundant in inoculated nurse plant rows compared to sterile control nurse plant rows. We then used a separate MANOVA to determine whether inoculated OTUs decreased with distance from inoculated nurse plant rows, to determine the percentage of OTUs fitting into particular spread categories. We categorized all present OTUs into the following particular spread categories, using contrasts statistics and marginal means between each distance.

We identified five distance categories. (1) No spread from the nurse plant row (OTU made up a significantly higher proportion of the AMF community in the nurse plant row compared to both 0.5-m and 2-m away). (2) Spread 0.5-m away from the nurse plant row (OTU relative abundance in nurse plant row and 0.5-m away were not significantly different, but the OTU made up a significantly higher proportion of the AMF community in the nurse plant row and 0.5-m away compared to 2-m away). We then have three categories involving varying levels of confidence in spread to 2-m away. (3) Potentially spread 2-m away from the nurse plant row (the nurse plant row and 0.5-m away were not significantly different, 0.5-m and 2-m away were not significantly different, but the OTU made up a significantly higher proportion of the AMF community in the nurse plant row compared to 2-m away), (4) Overall decrease with distance from the nurse plant row (though there was no statistically significant trend with distance, an overall distance affect was present, with the OTU decreasing in proportion to the rest of the community with distance). (5) Spread to 2-m away (i.e. showed no significant affect across distance). (6) inconclusive distance effects (significant differences between different distances,

but not displaying any clear distance effects). This last category includes OTUs in which the nurse plant row was significantly lower than either/or 0.5-m or 2-m away. This pattern was particularly common at Ft. Riley and Tinker sites, suggesting that spread from outside the plot into the plot was common.

We counted each OTU present in either the AMF or whole soil inocula as one trial. If an OTU was present in both, it got one test for the AMF inoculated plots and one test for the whole soil inoculated plots. This resulted in 185 trials at Chanute (61 and 124 OTUs in the AMF and whole soil inocula respectively), 133 trials for each Ft. Riley site (46 and 87 OTUs in the AMF and whole soil inocula respectively), and 103 trials for Tinker (15 and 88 OTUs in the AMF and whole soil inocula respectively).

METHODS—SUPPLEMENTAL FIELD INOCULATION STUDY 1

This study was located in eastern Kansas in an area dominated by *Bromus inermis,* a cool season non-native invasive grass species. As in our core inoculation studies, native grassland microbes were introduced to plots through freshly collected whole soil or mixtures of AMF cultures isolated from the same location. Nurse plants were inoculated with AM fungi isolated from Rockefeller Prairie, Lawrence, KS, whole soil from Rockefeller prairie, or were not inoculated and grown in sterile background soil. Nurse plants were propagated at Indiana University and seedlings of each species were inoculated with one of the three inoculum treatments at 10% by soil volume. Background soil was collected from near the remnant prairie and sterilized by heating it to 212°C for 2 hours, resting the soil for 24 hours and repeating the 2 hours heating, and mixed 1:1 with sterile sand to improve drainage before inoculation.

Field plots were either cleared of brome or remained uncleared to evaluate the effect of brome removal on the success of prairie plant establishment, with and without inoculation of native soil microbes. Each plot had a 'runway' (a row of uninoculated phytometer plants spaced in 0.5 m increments up to 2.0 m) on one side of the plot and no 'runway' on the other side of the plot (phytometer at 0.5 and 2.0 m only) in order to track the rate of spread of AM fungi from nurse plant to phytometer plant, with and without suitable hosts along the way. Each inoculation treatment x field treatment was replicated six times for a total of 36 plots in the experiment. Plots were spaced 3 m apart to maintain treatment effects. Along the center of each 4 x 4 m plot, a row of 16 inoculated native prairie seedlings (nurse plants) were planted at 0.25 m intervals, with four individuals of four nurse plants in each plot. Four late-successional native prairie species were chosen as nurse plants, *Lespedeza virginica* (Slender lespedeza; legume), *Asclepias tuberosa* (Butterfly milkweed, forb), *Echinacea pallida* (purple cone flower; forb), and *Schizachyrium scoparium* (Little bluestem; grass), selected for their dependence on AM fungi and their importance in native prairie ecosystems. All plots were seeded with diverse native prairie seed mix (60% forbs, 40% grasses) at the time nurse plants were planted.

Data were collected on native seedling survival and growth in each plot over two years (2016, 2017) without destructive sampling (i.e. plant height, number of leaves or tillers, length and width of the longest leaf, and flower number). Data on plant community composition and diversity in each plot was collected using a pin-drop method and by estimating percent cover of each species.

METHODS—SUPPLEMENTAL FIELD INOCULATION STUDY 2

We took advantage of a large area of the Konza Prairie Biological Station near Fort Riley Army Base that was invaded by the warm-season non-native grass, *Bothriochloa bladhii*. Eight years of glyphosate applications have removed much of the invasive grass, but there has been little to no success in establishing native plant species. We tested the efficacy of native AM fungal inocula for establishment of native plant species and suppression of non-native invasive plant species using a design similar to our core inoculation studies.

METHODS—SUPPLEMENTAL FIELD INOCULATION STUDY 3

This restoration occurred at the Hilltop Garden and Nature Center in Bloomington, IN. The site was mowed turf grass for the past several decades. The site was prepared by installing a four millimeter thick black plastic over the area to solarize the existing vegetation in April of 2014. To assess the effects of AM fungal composition within grassland restorations, we inoculated plots with six different AM fungal community treatments including one of four different AM fungal species isolated from a prairie, a mixture of all four fungal species, and a non-inoculated control. AM fungal species were isolated from a remnant prairie in Morocco, Indiana and included *E. infrequens, C. lamellosum, C. claroideum,* and *A. spinosa.* AM fungi were introduced by planting sixteen different inoculated nurse plants into replicated plots. We also seeded the restoration with a diverse, 54-species prairie seed mixture. During year one, we monitored the growth, survival and fecundity of nurse plants. At the end of year two, we harvested the above-ground biomass of each plot for species abundance analyses. We calculated mean species richness, abundance, and diversity.

We analyzed nurse plant growth in response to AM fungal inoculation using a mixed model with plant species within successional stage identified as a random effect to test for general patterns across plant species. We analyzed plant survival using Glimmix in SAS 9.4. We used the log transformed number of leaves and the log transformed plant height (cm) to analyze nurse plant growth. We deconstructed plant growth in response to inoculation treatments using six *a priori* orthogonal contrasts comparing inoculated versus non-inoculated plant growth, plant growth differences among the individual fungal species, whether the diverse mixture affected plants differently than the average effect of the individual species and each of these contrasts by successional stage within the model. Contrasts assessing the fungal inoculation by successional stage interactions tested for consistent differences between early and late successional plant species in their specificity of response to inoculation with different AM fungal composition.

METHODS-- CLIMATE PERTURBATION EXPERIMENT

Study 1: *Bothriochloa ischaemum* and *Bromus inermis* seedlings were established and subjected to elevated temperatures and reduced soil moistures to examine the growth responses of these two invasive grasses to projected climatic conditions. For these experiments, *B. ischaemum* was paired with the functionally similar native *Schizachyrium scoparium*, while *B. inermis* was paired with *Pascopyrum smithii*. Two temperature treatments (ambient, ambient + 5°C) and four soil moisture treatments (100% field capacity [FC], 85% FC, 75% FC, and 65% FC) were used

in these experiments. Due to the differences in growing seasons, the warm- and cool-season experiments were conducted separately, with ambient photoperiods closely replicating that which the plants would be experiencing under natural conditions. Following the seedling experiment, we further examined the effects of drought and elevated temperatures on these species at different life stages. We assessed seed germination in response to drought and elevated temperatures, followed by the examination of the responses of established crowns of the aforementioned species.

Study 2: Using conditioned soils from the seedling experiment, a plant-soil feedback test was performed. In this experiment, individual seedlings were planted into soil conditioned by either the non-native or native grass that had been conditioned under all combinations of drought and temperature treatments in the previously described experiment. As with the baseline experiments, *B. ischaemum* was paired with native *S. scoparium*, while *B. inermis* was paired with native *P. smithii*, based on growing season functional groups.

METHODS-- MYCORRHIZAL FUNGI LOCAL ADAPTATION STUDY

Study System

From our study sites in Illinois, Kansas, and Oklahoma, we isolated mycorrhizal fungi from soils collected from remnant tallgrass prairies. We then established mesocosms containing soil from each of our study sites and inoculated each of these soil types with fungi from each of our study sites in a fully factorial 3 x 3 design. These mesocosms were then planted with nine species of tallgrass prairie plants common to each of the study regions. Half of our mesocosms were watered to near field capacity and the remaining received half as much water, corresponding to typical drought conditions in our study area. In total, each soil*fungi*drought treatment included 10 replicates, and our experiment included 240 total mesocosms (3 soil treatments x 4 fungi treatments x 2 drought treatments x 10 replicates = 240).

Fungi Treatments

Fungi were isolated from remnant prairies soils collected in 2012 from IL, OK, and KS. We created fungal cultures by sorting spores microscopically by morphotype. Morphotypes were grown with *Sorghum bicolor* host plants for six months in their relevant sterilized background soils. To initiate this experiment, single species fungal cultures were mixed together to create diverse mixtures of fungi isolated from each of the three sites. Mesocosms were filled with 6 L of sterilized soil. Then, mesocosms were inoculated with 150 mL of mixed AMF cultures from one study location spread evenly over the sterilized soil. Additional mesocosms were maintained as sterilized controls. Then mesocosms were capped with sterilized soil. Fungi treatments were imposed in a fully-factorial design with soil treatments (3 soil types x 4 fungal treatments, including 3 fungi sources and a sterile control).

Plant Treatments

We identified nine species of tallgrass prairie plants that occur near each of our study sites. These were chosen to represent a range of species life histories and known responsiveness to mycorrhizal fungi (Koziol and Bever 2015; Bauer et al. 2018). We selected Missouri as an intermediate location to collect seed because they would not have an evolutionary history with any of the fungi in our study. All seed was purchased from Hamilton Native Outpost (Missouri).

Our study species included *Elymus canadensis, Panicum capillare, Panicum virgatum, Sorghastrum nutans, Schizachyrium scoparium,* and *Andropogon gerardii* (Poaceae); *Rudbeckia hirta* and *Liatris aspera* (Asteraceae); and *Monarda fistulosa* (Lamiaceae). Seedlings of each were germinated in sterilized potting soil and allowed to grow for two weeks. Then a single individual of each species was transplanted into each mesocosm in a 3 x 3 grid.

Drought Treatments – After transplanting, mesocosms were watered to field capacity for four weeks to allow seedlings to establish. Then, watering was reduced over two weeks. Control mesocosms received 250 mL of water daily (3% of total soil volume), and the drought treatment received half of this (125 mL/day, 1.5% of total soil volume). Control treatments remained above 25% soil moisture, and the drought treatments remained between 15% and 20% soil moisture. These levels correspond to relatively high and low soil moisture conditions in tallgrass prairie, with well-documented effects on primary productivity (Fay et al. 2003; Knapp 2001).

Data Analysis – We tested for effects of soil, fungi, and drought treatments and their interactions on the total productivity of our mesocosms using ANOVA, and we tested these effects on the productivity of each species using MANOVA. In both cases, we used follow-up linear contrasts within the soil*fungi and soil*fungi*drought interactions terms to test for mycorrhizal mediation of plant adaptation to soil and drought conditions (following recommendations in Blanquart et al. 2011).

RESULTS AND DISCUSSION

RESULTS—SURVEY OF AM FUNGAL COMMUNITY COMPOSITION

Strong AM fungal community differentiation in remnant sites

The interaction between site disturbance history (remnant/disturbed) and site location (West/East) explained general trends in AM fungal community differentiation (p=0.0004). This interaction was driven by AM fungal communities from remnant sites being significantly different compared to those from disturbed sites overall (p=0.0016; Fig. 1A), as well as communities from remnant sites being strongly differentiated across the precipitation gradient (p=0.004; Fig. 1B). In contrast, AM fungal communities in disturbed sites were not significantly differentiated across the precipitation gradient, nor were communities significantly differentiated in disturbed sites that had different histories of mechanical soil disruption (Fig. 1C).

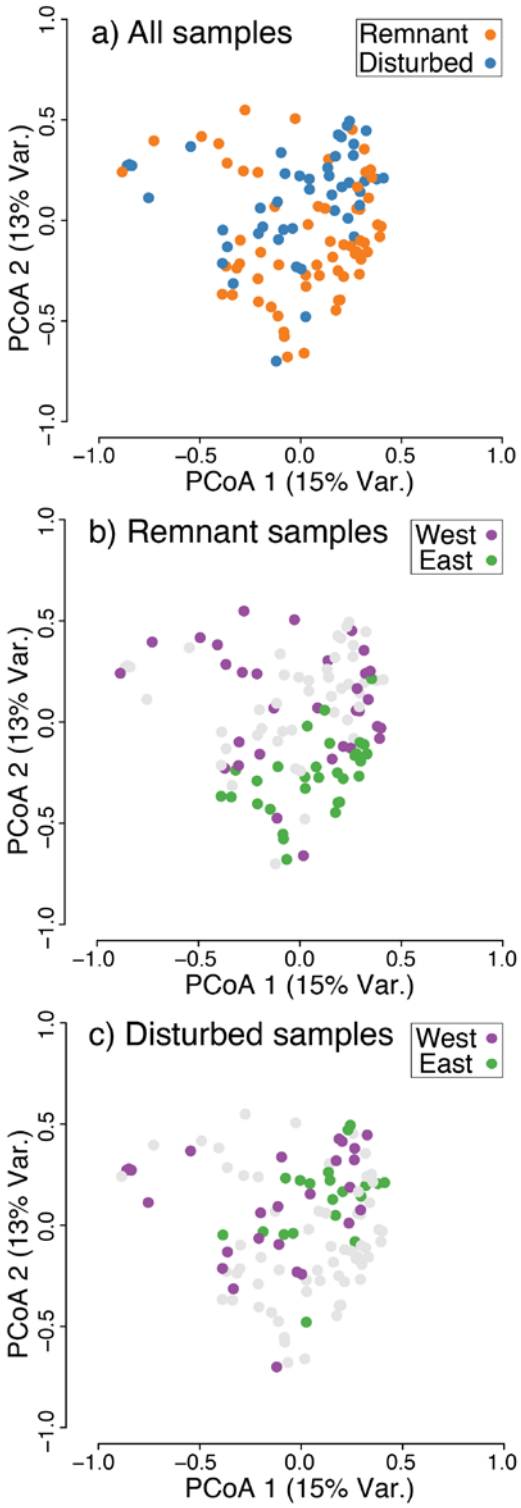


Figure 1. *Principal coordinates analysis (PCoA) of AM fungal community composition in all samples using Morisita's dissimilarity index calculated from the OTU table counts, with highlighted comparisons between a) site histories for all samples, and comparisons between the two sides of the precipitation gradient for: b) only remnant samples, and c) only disturbed samples. For panels b and c, samples from groups not being compared are denoted by gray dots. PCoA axis 1 explained 15% of the variance; PCoA axis 2 explained 13% of the variance.*

The differential abundance of OTUs with either site disturbance history (for all samples) or side of the precipitation gradient (for remnant or disturbed samples) revealed differences both in the number of OTUs with significant abundance skews as well as phylogenetic clustering (Fig. 2). Although there was not a significant skew in the number of particularly abundant OTUs when comparing remnant and disturbed sites for all samples (Fig. 2A), there was significant phylogenetic clustering among the OTUs that were more abundant in remnant sites (NRI=2.38, p=0.005). However when considering only samples from remnant sites, there was no difference

in the number or the phylogenetic clustering of OTUs that were particularly abundant on either side of the precipitation gradient (Fig. 2B). For samples only from disturbed sites, there were significantly more OTUs that had increased abundance in sites from the eastern side of the precipitation gradient compared to the western side (Fig. 2C, Binomial test $p < 0.001$), and this was also true for OTUs from the genera *Rhizoglomus* (Fig. 2C; Binomial test $p = 0.002$) and *Claroideoglomus* (Fig. 2C; Binomial test $p = 0.016$) in particular. Although there were relatively few OTUs that were more abundant in western disturbed sites, they showed significant phylogenetic clustering (NRI= 2.30, p=0.008), due to the absence of OTUs representing genera in the families Diversisporaceae, Acaulosporaceae, Gigasporaceae, and Claroideoglomeraceae (Fig. 2C), while the OTUs with greater abundance in eastern disturbed sites were not phylogenetically clustered. When we tabulated the joint abundance for each of the 199 OTUs across the comparison with all samples (Fig. 2A) and the comparison with only remnant samples (Fig. 2B), a disproportionate number of remnant OTUs that were more abundant in eastern sites were also sensitive to anthropogenic disturbance, while OTUs that accumulated with disturbance were not differentially distributed along the precipitation gradient ($\chi^2 = 51.6$, df = 4, $p < 0.0001$).

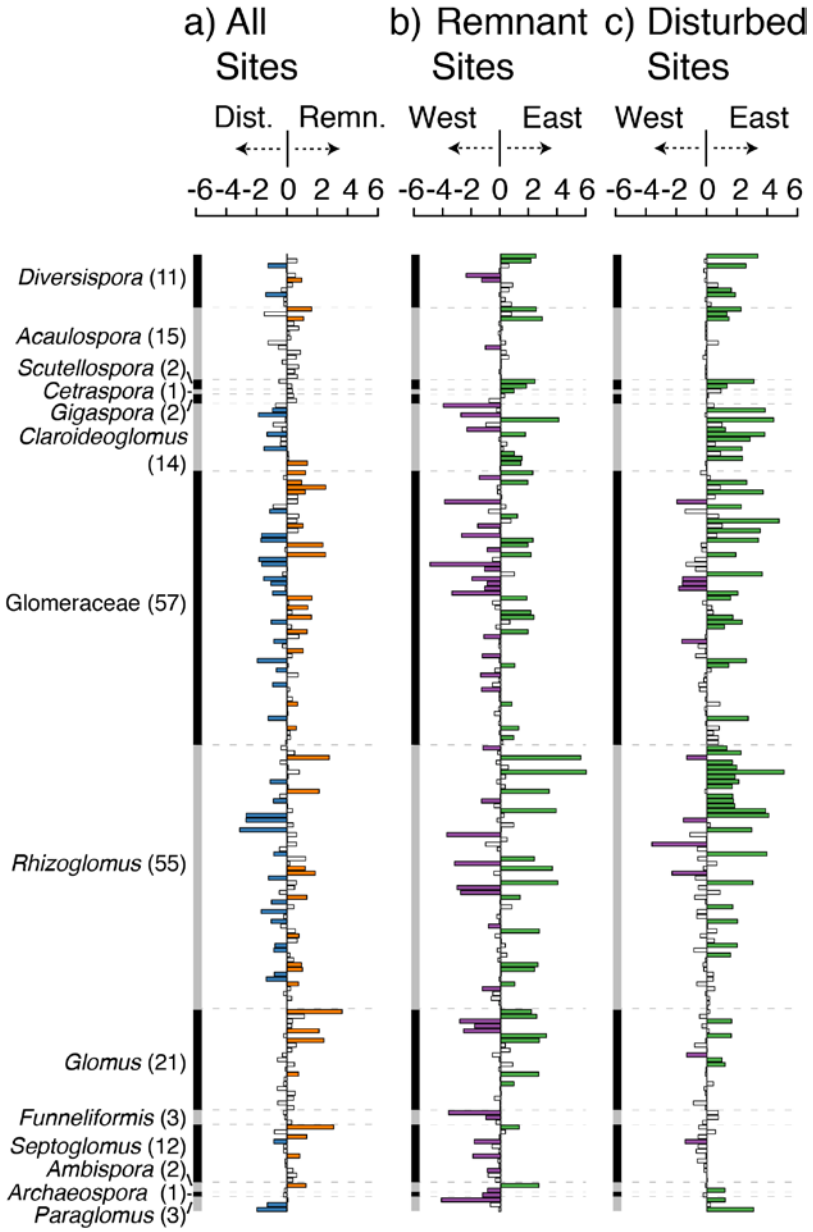


Figure 2. *Differential abundance (on log_2 scale) for each of the 199 OTUs (bars) between a) remnant (orange) or disturbed (blue) site histories for all sites, and between the western (purple) or the eastern (green) side of the precipitation gradient for: b) only remnant sites, and c) only disturbed sites. Filled bars denote OTUs with a significant difference in abundance between the two groups being compared. The order of bars is the same for all panels, and is sorted by the phylogenetic relationships of each OTU's taxonomic attribution (genus-level except family Glomeraceae), with the number of OTUs represented in each taxon in parentheses. OTUs from different taxonomic groups are denoted by alternating black and gray vertical bands on the left side of each panel, and by dashed horizontal lines.*

Despite samples from remnant and disturbed sites having contrasting amounts of AM fungal community differentiation across the precipitation gradient, this was generally not the case at the level of individual OTUs. For samples from both remnant and disturbed sites, both the random forest classifier and the Linear Discriminant Analyses were able to use OTU composition to correctly assign samples to the western or the eastern side of the precipitation gradient with at least 80% accuracy. Overall, this was similar to the accuracy in correctly assigning all samples to their site disturbance history (remnant/disturbed). Finally, combining the OTU-level random forest classification accuracies with the differential abundance analysis allowed us to better identify OTUs that were closely associated either with site history for all samples (Fig. 3A), or with the side of the precipitation gradient for only remnant samples (Fig. 3B) or only disturbed samples (Fig. 3C). Using these combined analysis results, we identified eight of the top 20 OTUs used by the random forest classifier as being significantly more abundant in remnant sites compared to disturbed sites (Fig. 3A). All of these OTUs represented the family Glomeraceae or genera within it, although other OTUs also representing the Glomeraceae were significantly more abundant in disturbed sites (Fig. 3A).

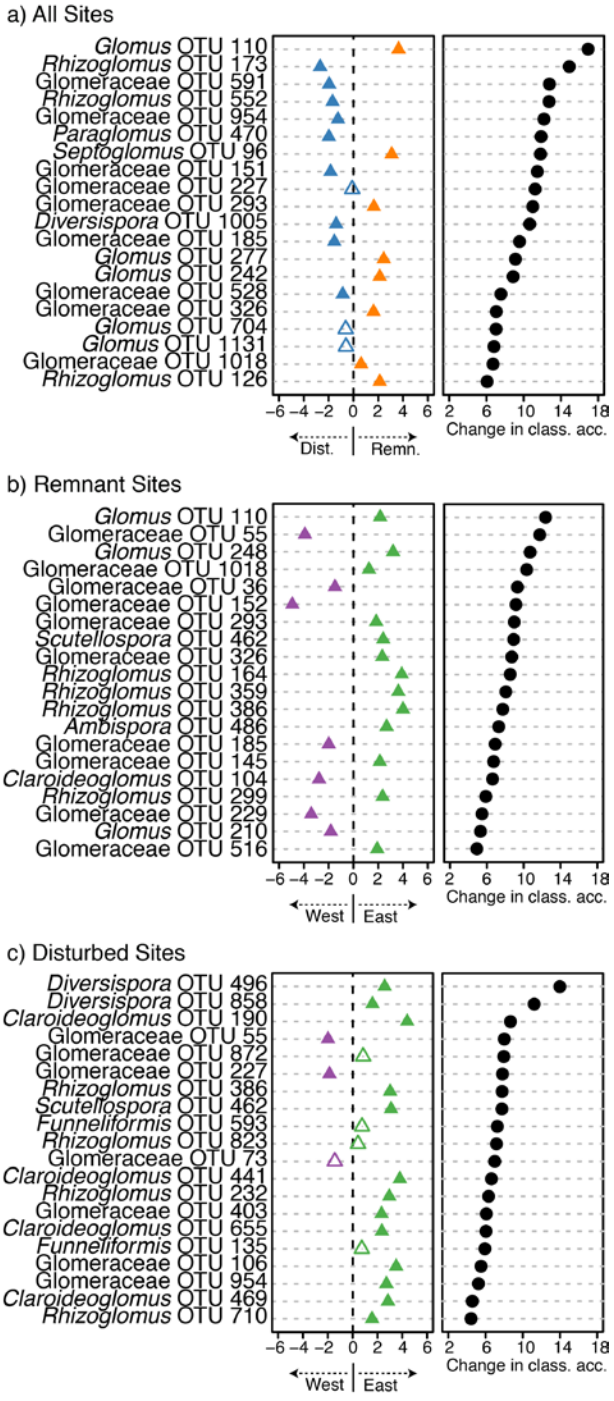


Figure 3. *The influence of specific OTUs on the classification accuracy ('class. acc.') of the random forest classifier using OTU presence/absence data (right side of each panel), and whether the same OTU was significantly more abundant in either of the two tested groups (left side of each panel) for comparisons between a) site histories for all sites, and comparisons between the two sides of the precipitation gradient for: b) only remnant sites, and c) only disturbed sites. OTU abundance is on a* $\log_2$ *scale. Filled triangles represent significant differential abundance for that OTU in one of the two tested groups (colors match those in Fig. 3); open triangles represent trends in abundances that are not significant.*

Contribution of environmental factors to AM fungal community differentiation

Precipitation, site history, and their interaction explained roughly 14% of the variation in AM fungal community composition after accounting for sequence number and site location (Table 4). Individual soil variables accounted for between 2.5% (magnesium) and 8% (Bray 2 phosphorus) of the variation in AM fungal community composition. However, this was primarily in place of variation explained by precipitation or site history due to correlations between them: disturbed sites were most strongly correlated with increases in both Bray 2 phosphorus and soil pH as well as with decreases in soil organic matter, while increased precipitation was most strongly correlated with decreases in soil potassium, calcium, CEC, and pH.

DISCUSSION AND INTERPRETATION—SURVEY OF AM FUNGAL COMMUNITY COMPOSITION

Differentiation of AM fungal communities in remnant sites

The AM fungal communities from remnant sites were significantly differentiated across the precipitation gradient (Fig. 1B). Because precipitation was strongly correlated with soil characteristics, especially potassium, calcium, CEC, pH, and Bray 2 phosphorus, it is possible that these soil characteristics also helped to drive the community differentiation. Although the OTUs comprising these communities were taxonomically and phylogenetically diverse, representing at least 12 genera (Fig. 2B), they nonetheless showed significant phylogenetic clustering across the precipitation gradient, and AM fungal communities in grassland ecosystems can also be phylogenetically clustered at more local scales (Horn et al. 2014). This phylogenetic clustering is consistent with filtering of AM fungal communities among all remnant sites compared to all disturbed sites, suggesting that site disturbance is associated with the loss of phylogenetic clustering, independent of precipitation. While other studies have also found variation in AM fungal community differentiation with precipitation in grasslands (Egerton-Warburton et al. 2007) or in sites with diverse environmental conditions and land use histories (Antoninka et al. 2015, Hazard et al. 2013), this study identifies that the differentiation of the remnant AM fungal communities along the precipitation gradient was weakened by disturbance. The OTUs that were abundant in the eastern remnant sites were particularly sensitive to disturbance, suggesting that the AM fungi of the highly fragmented and rare eastern prairies are of particular conservation concern.

This AM fungal community differentiation and the phylogenetic clustering of OTUs in remnant prairies is consistent with the role of native AM fungi in promoting local adaptation of prairie plant communities. Experimental support for this has been provided by observations that AM fungal isolates from drier sites conferred greater drought tolerance to their hosts than AM fungal isolates from wetter sites (Stahl and Smith 1984). In addition, prairie grasses grown with their sympatric AM fungal community consistently had increased biomass and reproductive output relative to their growth with AM fungal communities from other prairie sites (Johnson et al. 2010), and these outcomes may be partially driven by the resource allocation strategies used by both plants and fungi (Revellini et al. 2016).

Functionally, AM fungal communities in grassland ecosystems help maintain diverse plant communities and improve soil stability. Inoculation experiments have demonstrated the importance of AM fungal isolates from remnant prairies in improving the establishment, growth,

and reproduction of late successional stage prairie plants and in increasing prairie plant diversity (Koziol and Bever 2016b, Middleton et al. 2015). Conversely, the experimental reduction of AM fungal abundance in remnant prairies using fungicide has strong effects on both plant species richness and diversity (Hartnett and Wilson 1999). The proportion of water stable aggregates, a measure of a soil's ability to resist erosion, has been shown to be significantly higher in remnants compared to sites with a history of soil disturbance (Duchicela et al. 2012, Jastrow 1987). There is also a strong correlation between the abundance of AM fungal hyphae in the soil and the amount of water stable aggregates (Wilson et al. 2009), suggesting AM fungal communities are critical in promoting soil stability either directly through hyphal meshes or indirectly through the glycoprotein glomalin (Rillig and Mummey 2006).

Reduced AM fungal community differentiation in disturbed sites

In contrast to remnant sites, AM fungal communities in disturbed sites were not significantly differentiated across the precipitation gradient (Fig. 1C), despite the various histories of mechanical soil disruption that are represented by the disturbed sites we sampled. However among samples from disturbed sites only, there were few OTUs that were more abundant in western sites (Fig. 2C) and they also had significant phylogenetic clustering, which would be consistent with the presence of strong environmental filtering of OTUs due to precipitation, its correlated soil characteristics, or other factors. Because our main focus here was not to comprehensively evaluate the effects of different disturbance types on AM fungal communities, we took a conservative approach when labeling the history of mechanical soil disruption at each of the disturbed sites by assigning any site not having a confident history of past disturbance to an 'unknown' disturbance category. Although classifications of disturbance histories are unavoidably at least somewhat subjective, these classifications can have substantial effects on the outcome of statistical tests. For instance, in this study, when we repeated the PERMANOVA analysis for disturbed samples but did not include the history of mechanical soil disruption as a predictor, the AM fungal communities in disturbed sites were then significantly differentiated across the precipitation gradient ($p = 0.04$). However, the AM fungal communities in remnant sites were always more strongly differentiated across the precipitation gradient compared to those in disturbed sites regardless of the model used. The early successional or invasive plant communities that dominated all of the disturbed sites sampled are less reliant upon AM fungi than the late successional stage plants found in remnants (Koziol and Bever 2015, Koziol and Bever 2016a, Pringle et al. 2009, Wilson and Hartnett 1998). These plant community characteristics in disturbed sites may allow a range of AM fungal taxa to persist compared to remnant sites where reliance of the plants on AM fungi may place functional constraints on the AM fungal communities. This expectation is supported by the phylogenetically even representation of OTUs from disturbed sites compared to the significant phylogenetic clustering of OTUs from remnant sites. While many factors can contribute to the local adaptation of plant communities, this lack of both AM fungal community differentiation and phylogenetic clustering across the precipitation gradient following anthropogenic disturbance may reduce the ability of these AM fungal communities to aid the local adaptation of plants.

Despite the lack of AM fungal community differentiation in disturbed sites, individual OTUs still had substantially different abundances across the precipitation gradient (Fig. 2C), facilitating the ability to classify communities on both sides of the gradient with high accuracy. Although we found no evidence of phylogenetic clustering of AM fungal communities from

eastern disturbed sites, there was strong phylogenetic clustering in communities from western disturbed sites. This clustering may be partly due to differences in the dominant plant species between western and eastern sites, with mainly smooth brome and yellow bluestem in western disturbed sites and mainly tall fescue in eastern disturbed sites, due to differences in precipitation or related soil characteristics, or due to other factors we did not measure here.

Soil nutrients also predict AM fungal community differentiation

Among measured soil nutrients, differences in soil phosphorus were most strongly correlated with changes in AM fungal community composition. This was not unexpected given the importance of phosphorus exchange in mycorrhizal associations. The fact that not all AM fungi provide the same growth benefit to plants over a range of phosphorus conditions (Vogelsang et al. 2006) may help drive shifts in AM fungal community composition between sites with different phosphorus levels. Concentrations of Bray 2 phosphorus in the soil most strongly separated samples from the two site histories, with disturbed sites consistently having more phosphorus, which is consistent with more intensive land use, including agricultural fertilization. Increased phosphorus levels in the soil can reduce plant preferential allocation of carbohydrates to the most mutualistic AM fungal strains (Ji and Bever 2016), which can allow the proliferation of less mutualistic, faster-growing strains of AM fungi (Bever 2015). In this study we cannot isolate the direct effect of increased soil phosphorus from that of site disturbance because the two were highly correlated. However, the significantly greater number of OTUs that were more abundant in eastern disturbed sites where the disturbance was primarily due to soil disruption (Fig. 2C) is consistent with the establishment of less mutualistic AM fungal strains following soil disturbance and concomitant agricultural phosphorus fertilization allowing their persistence. In contrast, several other studies failed to find significant correlations between AM fungal community composition and soil phosphorus concentrations (Jansa et al. 2014, Moora et al. 2014, Oehl et al. 2010). This discrepancy could be due to differences in host plant communities, the form of available soil phosphorus, or how AM fungal communities were assayed (e.g. spores or molecular methods).

Conclusions

AM fungal communities in remnant grasslands were strongly differentiated across a precipitation gradient, while those in disturbed sites across the same gradient were not differentiated despite these sites having a range of disturbance histories. Late successional stage prairie plants are generally reliant upon AM fungi (Koziol and Bever 2015), and the differences in AM fungal communities that occur in remnant prairies across the precipitation gradient are consistent with the role of AM fungi in aiding local adaptation in plant communities. We specifically found that AM fungi characteristic of eastern prairie remnants are especially vulnerable to anthropogenic disturbance. Those taxa are of particular conservation concern given the small size of existing prairie remnants and evidence that native prairie fungi can provide strong growth benefits to late successional prairie plants.

RESULTS: MICROBIAL BIOMASS AND SOIL AGGREGATE STABILITY

Quantification of Soil Microbial Communities:

PLFA and NLFA analyses indicated total microbial biomass was similar across all native and invaded sites at the DoD installations in Illinois, Kansas and Oklahoma. Furthermore, relative abundance of AM fungal biomass was similar across all sites, as determined by PLFA or NLFA. However, within Konza, where a cleaner comparison of the effects of invasion by Old World Bluestem was possible, we observed a decreased total microbial biomass driven by loss of AM fungal biomass with invasion compared to native grasslands (Fig. 4).

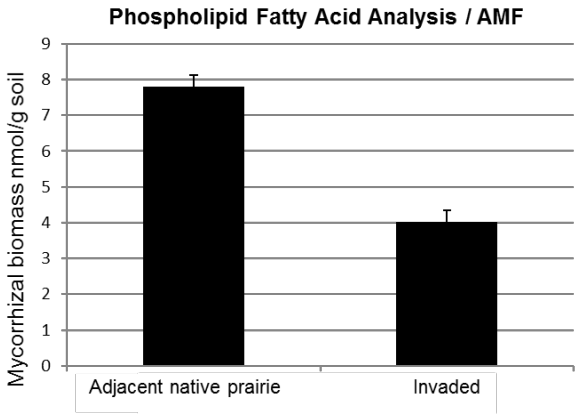


Figure 4. *Soil AMF biomass is reduced following invasion by non-native invasive plant species, Bothriochloa bladhii.*

Aggregate size distribution:

Across all sites in our study, water-stable macroaggregates (> 250 μm in diameter) comprised the largest aggregate proportion of the soil (63-70% regardless of site or disturbance); with a lower proportion of microaggregates (30-37% by volume). This soil macroaggregate:microaggregate ratio was not significantly different across site or year (p> 0.05), and is typical for soils of warm-season dominated tallgrass prairies.

However, at Konza Prairie, were a cleaner comparison of the effect of Old World Bluestem invasion, we were again able to detect a significant decrease in soil aggregate stability and in soil carbon with invasion by *Bothriochloa bladhii*.

DISCUSSION AND INTERPRETATION: MICROBIAL BIOMASS AND SOIL AGGREGATE STABILITY

These results from Konza prairie identify that disturbance and invasion of native grasslands by non-native invasive plant species can reduce AM fungal density with resulting degradation of soil aggregate structure, but this effect was not consistently observed across all grasslands. In contrast, the degradation of AM fungal composition observed with the environmental sequencing was consistent across grasslands across the central US plains. Together, this suggests that the degradation in AM fungal composition represents the primary negative effect of disturbance and invasion of native grasslands.

RESULTS—GREENHOUSE ASSAYS OF RESPONSIVENESS TO AMF

We found that both plant natives status and plant successional stage were strong predictors of mycorrhizal responsiveness. Native plants were more strongly responsive to mycorrhizal fungi ($F_{1,148}$=6.9, p=0.01, Fig. 5a). Plant successional category was an even stronger

predictor of mycorrhizal responsiveness ($F_{3, 146}$=5.5, p=0.001, Fig. 5b). We found that average mycorrhizal responsiveness increased consistently across plant successional stage and that average late successional plant growth was improved more than 300% when grown with prairie mycorrhizal fungi.

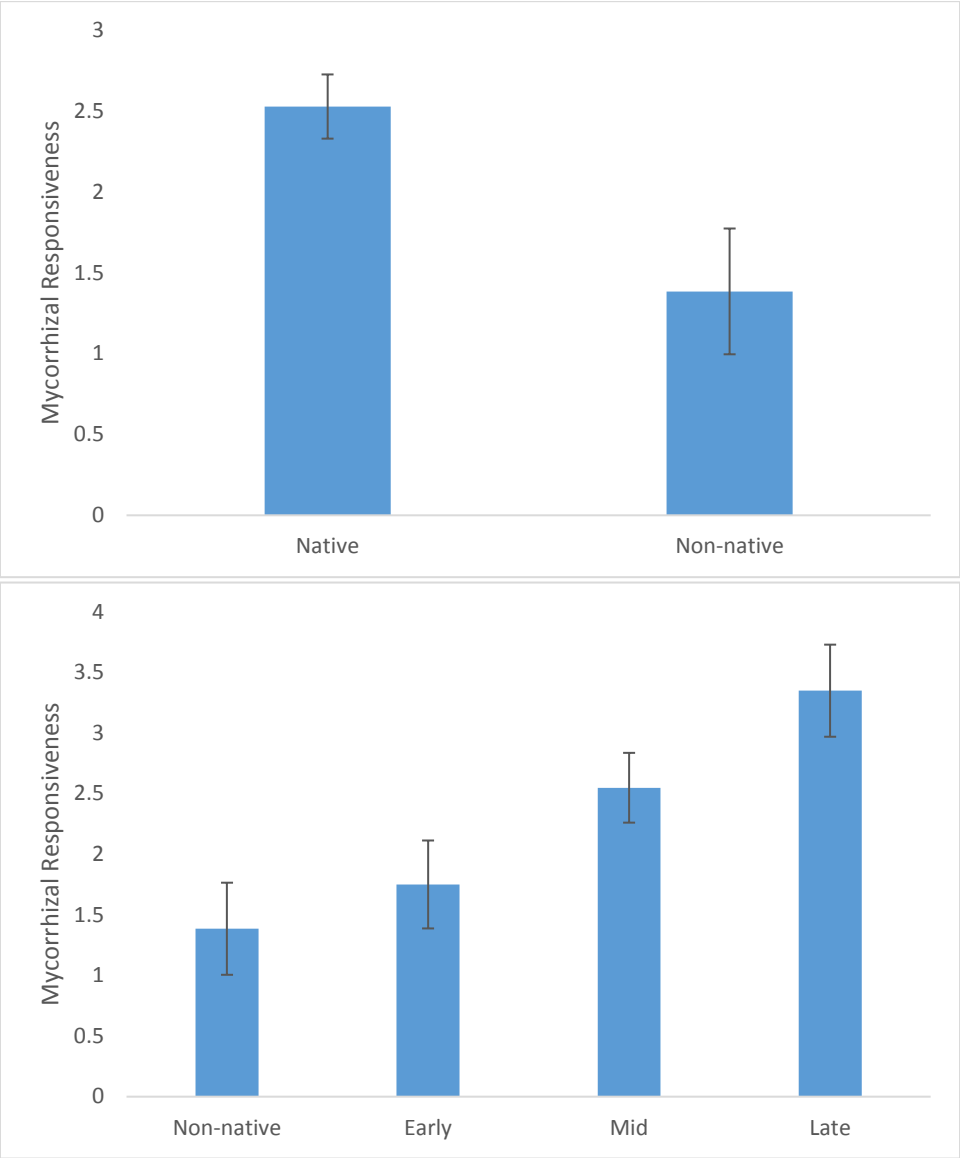


Figure 5. *Native plant species generally benefit more from AMF than non-native invasive plant species (5a). Amongst native plant species, successional stage was a good predictor of plant response to mycorrhizae, where non-native and early successional (CC 1-2) were less responsive than middle (CC 3-6) and late (CC 7-10) successional plants (5b).*

RESULTS—GREENHOUSE ASSAYS OF SPECIFICITY OF PLANT RESPONSE TO AMF

We found that plant successional stage was the strongest predictor of mycorrhizal responsiveness in grassland plants of North America ($F_{2,22} = 24.45$, $P < 0.0001$; Table 1, Fig. 6a). Late successional native plants grew an average of 322% larger with AM inoculation relative to controls (Fig. 6a), and had a much stronger growth response to AM fungi than early successional native or non-native plant species, which were often inhibited by AM fungi ($P < 0.0001$). We found no difference in overall mycorrhizal responsiveness between non-native and early successional native plants ($F_{1,22} = 0.35$, $p = 0.56$).

Variation in mycorrhizal responsiveness differed across successional stage ($F_{2,22} = 21.85$, $P < 0.0001$) and was higher in late successional plant species than non-native ($F_{1,22} = 35.32$, $P < 0.0001$; Fig. 6b) and early successional plant species ($F_{1,22} = 40.80$, $P < 0.0001$; Fig. 6b). Variation in mycorrhizal response did not differ between non-native and early successional native plants ($F_{1,22} = 0.14$, $P = 0.71$).

Meta-analysis: Plant-fungal specificity

The meta-analysis showed that the coefficient of variation, a measure of plant-fungal specificity, was highest in late successional native plant species ($F_{2,22} = 22.77$, $P < 0.0001$; Fig. 6c) and was strongly correlated with mycorrhizal responsiveness ($R^2 = 0.66$, $P < 0.05$; Fig. 7). The coefficient of variation in mycorrhizal responsiveness was very low (little specificity towards fungal isolates) and did not differ between non-native and early successional plants ($F_{1,22} = 0.65$, $P = 0.43$).

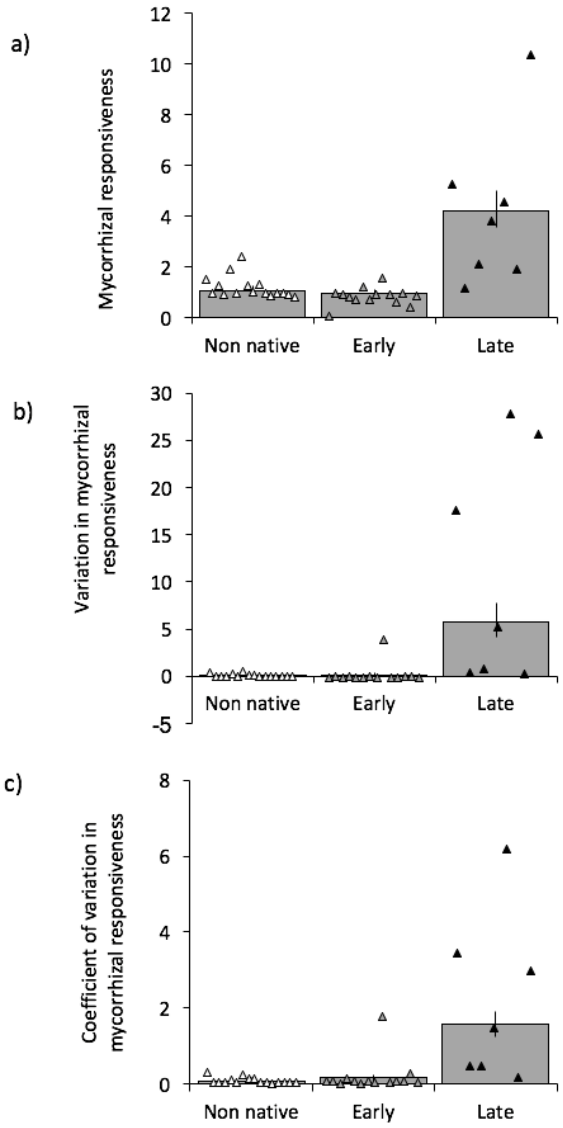


Figure 6. *Plant successional stage was a strong predictor of (a) plant growth response to arbuscular mycorrhizal fungi (i.e. mycorrhizal responsiveness), (b) variation in mycorrhizal responsiveness, and (c) coefficient of variation in responsiveness among different fungal species. Bars and error bars represent plant successional stage means (+/- SEM); symbols represent individual non-native plant species (white symbols), early successional native prairie plant species (grey symbols), and late successional native prairie plant species (black symbols).*

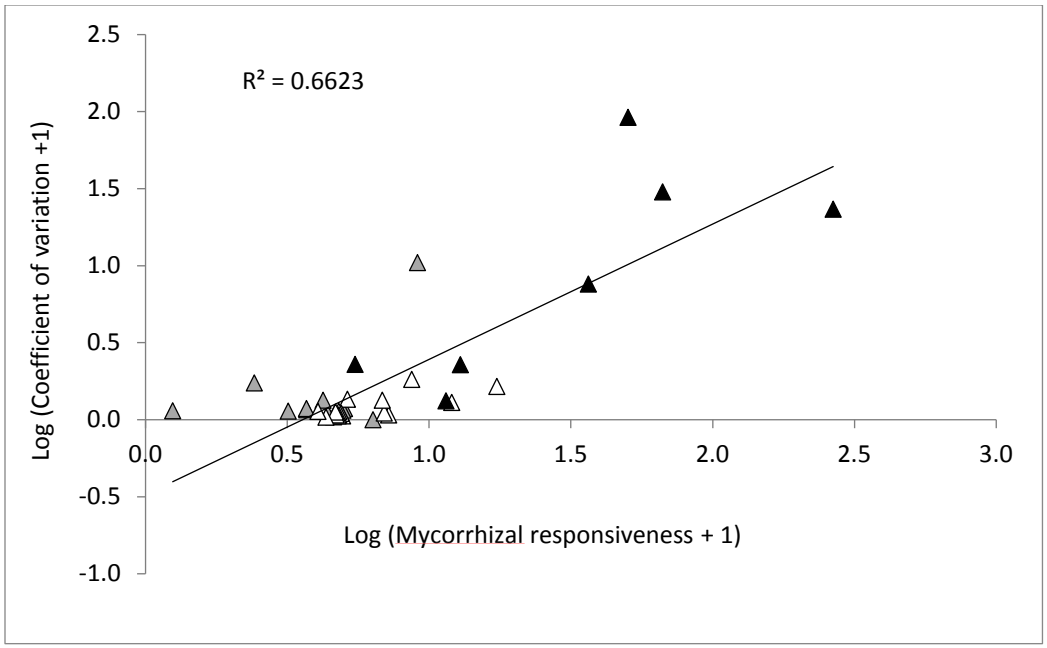


Figure 7. *Late successional native plant species (black symbols) had higher mycorrhizal responsiveness and higher coefficient of variation in mycorrhizal responsiveness than early successional native plant species (grey symbols) and non-native plant species (white symbols). Coefficient in variation of mycorrhizal responsiveness – a measure of plant-fungal specificity – was highly correlated with mycorrhizal responsiveness.*

DISCUSSION AND INTERPRETATION—GREENHOUSE ASSAYS OF RESPONSIVENESS TO AMF

We find that non-native invasive plant species have low responsiveness to mycorrhizal fungi, and are not particular to AM fungal species. Weedy, early successional native species have similarly limited responsiveness and sensitivities to AM fungi. This low dependence of AM fungi is consistent with the competitive dominance that non-native invasive plant species and weedy native plant species show following disruption of the AM fungal community by mechanical disturbance, such as military training, road construction or agriculture.

In contrast, we show that late successional grassland plant species have strong positive growth responses to AM fungi, exhibit high variation in response to individual AM fungal taxa, and are more sensitive to variation in AM fungal community composition than early successional or non-native plant species. The sensitivity of late successional grassland plants to variation in fungal community composition could be especially important for plant community dynamics in landscapes where mycorrhizal community composition has been disrupted by disturbance, such as fertilizer applications, tillage, or invasive species. Although previous experimental work showed the potential importance of AM fungal community composition on plant community diversity (e.g. Koziol and Bever 2017), our meta-analysis demonstrates that the impact of variation in AM fungal composition on plant community dynamics may depend on the strength and specificity of plant response to individual AM fungal species.

Importance of plant-fungal specificity for ecological restorations

With increasing levels of disturbance in grassland ecosystems due to climate change, invasive species, and agricultural management practices, understanding how plant-fungal interactions shape above and belowground communities is critically important. Although plant relationships with mycorrhizal fungi are known to range from parasitism to mutualism (Johnson et al. 1997) and can vary among plant species as we demonstrate in this study, variation in growth response to specific fungal taxa could be an important driver of plant diversity and productivity (van der Heijden et al. 1998, Vogelsang et al. 2006, Wagg et al. 2011). Our meta-analysis showed that many exotic plant species, including common forage crops in the USA such as *Bromus inermis* (smooth brome) and *Festuca arundinacea* (tall fescue), exhibited little growth response or specificity of response to AM fungi. Plant species that are poor hosts or have chemical or microbial constituents that negatively impact AM fungi (e.g. allelopathy in mustard, fungal endophytes in fescue) (Matthews and Clay 2001, Mack and Rudgers 2008, Bainard et al. 2009, Harnden et al. 2011), can significantly alter fungal communities in soil, to the benefit of early successional and non-native plants that do not depend on AM fungi. Late successional grassland plant species may then be at a competitive disadvantage in landscapes where belowground communities have been disrupted. Poor host plants, combined with agricultural management practices that negatively impact AM fungal abundance and diversity (e.g. tillage, pesticides, and chemical fertilizer), make ecological restorations of former agricultural fields particularly challenging.

We show through greenhouse studies and meta-analyses that late successional grassland plant species not only have strong positive growth responses to AM fungi, but are also more sensitive to variation in AM fungal community composition than early successional or non-native plants, as evidenced by their strong differences in growth response to individual AM fungal taxa. The sensitivity of late successional plants to variation in fungal community composition could have important consequences for plant coexistence, especially in disturbed ecosystems where legacies of management practices have had a negative impact on soil communities. Our study highlights the fact that not all AM fungi confer the same benefits to plants (Fig. 6). Thus, targeted inoculations with AM fungal species that are known to improve the growth of a particular plant species may also be important in ecological restorations and agroecosystems. Taken together, our work supports previous reports of higher mycorrhizal responsiveness and specificity of response for late successional plant species (Koziol and Bever 2016a) and indicates that plant-fungal specificity may be a mechanism driving plant community dynamics in North American grasslands.

RESULTS-NURSE PLANT GROWTH AND SURVIVAL

Here we report the effects of inoculation with prairie soil or AMF isolated from prairies in five native grassland restorations at Ft. Riley Army Base (KS), Tinker Airforce Base (OK), and the retired Chanute Airforce Base (IL).

The effects of native AMF inoculation on plant survival

We found that the inclusion of living microbiome biota (either whole soil or AM fungi) isolated from remnant prairie communities improved native plant survival relative to controls during years one, two and three ($F_{1,235}$= 27.9, p= <0.0001, $F_{1,235}$=23.1, p<0.0001, $F_{1,89}$=3.4, p=0.07, respectively). Average nurse plant survival was 28% and 38% greater than the controls

when plants were inoculated with arbuscular mycorrhizal (AM) fungi and whole prairie soil, respectively, during the first and second growing season (Fig. 8). Plant survival was not statistically different after inoculation with either whole microbiome soil or solely with AM fungal during years one, two or three ($F_{1,235}$=1.6, p=0.2, $F_{1,235}$=1.7, p=0.2, $F_{1,89}$=0.3, p=0.6, respectively), indicating that AM fungi isolated from native prairies provides the majority of the growth benefit of the native soil microbiome to native plant survival. There was significant variation between the restoration locations (Ft. Riley, Tinker, or Chanute), but the benefits of inoculation were consistent across all restoration experiments (Fig. 8c).

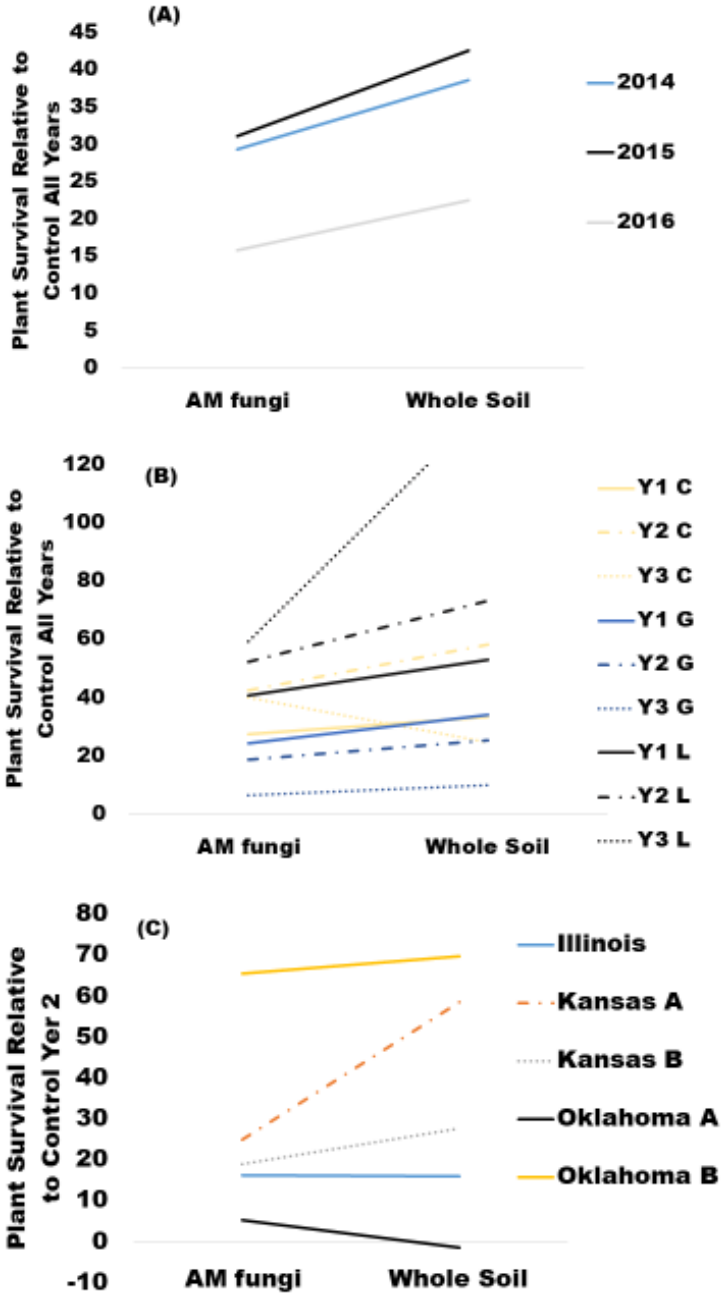


Figure 8. *Effects of inoculation relative to control on plant survival across all years where Y stands for year, C stands for composites, G stands for grasses, L stands for legumes. Nurse plant survival (A) was improved more than 30% when plants were inoculated during year one and two, and there was a non-significant trend for higher survival in whole rhizosphere soil relative to solely with AM fungal inoculated soil (p=0.2). Plants in all functional groups benefited from rhizosphere inoculation whether it be from AM fungi or whole soil inoculum (B). Grasses tended to respond less to inoculation over time whole legumes tended to respond more positively over time although there were not significant differences in plant survival among functional groups between living inocula types any year. Most sites showed improved plant survival with inoculation except for Oklahoma A (C).*

The survival of plants in all functional groups (grasses, legumes and composites) benefited from rhizosphere inoculation similarly whether it be from AM fungi or whole soil inoculum, as indicated by a non-significant contrasts comparing survival with the two living inocula types across functional groups the first growing season ($F_{2,235}$=0.1, p=0.9) and later sampling dates. Inoculation improved legume and composite survival 30-35 % relative to the controls year 1 and beyond (Fig. 9).

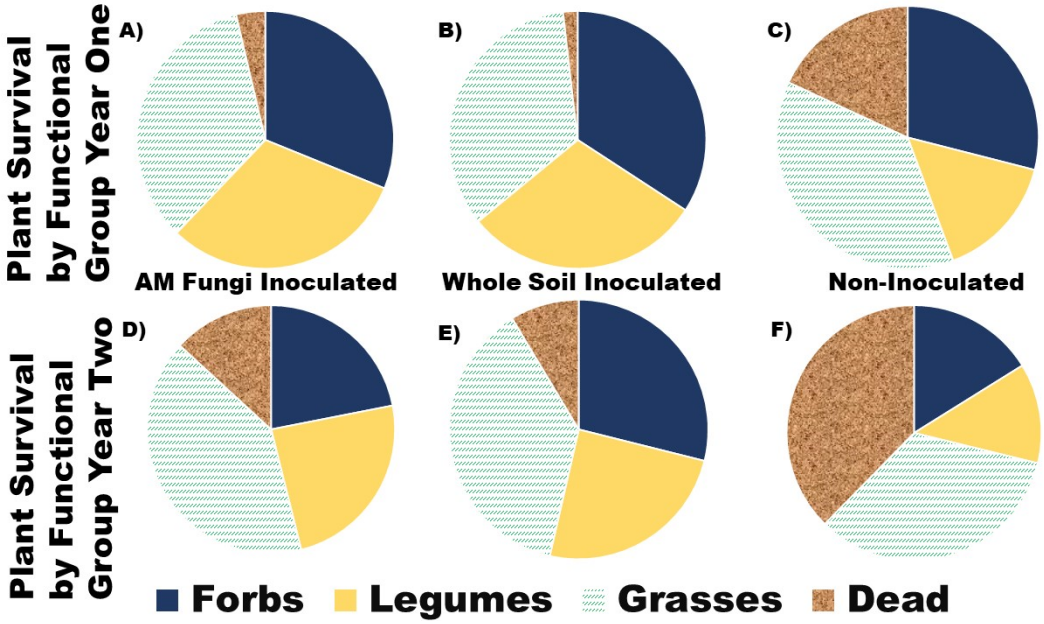


Figure 9. *The effect of microbiome inoculation on different plant functional groups across years. Inoculation with AM fungi and whole soil microbiomes resulted in greater first year (A-C) nurse plant seedling survival at Tinker Air Force site B (as shown here) and across all restorations. Plants from all functional groups responded similarly positive to the different inoculations, although forbs and legumes were more strongly affected than grasses. Around 95% of AM fungi or whole soil inoculated forbs and legumes survived whereas less than 70% of non-inoculated forbs or legumes survived. This pattern continued and was amplified during the second year (Fig. 9 D-F). Grass seedling survival tended to be high regardless of inoculation at Tinker Air Force site B and across all restorations, although inoculation did improve grass survival as well.*

The effect of native AMF inoculation on Plant Productivity

Surviving plants were more productive with microbiome restoration during year one (Fig. 10), where plants averaged 18% taller ($F_{1,120}$=32.4, p,<0.0001) and had 30% more leaves with AM fungal or whole soil inoculation ($F_{1,120}$=5.6, p<0.0001) relative to controls. Inoculation remained one of the strongest predictors of plant productivity during the following growing seasons. This effect was not dependent on site, as all sites showed improvement in plant productivity after inoculation with both AM fungi and whole soil relative to the non-inoculated controls year one. The productivity of all plant functional groups was similarly improved with living microbiome inocula from reference grasslands, as AM fungi inoculation and whole

rhizosphere soil inoculation were not statistically different in growth promotion year one or later (Fig. 10b, leaf and height $F_{1,120}$=0.3, p=0.7).

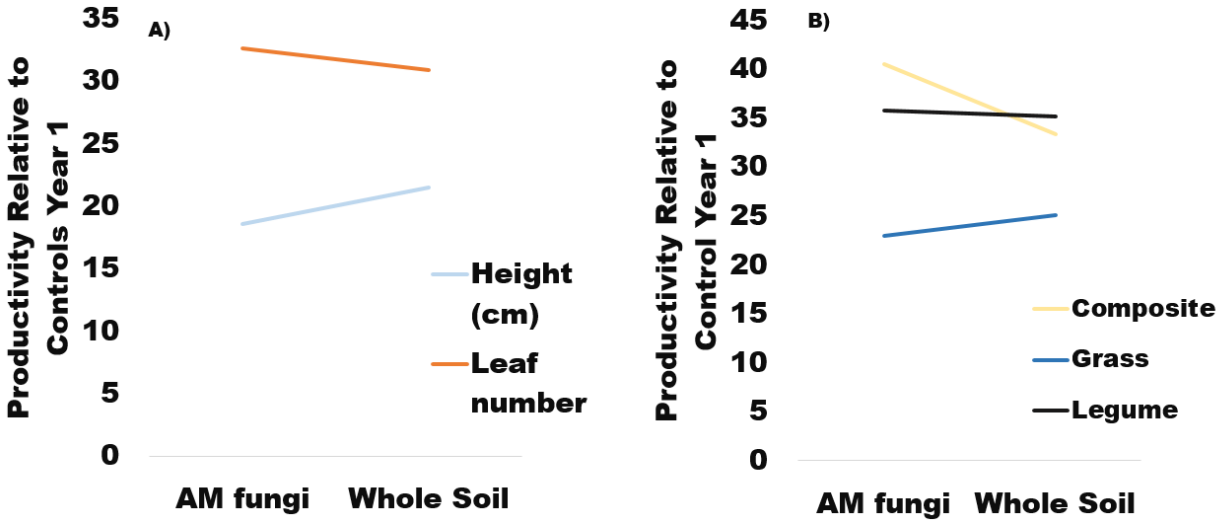


Figure 10. *The effect of inoculation on plant productivity during year one. Plant productivity was improved after microbiome restoration during year one (a), where plants averaged 18% taller and had 30% more leaf productivity with AM fungi or whole soil inoculation relative to controls. This effect was not dependent on site, as all sites showed improvement in plant productivity after inoculation with both AM fungi and whole soil relative to the non-inoculated controls. Inoculation with AM fungi or whole microbiome inocula improved plant leaf productivity similarly regardless of plant functional group (b). These patterns indicate that AM fungi and whole soil microbes can be interchangeably used to improve prairie seedling growth in restorations across a wide range of site conditions and for plants across a range of plant families and plant functional groups.*

The effect of native AMF inoculation on Plant Productivity

During the second year of the study, across all sites, inoculation reduced the density of non-native invasive plant species. Abundance of non-native species was lower in AMF inoculated plots to compared to the other two inoculation treatments ($F_{2,120}$=2.9, p=0.06, Fig. 11).

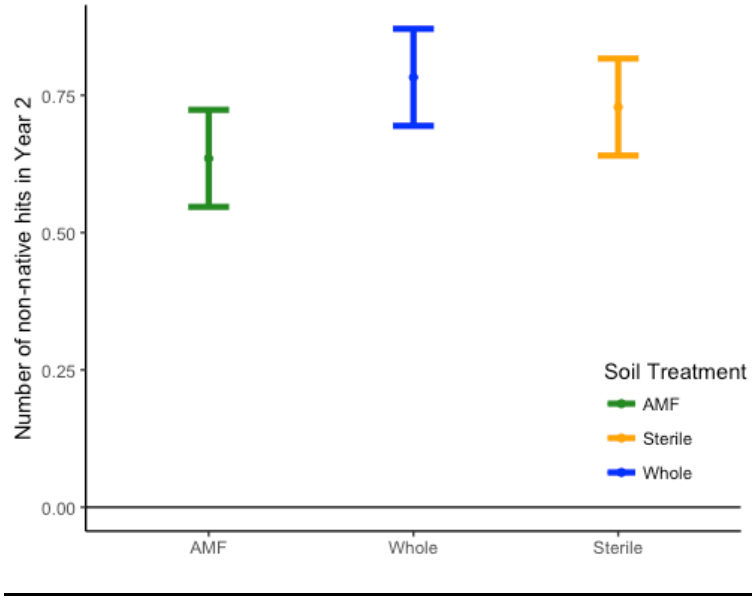


Figure 11. *Inoculation with native AM fungi reduced the density of non-native plant species compared to control plots across all five restorations.*

AMF Spread from inoculation point

At each site, we observed OTUs from our inocula near the inoculated nurse plants. Overall at Chanute Air Force Base, inocula OTUs made up a higher proportion of the AMF community in whole soil nurse plant rows compared to sterile controls overall ($F_{1, 50}$=5.6, $p<0.05$), and there was a large decrease in this proportion with increasing distance from the nurse plant row for all inoculated plots ($F_{5, 250}$=6.5, $p<0.0001$).

Though these overall effects were not as clear in the second year at Chanute or in either year at other sites, specific OTUs did show varying degrees of distance effects, and these OTUs are represented in Fig. 12. Individual OTUs varied in their rate of spread from the nurse plant rows. This was most easily illustrated with the restoration at the Chanute Air Force Base. We observed 62 AMF OTUs in the AMF inocula and 125 AMF OTUs in the whole prairie soil inocula. Of these, in the first year 13 or 9% were significantly higher in the inoculated row than the control row and showed no evidence of spread to 0.5m. Twenty or 14% showed no evidence of spread to 0.5m, but did not show a statistically significant difference with the control. Two or 1% of the OTUs were significantly higher in the inoculated row than the control row and showed evidence of spread to 0.5m but not 1.5m and four or 3% showed the same pattern with distance, but did not have significant differences between inoculated and control nurse plants. One or 0.6% of the OTUs were significantly higher in the inoculated row than the control row and showed evidence of spread to 1.5m and three or 2% showed the same pattern with distance, but did not have significant differences between inoculated and control nurse plants.

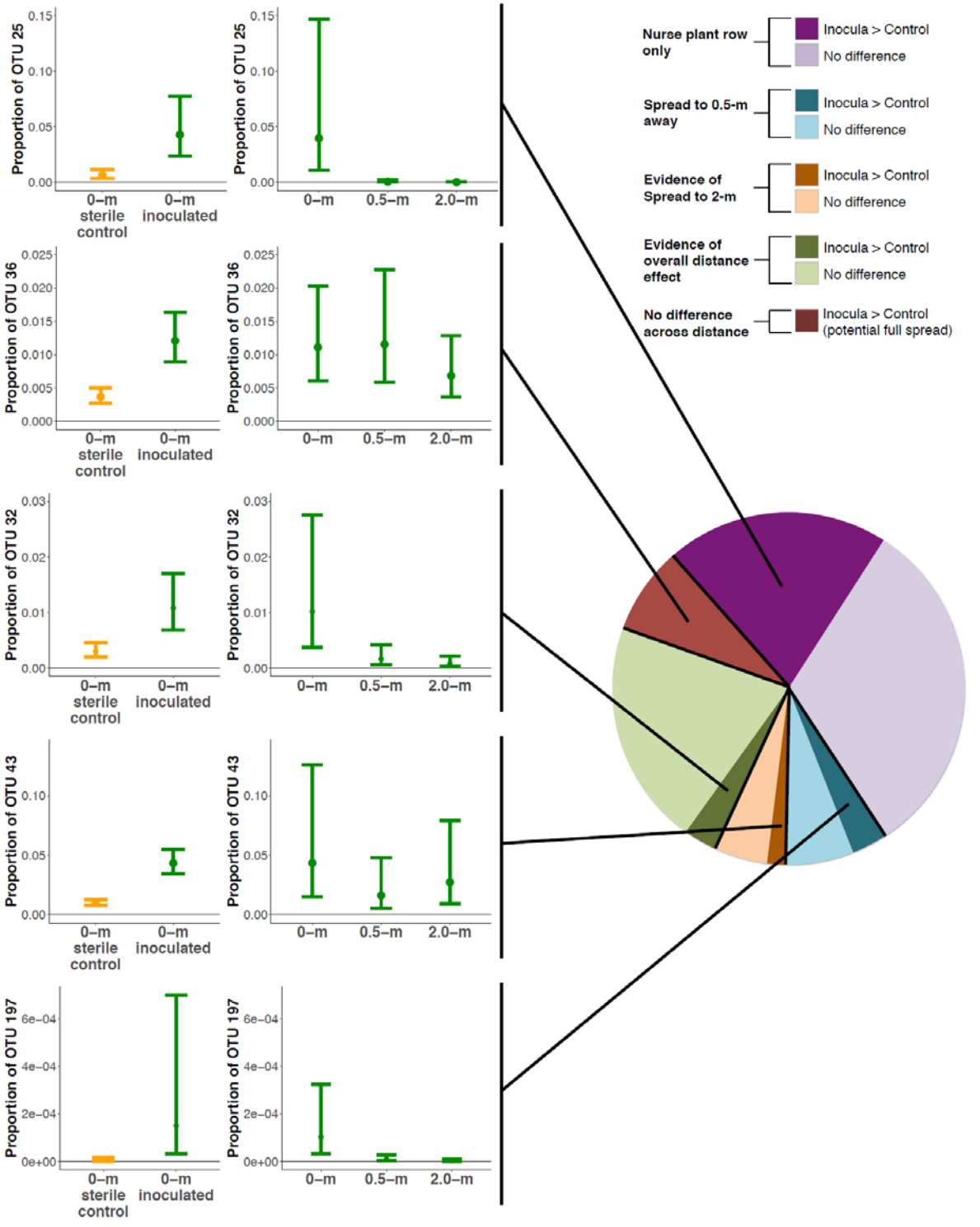


Figure 12. *AM fungal taxa varied in their level of dispersal away from their points of inoculation at the Chanute Air Force Base Restoration. Some of the AM fungal taxa, such as OTU 26, did not disperse away from the nurse plant row (a). Other taxa, including OUT 197, showed statistical evidence of spreading to 0.5 meters, but not to 2 meters. Other taxa, including OUT 43, showed statistical evidence of spreading to 2 meters.*

Though these overall effects were not as clear in the second year at Chanute or in either year at other sites, specific OTUs did show varying degrees of distance effects, and these OTUs are represented in Fig. 13. It is worthy of note that across all sites, individual AM fungal OTUs spread at least 1.5 meters within the first 16 months of inoculation.

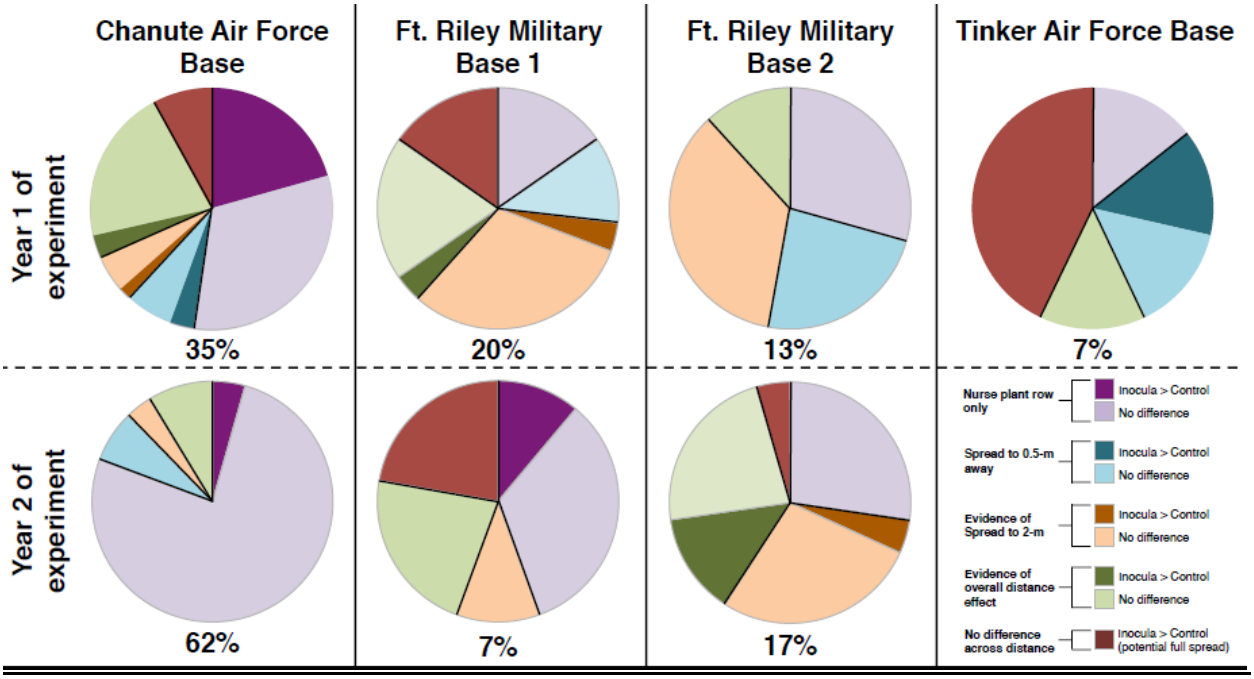


Figure 13. *The patterns of spread from points of inoculation in four experiments. Across all sites and all years measured, individual AM fungal OTUs varied in their rates of spread from points of inoculation. Across all sites, individual AM fungal OTUs spread at least 1.5 meters within 16 months following inoculation.*

RESULTS—SUPPLEMENTAL FIELD INOCULATION STUDY 1

As in our core inoculation studies, survival of native prairie plants was higher in plots inoculated with whole prairie soil or AM fungi isolated from prairie soil compared to the uninoculated plots (Fig. 14). Within the first four months of the experiment, more than 50% of the uninoculated prairie plants had died, highlighting the importance of soil microbes in facilitating native plant establishment in invaded ecosystems ($P < 0.001$, Fig. 14). Native prairie plants also grew larger in inoculated plots compared to uninoculated plots (Fig. 15).

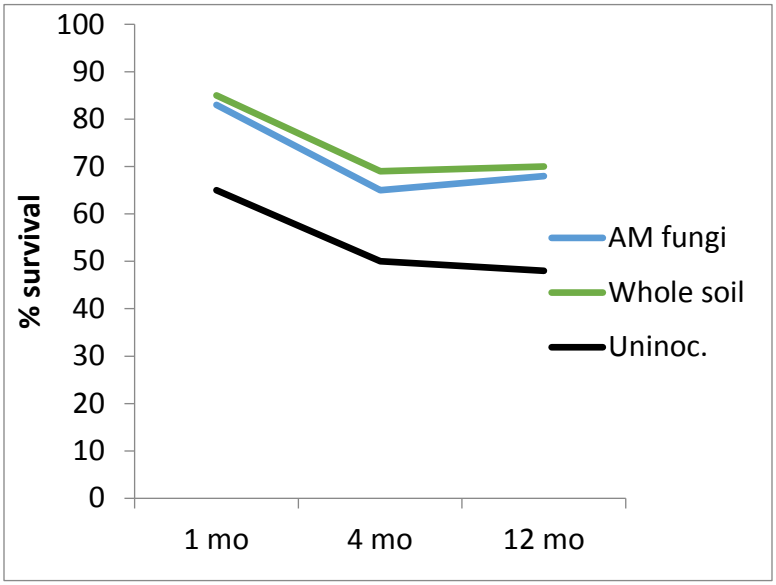


Figure 14. *Inoculation with soil microbes improved native plant survival as more than 50 percent of the uninoculated plants died within the first four months.*

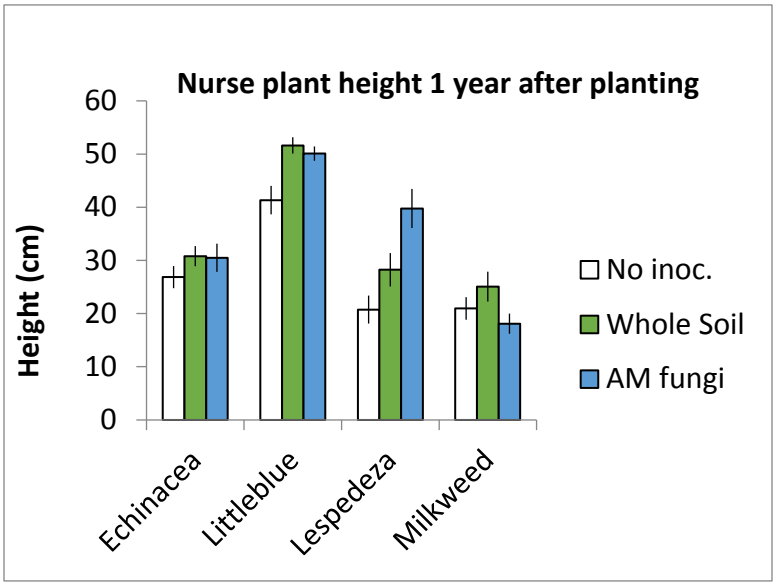


Figure 15. *The native nurse plants Echinacea pallida (purple cone flower), Schizachyrium scoparium (Little bluestem), Lespedeza virginica (Slender lespedeza), and Asclepias tuberosa (Butterfly milkweed) grew larger when inoculated with prairie microbes (AM fungi; blue bar or whole prairie soil; green bar) compared to uninoculated controls (white bar).*

This experiment offered an opportunity to test the spread of AMF inocula from the nurse plant row to neighboring uninoculated test plants (phytometers). Over the course of one year, AM fungi appeared to have spread at least 0.5m from the inoculated nurse plant row as indicated by larger growth responses of uninoculated phytometers planted closer to the site of inoculation compared to plants at the edge of the plot (growth at 0.5 vs 2.0 m $P = 0.02$; Fig. 16). In contrast, there was no difference in phytometer plant growth at different distances from the nurse plant row in uninoculated plots (growth at 0.5 vs. 2.0 m, $P = 0.82$; Fig. 16).

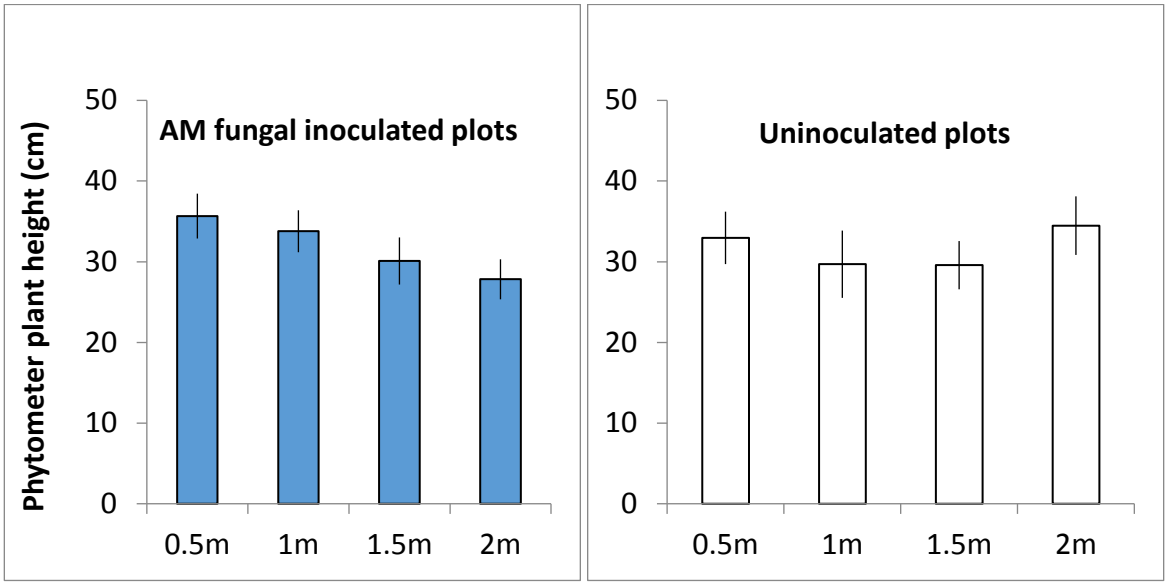


Figure 16. *After one year, uninoculated phytometer plants were larger when they were planted closer (0.5 m) to the source of inoculation in AM plots than plants grown further away from the source of inoculation (0.2 m), P = 0.02). In uninoculated plots, there was no difference in growth of phytometer plants and any distance from the nurse plant row (P = 0.82).*

Native plant survival was increased from 20-40% in plots that were cleared of the invasive non-native species, *Bromus inermus*. However, little bluestem, a native grass, had 90% survival with and without competition by brome. Thus, little bluestem may be an ideal candidate for re-introducing native soil microbes into disturbed systems as it is a good host for AM fungi and is also able to compete well with invasive grass species. For other plant species, inoculation with prairie microbes improved their survival in competition with brome (P=0.02). Within the first year of the experiment, species richness and diversity of native plants from the seed mix was higher in plots inoculated with soil microbes compared to the uninoculated plots (Figs. 17, 18).

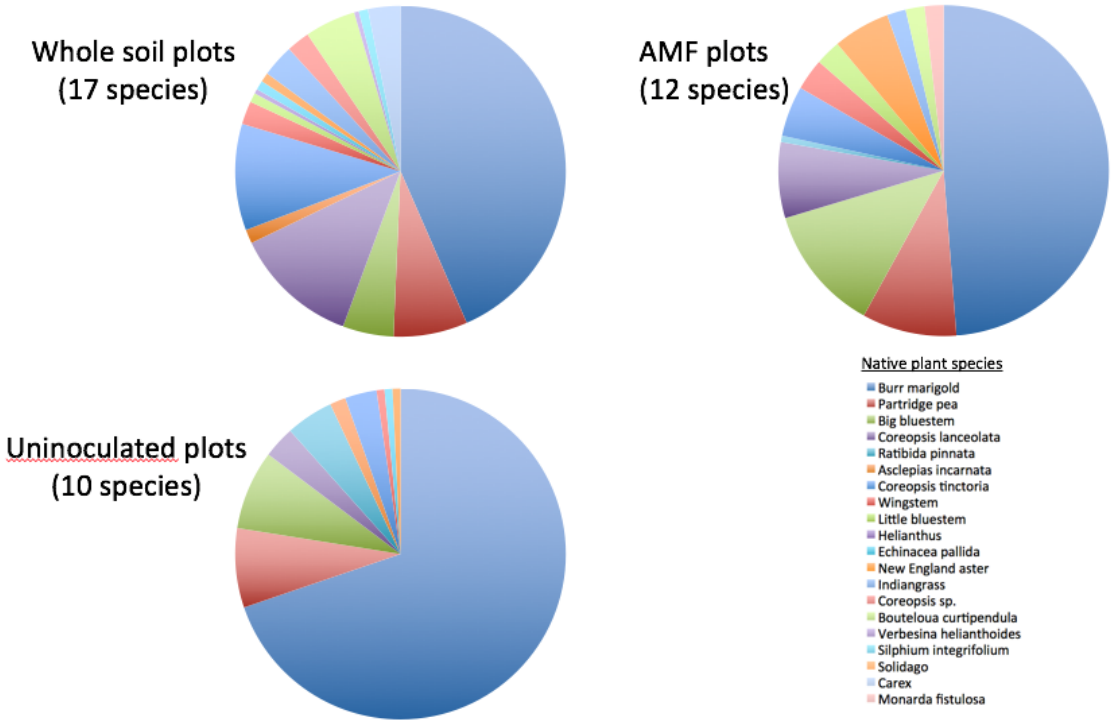


Figure 17. *Species richness of native plants from the seed mix was higher in plots inoculated with whole prairie soil compared to uninoculated plots.*

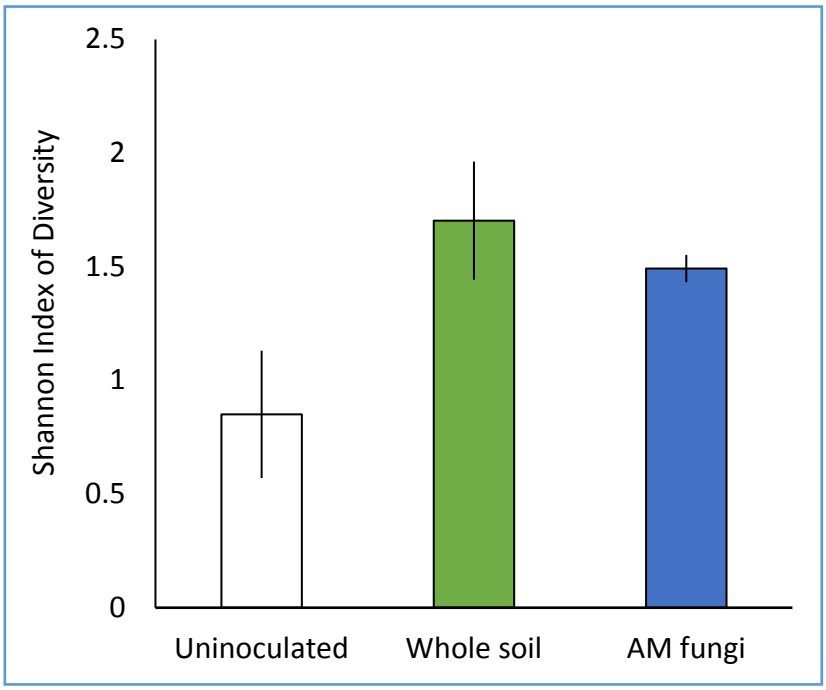
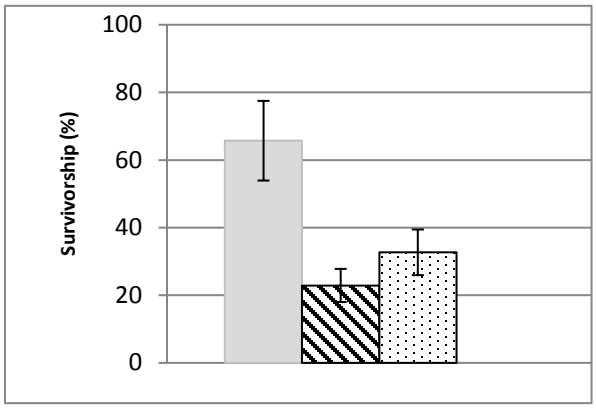


Figure 18. *The diversity of desirable native plant species that came up from seed was higher in inoculated versus uninoculated plots.*

RESULTS—SUPPLEMENTAL FIELD INOCULATION STUDY 2

We repeated the inoculation study in central Kansas in areas previously dominated by Old World Bluestem (*B. bladhii*). Following *B. bladhii* eradication with glyphosate applications, native seedling survivorship was greater when inoculated with 'live' native soil inoculum (Fig. 19).

Figure 19. *Seedling survivorship was greater following inoculation with native prairie soil (grey bars), compared to inoculation with autoclaved soil (hashed), or no soil (stippled).*



Inoculation with native 'live' soil also suppressed re-invasion by the non-native invasive *B. bladhii*. At the end of the growing season, plots receiving no soil inoculum averaged 63-70% cover by the non-native invasive species *B. bladhii*, while plots receiving 'live' native soil inoculum, averaged 38% cover by *B. bladhii.*

RESULTS—SUPPLEMENTAL FIELD INOCULATION STUDY 3

This study complements our core study by testing the benefits of inoculation with individual species of native AM fungal taxa. All AM fungi were derived from Indiana prairies and we test their benefits for native plant establishment in disturbed grasslands in Indiana.

Nurse Plant Growth, Survival, and Fecundity

Soil treatment was a strong predictor of nurse plant survival (Fig. 20A, $F_{5,21}=16.3$, $p<0.0001$), where inoculated nurse plants were 40% more likely to survive with AM fungal inoculation (Fig. 1A, $F_{1,21}=74.8$, $p<0.0001$) during year one. These patterns continued through the end of the second year, when inoculated nurse plants were about three times more likely to survive with AM fungal inoculation than controls ($F_{1,21}=20.7$, $p=0.0002$) and nurse plants were more likely to survive when inoculated with certain fungal species ($F_{3,21}=4.4$, $p=0.01$). We found that plants inoculated with the diverse fungal mixture grew 10% more leaves (Figure 20B, $F_{1,21}=2.8$, $p=0.1$) and grew 40% taller ($F_{1,21}=4.25$, $p=0.05$) than expected based on the average plant growth observed among plots inoculated with individual fungal species. We found that the ability to produce flowers for late successional plants was strongly dependent on inoculation ($F_{1,41}=16.0$, $p=0.0003$), late successional nurse plants rarely flowered without inoculation while early successional nurse plants were sometimes less likely to flower with AM fungal inoculation relative to the controls.

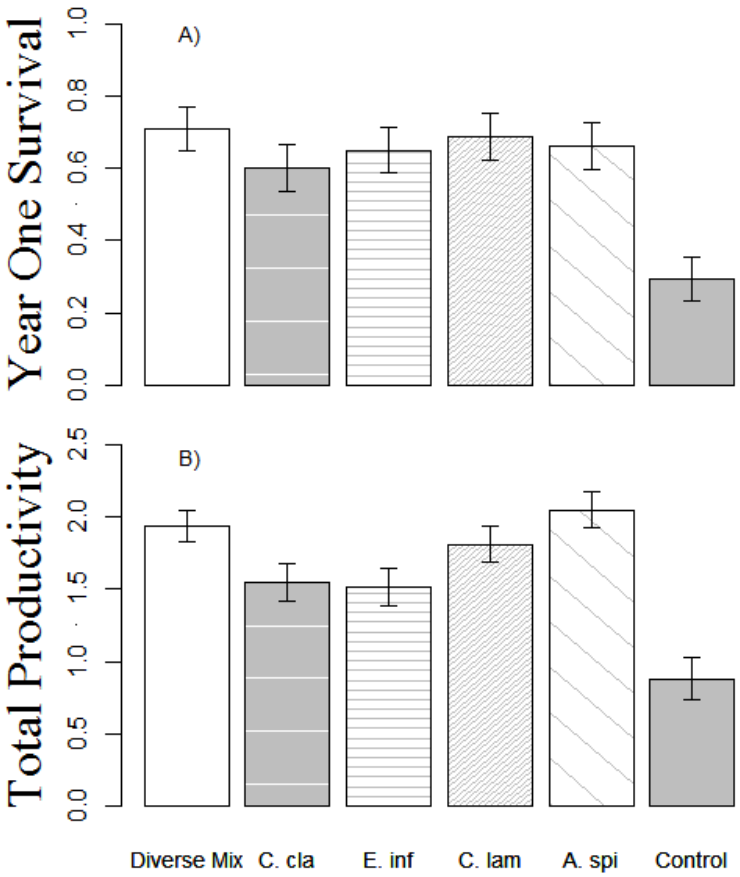


Figure 20. *Inoculation improved nurse plant survival by nearly 40% during year one (A). Inoculated plants were also significantly larger, though this effect as strongly affected by the AM fungal species identity (B). Bars represent the average proportion nurse plant survival (20A) and the log transformed number of leaves or tillers (20B) among plots inoculated with the six different fungal communities and error bars are standard error. Plants inoculated with E. infrequens, C. lamellosum, C. claroideum, and A. spinosa are represented by E. inf, C. lam, C. cla and A. spi, respectively.*

Year Two: Total Plant Community Richness, Abundance & Diversity

Inoculated plot richness averaged five more species per plot (Fig. 21A, $F_{1,21}$=10.6, p=0.004) with the highest plot richness of the inoculated plots was about 55% greater than the control. Many highly conservative late successional forbs and legumes only established within plots that had been inoculated with native prairie AM fungal amendments, including *Allium cernuum*, *Amorpha canadensis*, *Asclepias tuberosa*, *Aster azureas*, *Baptisia austrailis*, *Dalea purpurea*, *Echinacea pallida Eryngium yuccifolium*, *Helianthus mollis*, and *Liatris pycnostachum*, while two conservative late successional forbs or legumes, *Cassia herbace* and *Parthenium integrifolium* established consistently in plots regardless of inoculation treatment. Thus, we found that inoculation significantly improved total late successional plant richness (Fig. 21B, $F_{1,21}$,=11.2, p=0.003). We found that soil treatment was not a good predictor of total early

successional richness or desirable early successional seed recruitment ($F_{5,21}$=0.8, p=0.6 and F_5=1.1, p=0.4), as many desirable early successional species, such as *Monarda fistulosa, Elymus canadesis,* and *Rudbekia hirta* established well regardless of plot inoculation treatment. These results indicate that fungal composition is highly important for the establishment of late successional plant species and that on average, the establishment of seeded early successional species is not sensitive to AM fungal composition.

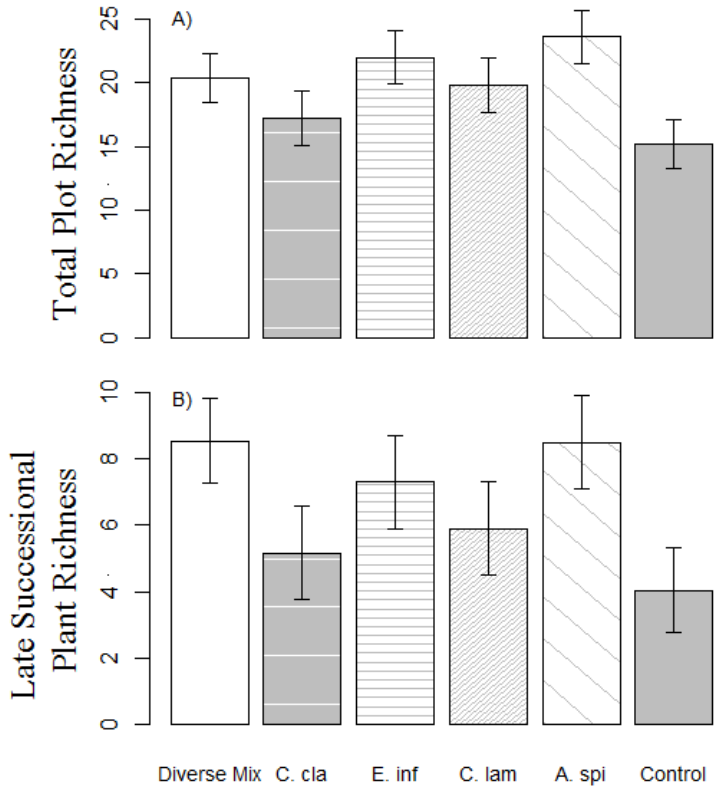


Figure 21. *Inoculation during year one resulted in increased plot richness through the second year (A). Late successional richness was twice as high after inoculation with the diverse AM fungal mixture relative to the control. Bars represent the total plot richness (A) and late successional plant richness (coefficient of conservatism of five or greater) (B) among plots inoculated with the six different fungal communities and error bars are standard error. Plots inoculated with E. infrequens, C. lamellosum, C. claroideum, and A. spinosa are represented by E. inf, C. lam, C. cla and A. spi, respectively.*

We found that inoculation increased biomass of desirable native prairie plant species and decreased the abundance of non-native invasive plant species by an average of 300% relative to the non-inoculated controls (Fig. 22A, $F_{1,21}$=6.9, p=0.02). We found that native plant diversity was about 70% greater in AM fungal inoculated plots (Fig. 22B, $F_{1,21}$=8.9, p=0.007), although some fungal species increased diversity up to 300% more than others by the end of the second growing season ($F_{3,21}$=5, p=0.009).

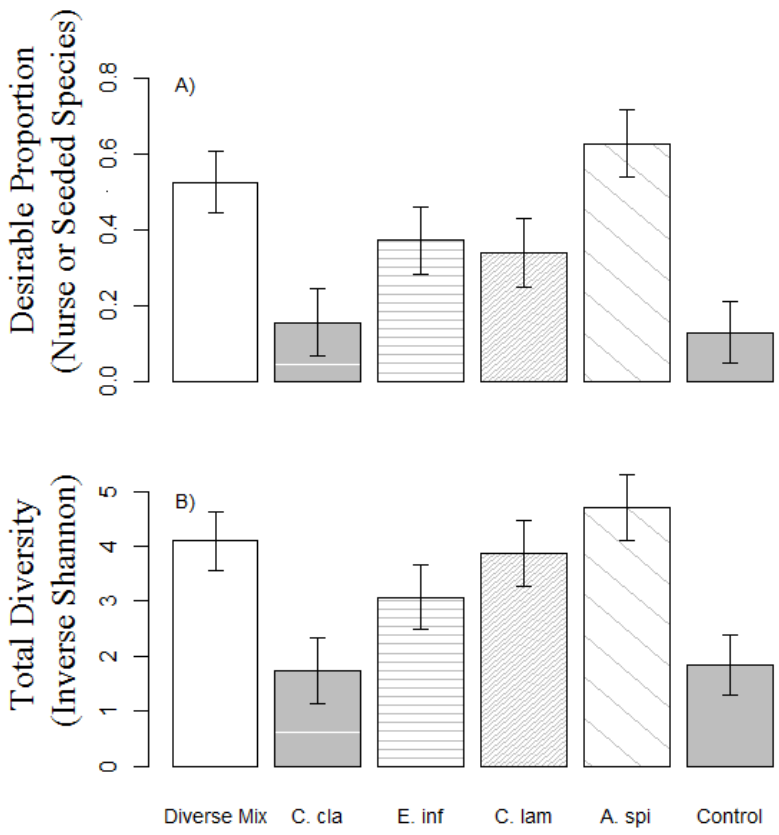


Figure 22. *Fungal composition greatly affected both the abundance of desirable species (A) and total plot diversity (B). Total diversity and desirable abundance were greatly improved with diverse AM fungal mixture and A. spinosa. Bars represent the average desirable proportion of plant biomass (A) and the average total diversity (B) among plots inoculated with the six different fungal communities and error bars are standard error. Plots inoculated with E. infrequens, C. lamellosum, C. claroideum, and A. spinosa are represented by E. inf, C. lam, C. cla and A. spi, respectively.*

DISCUSSION—FIELD INOCULATION STUDIES

Across five core inoculation experiments and three supplementary inoculation studies, we consistently found that inoculation with native AM fungi increases the survival and growth of native grassland plant species. This benefit held whether the AM fungi were isolated and cultured from native prairies or introduced through inoculation with fresh soil from native prairies. The benefit was robust across four states (Kansas, Illinois, Indiana, and Oklahoma) involving four independent isolations of native AM fungi and 15 different native plant species. The benefit held whether or not non-native invasive plants were initially knocked back. Moreover, we show that inoculation increased the competitive ability of the native plants against the non-native invasive plant species. While previous studies have shown the benefits of native AM fungi for native plant establishment (Bever et al. 2003, Middleton and Bever 2012, Middleton et al. 2015, Koziol and Bever 2016b), the benefits of inoculation with AM fungi was recently questioned (Hart et al. 2017). The present study work provides robust and definitive proof of the benefits of re-establishment of native AM fungi.

Moreover, our supplementary studies show clearly that the benefits of inoculation extends beyond the inoculated plants to neighboring desirable native seedlings. Inoculated plots had higher establishment and growth of native prairie plant seedlings species and as a result these plots had greater native plant species richness and greater native plant diversity. Inoculation with native AM fungi also suppressed undesirable plant species, including non-native invasive plant species.

AMF spread from points of inoculation

The documentation that AM fungi spread up to 2 meters within a year of inoculation is consistent with neighboring plants benefiting from inoculated nurse plants, as has been observed previously (Middleton and Bever 2012, Middleton et al 2015, Koziol and Bever 2016b), and as was shown in our supplemental inoculation studies. As AM fungal species have been shown to vary significantly in their rate of spread and also vary in their effect on native plant species (Koziol and Bever 2016a), neighboring plants may not receive the same benefits from inoculated AM fungi as nurse plants. Nevertheless, we repeatedly observed benefits to uninoculated neighboring plants.

The overall rates of spread varied between restorations. Factors that likely affect the rate of spread include the rate of establishment of plant species that are good hosts for AM fungi (Bever et al. 1996, Bever 2002). As non-native invasive plant species can be poor hosts for AM fungi (Vogelsang and Bever 2009), dominance of non-native invasive plant species could inhibit spread of AM fungi, as was observed in our supplementary inoculation study 1 (Fig. 16). This could also explain the limited spread of AM fungi in the Chanute inoculation study, where we observed poor establishment of native plants, perhaps because of flooding. The limited spread of beneficial AM fungi with dominance of non-native invasive plant species is consistent with a positive feedback that inhibits the restoration (Bever et al 2013).

RESULTS AND DISCUSSION—CLIMATE PERTURBATION EXPERIMENT

Results from Study 1 Our data show non-native warm- or cool-season grasses produced significantly greater vegetative and reproductive biomass, and increased germination when grown under elevated temperature and drought, compared to their paired native counterparts. Mycorrhizal root colonization of the non-native grasses was generally greater than native grasses regardless of soil moisture or elevated temperature. Our results suggest non-native grasses may increase in native grasslands under current global climate change scenarios. Results from Study 2 (feedback experiment) indicate that drier, warmer environments will decrease the likelihood that *B. ischaemum* will coexist with *S. scoparium*, as the soil microbial feedbacks are positive (indicating likely competitive exclusion) under warmer and drier climate, and negative (indicating stable coexistence of the native and invasive plant species) in the current environment.

This study illustrates that problems of non-native invasive plant species may increase with changes in climate. Climate perturbations may be particularly problematic because they alter plant-soil feedbacks in ways that disadvantage native plant species.

RESULTS AND DISCUSSION—LOCAL ADAPTATION EXPERIMENT

We found significant main effects of soil ($p < 0.0001$) and drought treatment ($p < 0.0001$) on the total aboveground productivity of our mesocosms. Fungi treatments did not have significant main effects or interact with drought treatments (Fig. 23). However, we did observe a soil × fungi interaction on plant productivity ($p = 0.0013$). Our follow-up tests for measures of local adaptation found significant positive effects, which were most pronounced in contrasts between soils from Oklahoma and our other two sites. Drought did not significantly affect these measures of local adaptation.

Our MANOVA results indicated that all main effects and interactions were significant in determining the plant species composition of our mesocosms, though our follow up tests indicate responses varied substantially between plant species.

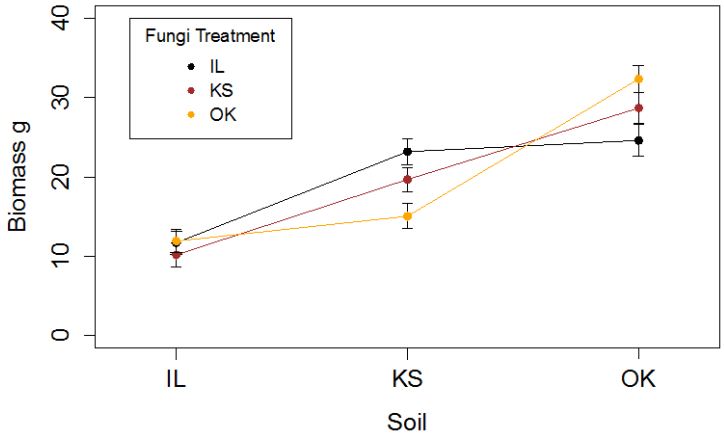


Figure 23. *Total productivity of our experimental mesocosms showed evidence that local adaptation of fungi to soil conditions increases the benefits provided to plant communities.*

Seven of our nine study species had significant, positive responses to the presence of mycorrhizae, while the weediest of the plant species *Elymus canadensis* ($p < 0.0001$) and *Panicum capillare* ($p = 0.1$) were negatively affected by mycorrhizae inoculation. Five of our study species responded negatively to our drought treatment, and four did not show a statistically significant response, although the above-ground biomass of all species except *Liatris aspera* was lower in the drought treatment. Despite significant effects of our drought and fungi treatments, we did not find that mycorrhizae reduced the effects of drought for any of our study species, even those that benefited strongly from mycorrhizae as compared to non-inoculated controls.

We found evidence that local adaptation of mycorrhizae to soils lead to greater benefits for two of our study species: *Panicum capillare* and *Panicum virgatum.* In contrast, three species that benefitted from the presence of mycorrhizae experienced reduced benefits of this symbiosis when fungi were in "home" soils (Fig 2). Drought modified the effects of local adaptation between fungi and soil for three of our study species. For *Panicum virgatum*, measures of local adaptation were stronger in our drought treatment, and for *Andropogon gerardii* and *S. scoparium* the negative effects of local adaption were reduced under drought conditions (Fig. 24).

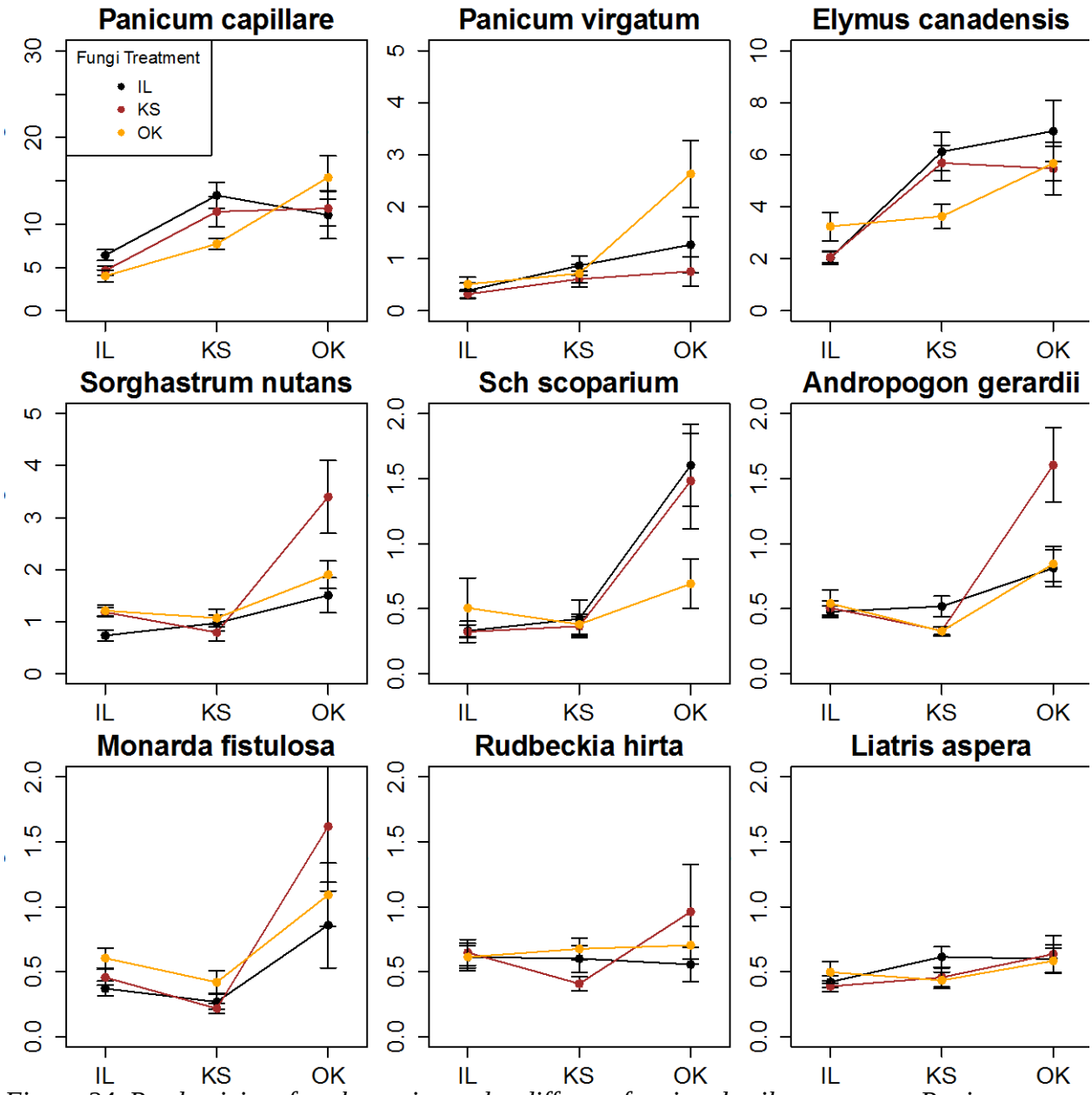


Figure 24. Productivity of each species under different fungi and soil treatments. Panicum capillare and P. virgatum each showed evidence of benefiting from local adaptation of fungi to soil conditions. However, negative effects were observed for S. nutans, S. scoparium, and A. gerardii.

Overall, we found support for the hypothesis that adaptation of arbuscular mycorrhizal fungi (AMF) to local soil conditions may increase the benefits these fungi deliver to plants. This effect was evident in measures of total plant community productivity. Unexpectedly, the presence of AMF did not appear to mitigate the negative effects of drought. Perhaps the ability of AM fungi to mitigate the effects of drought were constrained by the pot volume. Future work should test the importance of drought adapted AM fungi in environments more similar to field conditions.

Individual species showed surprising responses to local adaptation of AMF, with two plant species that are relatively less responsive to AMF showing benefits of associating with locally adapted fungi and plant species that are more responsive to AMF being negative affected by local adaptation of their fungal symbionts. This pattern likely emerged because high relative growth rates of non-responsive plant species allowed these ruderal species to dominate our experimental mesocosms, overwhelming the potential positive effects of local adaptation of fungi on the late successional plant species that are most reliant on these fungi (Koziol and Bever 2015). We might expect that the importance of local adaptation of AM fungi would increase during plant succession. Future work should test this possibility.

CONCLUSIONS AND IMPLICATIONS

Our work identifies AM fungi as keystone components of the plant microbiome and illustrates the potential value of reintroduction of native AM fungi as a grassland management strategy to facilitate recovery and control of non-native invasive plant species. Specifically, we use robust sampling of many independent land-use histories across broad spatial areas to prove that the composition of the AM fungal community is degraded by anthropogenic disturbance such as military training. We show through assays of more than a hundred plant species that non-native invasive plant species are generally less responsive to AM fungi than native plant species and that the very desirable late successional plant species are particularly responsive to native AM fungi. Through nine independent field inoculation studies, we show that reintroduction of native AM fungi accelerates grassland recovery by improving establishment and growth of highly desirable native plant species, and suppressing undesirable plant species including non-native invasive plant species. As the reintroduction of native AM fungi increases competitive ability of late successional long-lived native plant species, the implementation of this management approach could offer long-term solutions to recovery of grasslands from a history of disturbance and dominance by non-native invasive plant species.

Our work suggests that re-establishment of beneficial native AM fungi can establish a self-reinforcing positive feedback with desirable native plant species (Vogelsang and Bever 2009, Bever et al. 2012). We show that late successional native plant species benefit more from native AM fungi and that these plant species are also better hosts for these beneficial fungi. As a result, establishment of native late-successional plant-native AM fungal combinations within a patch creates benefits that can spread to neighboring areas (Molofsky et al. 2001, Molofsky and Bever 2002, Abbott et al. 2016).

This positive feedback dynamic has important implications for grassland management. Establishment of patches of late successional plant species with native AM fungi can reduce future management costs by limiting non-native invasive plant species. Once established, the suppressive effects can spread to neighboring areas as AM fungi spread and late successional native grassland species establish (Middleton et al. 2010). These long-term benefits may make inoculation with native AM fungi a cost-effective management strategy for areas that are not undergoing repeated physical disturbance. Moreover, this work suggests that in areas such as Fort Riley, where disturbance from military training occurs within expanses of late successional native grassland, management approaches could focus on spread of native AM fungi from undisturbed areas.

The long-term benefits of inoculation with native AM fungi will depend upon the rate of spread of native AM fungi after inoculation. Our work identifies that this spread is context-dependent. In particular, we find that the spread may be most rapid following initial knocking back of non-native invasive plant species. In our studies, non-native invasive plant species were inhibited through a variety of management approaches, including solarization via tarps, tillage, and herbicide. It is possible that appropriately timed burns would also be sufficient. Further work is required to robustly test these management approaches along with the context dependence and rate of spread of native AM fungi from areas of establishment.

Re-establishment of native AM fungi should have the additional benefits of improved aggregate stability along with the improved soil carbon sequestration (Duchicela et al. 2012,

Wilson et al. 2010). While individual studies found improvement in soil aggregate stability due to establishment of native AM fungi, we did not find these effects consistently across all studies. It is quite possible that these benefits will become stronger over time. Further work should follow up on these inoculation studies to document the long-term benefits of reintroduction of native AM fungi.

The example of reintroduction of native AM fungi illustrates the value of a holistic view of plant communities and restoration practices that embrace the intricacies and dynamics of native microbial communities. The implementation of the inoculation approach on a large scale would require investment in the development of native AM fungal cultures from native grasslands across the US. It is also possible that additional benefits could be gained from re-introduction of other components of the native plant microbiome such as rhizobia.

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APPENDIX A. SUPPLEMENTARY METHODS AND RESULTS

APPENDIX A1. METHODS OF ENVIRONMENTAL SEQUENCING, PLFA ANALYSIS AND MEASUREMENT OF AGGREGATES STABILITY

DNA Extraction, Sequencing and Processing

Roots from each sample were finely chopped on disposable weighing paper using forceps and scissors that were ethanol treated and flamed between samples to minimize cross-contamination; 35 mg of chopped roots were used for DNA extraction (PowerSoil kit, Qiagen Carlsbad, CA). A roughly 850 bp portion of the nuclear large subunit ribosomal RNA (rRNA) gene was amplified from the DNA extractions using PCR with forward primer LROR (Bunyard et al. 1994) and barcoded reverse primer FLR2 (Trouvelot et al. 1999). PCR amplification proceeded as follows: 94°C for 5 min, then 35 cycles of: 1) 94°C for 30 sec, 2) 48°C for 30 sec, and 3) 72°C for 45 sec; ending with 72°C for 10 min. PCR products were purified (AMPure XP Beckman Coulter, Indianapolis, IN) and the resulting concentration was quantified using PicoGreen (Thermo Fisher Scientific, Waltham, MA). An equimolar amount of PCR product from each sample was pooled and sequenced using Illumina MiSeq (Center for Genomics and Bioinformatics, Indiana University) to produce paired 300 bp non-overlapping reads. We used this approach to generate paired sequences that covered portions of both the phylogenetically informative D1 and D2 variable regions of the large subunit rRNA gene (Hassouna et al. 1984).

Raw sequences were truncated to only retain bases with quality scores of at least 10 and were screened allowing a maximum of one expected error (Edgar and Flyvbjerg 2015) per read pair using a custom Python script. To keep roughly equal numbers of forward and reverse reads after standardizing their lengths, all forward reads were truncated to 235 bp and all reverse reads to 165 bp. Reads with shorter lengths were discarded and any previously paired reads where only one read remained after length trimming were removed. Remaining read pairs were then concatenated after taking the reverse complement of the reverse read, and identical sequences were de-replicated before identifying chimeric sequences using the –uchime_denovo function of VSEARCH (Rognes et al. 2016). Chimeric sequences were removed and the remaining concatenated read pairs were re-replicated before clustering.

Concatenated read pairs were clustered into OTUs with AbundantOTU (Ye 2010) using a 97% sequence similarity threshold; this method performs well for AM fungi although no clustering method can generate OTUs that consistently match morphologically defined AM fungal species (House et al. 2016). The consensus sequence representing each OTU was added to a reference alignment of AM fungal sequences (House et al. 2016) using MAFFT (Katoh and Standley 2013), and the gap in the aligned consensus sequences that represented the non-overlapping forward and reverse reads was manually deleted. Consensus sequences that aligned poorly with the reference sequences were removed before creating a rooted phylogeny containing both the consensus and reference sequences using RAxML (Stamatakis 2014) with the GTR-GAMMA model and using *Mortierella elongata* as an outgroup. Consensus sequences falling outside the AM fungal clade in the phylogeny were considered non-AM fungal and were discarded, leaving 199 AM fungal OTUs representing 499,507 sequences. Another rooted phylogeny was created using only the consensus sequences of these AM fungal OTUs with the same reference sequences as above, and was used to attribute each OTU to a taxonomic group using its position in the phylogeny (Appendix S1: Fig. S1). Most OTUs were identifiable to the genus level except 57 OTUs that could only be assigned to the family Glomeraceae (See Data S1 for OTU consensus sequences). After quality screening, the number of sequences per sample varied by more than an order of magnitude (min = 451; max = 26,254; median = 3,571) and we accounted for this in various ways during our analysis, as described below.

Quantification of Soil Microbial Communities:

Relative abundances of soil microbial functional groups (gram- positive and negative bacteria, AM and saprophytic fungi), and total microbial biomass were assessed using phospholipid fatty acid (PLFA) and neutral lipid fatty acid (NLFA). PLFAs are constituents of biological membranes that can be used to estimate the biomass of fungi, because biovolume and cell surface area are well correlated (Tunlid & White 1992). The NLFAs are the basic storage product of many fungi and serve as the primary energy reserve in fungi (Larsen & Bødker 2001). PLFA and NLFA were extracted from rhizosphere soil collected from each plot using a modification of White and Ringelberg (1998). Total lipid extracts were separated into PLFA's and NLFA's using silicic acid chromatography; the fatty acids cleaved from the glycerol backbone using KOH saponification; and the harvested fatty acids methylated to form fatty acid methyl esters (FAME) (Allison and Miller, 2005). The FAME's were then analyzed by gas chromatography and mass selection detection using a GCMS unit Agilent MS 5975C/GC 7890A. Biomarkers used to select for the functional group of gram-positive bacteria consisted of i-15:0, a-15:0, i-17:0, and i-16:0. For gram-negative bacteria, selected biomarkers were 16:1ω7, cy19:0, cy17:0ω9, 2-OH 14:0, 2-OH 16:0, 3-OH 14:0, and 18:1ω9 trans. For AM fungal biomass, biomarkers consisted of 16:1ω5c, 20:1ω9, and 22:1ω13. Biomarkers selected for the functional group of saprophytic fungi were 18:2ω9,12 and 18:1ω9c. The abundances associated with these biomarkers were used to calculate a total nmol per gram of soil for each functional group and for total microbial biomass when all functional groups were added with non-specific markers (14:0, 15:0, 16:0, 17:0, 18:0, and 20:0).

Aggregate size distribution:

All soil samples were pre-sieved (6 mm diameter) prior to wet-sieving to remove stones and coarse organic matter. Water-stable aggregates (WSA) were separated using an instrument similar in principle to a Yoder wet-sieving apparatus. The apparatus was modified to handle stacked sieves (12.7 cm diameter) and to allow complete recovery of all particle fractions from individual samples. Four aggregate size classes were collected from each sample (>2000, 250-2000, 53-250, and 20-53 μm diameter). The 20-53 and 53-250 μm size fractions were defined as microaggregates, while the 250-2000 and >2000 μm size fractions were designated as macroaggregates. Following wet-sieving action, material remaining on each sieve was collected and dried at 50 °C for 24 hours prior to weighing. Sand-free WSA was measured using a subsample of intact aggregates (2-5 g) and combined with fivefold volume (10-25 mL) of 5 g L^{-1} sodium hexametaphosphate for approximately 16 hours, then shaken on an orbital shaker at 350 RPM for 4 hours. The dispersed organic matter and sand were collected on a 53 μm mesh sieve, washed with distilled water, and dried at 105 C for 24 hours. The aggregate weights were then recorded for estimating sand-free correction.

Appendix A2 Table of Mycorrhizal Responsiveness of native and non-native invasive plant species

Table of plant species dependence on native AM fungi. Here we highlight the percent prairie plants and restoration colonizers were improved or inhibited by prairie mycorrhizae relative to non-inoculated plants based on total plant weight (Myc Resp). We color code each plant species based on its conservation coefficient (CC) value. BLUE indicates species with of CC 7-10. YELLOW indicates species with moderate coefficient of conservation with CC 3-5. RED indicates non-native species. BLACK are species with low conservation concern with CC 1-2. Generally, we found that later successional species are strongly responsive to prairie mycorrhizae, whereas non-native species and species of low conservation value tend to be less responsive to mycorrhizae.

Genus	Species	Native Status	Common Name	Myc Resp
Rumex	patientia	non-native	patience dock	-16
Silphium	laciniatum	native	compass plant	-8
Poa	pratensis	non-native	kentucky blue grass	-8
Polygonum	lapathifolium	native	heartsease	-7
Hordeum	pusillum	native	little barley	-7
Koeleria	macrantha	native	june grass	-7
Erigeron	annuus	native	annual fleabane	-6
Hordium	jubatum	native	fox-tail barley	-4
Geum	triflorum	native	prairie smoke	-3
Plantago	lanceolata	non-native	english plantain	-2
Bromus	japonicus	non-native	japanese chess	-2
Liatris	aspera	native	button gayfeather	0
Dactylis	glomerata	non-native	orchard grass	0
Amaranthus	spinosus	non-native	thorny amaranth	1
Helianthus	annuus	native	common sunflower	1
Vernonia	fasciculata	native	common ironweed	2
Bromus	tectorum	non-native	downy chess; downy brome	2
Pediomelum	esculenta	native	bread-root scruf-pea	2
Agropyron	elongatum	non-native	tall wheat grass	2
Capsella	bursa-pastoris	non-native	shepherds purse	4
Erigeron	strigosus	native	daisy fleabane	5
Agropyron	smithii	non-native	western wheat grass	5
Viola	rafinesquii			6
Setaria	glauca	non-native	yellow foxtail	6
Agrostis	stolonifera	non-native	bent grass	6
Agropyron	cristatum	non-native	crested wheat grass	7
Erechtites	hieraciifolius	native	american burnweed	8
Lolium	perenne	non-native	perennial rye grass	8
Setaria	viridis	non-native	green foxtail	9
Kuhnia	eupatorioides	native	false boneset	11
Achillea	millefolium	native	western yarrow	12
Cynodon	dactylon	non-native	bermuda grass	12
Setaria	faberi	non-native	giant foxtail	13
Digitaria	sanguinalis	non-native	hairy crab grass	13
Penstemon	digitalis	native	foxglove beard tongue	14
Elymus	canadensis	native	canada wild rye	15
Daucus	carota	non-native	queen annes lace	15
Tradescantia	ohiensis	native	common spiderwort	16
Baptisia	alba	native	white wild indigo	17
Solidago	rigida	native	stiff goldenrod	19
Leymus	cinereus	non-native	basin wildrye	20
Oenothera	biennis	native	common evening primrose	20
Mimosa	quadrivalvis	native		25
Artemisia	ludoviciana	native	white sage	25
Rumex	obtusifolius	non-native	bitter dock	26
Bromus	inermis	non-native	smooth brome	26
Linum	sulcatum	native	wild flax	28
Panicum	capillare	native	old witch grass	29
Dichanthelium	clandestinum	native	dear-tongue dichanthelium	31
Monarda	fistulosa	native	wild bergamot	31
Carex	scoparia	native	lance-fruited oval sedge	34
Schedonorus	arundinaceus	non-native	tall mountain-fescue	35

Genus	Species	Native Status	Common Name	Myc Resp
Verbena	hastata	native	simplers-joy; blue vervain	36
Persicaria	pennsylvanicum	native	pennsylvania smartweed	38
Carex	vulpinoidea	native	common fox sedge	38
Carex	tribuloides	native	awl-fruited oval sedge	39
Asclepias	syriaca	native	common milkweed	40
Aster	ericoides	native	heath aster	44
Asclepias	incarnata	native	swamp milkweed	45
Eragrostis	spectabilis	native	purple love grass	46
Bouteloua	gracilis	native	blue grama	49
Apocynum	cannabinum	native	indian hemp	51
Tripsacum	dactyloides	native	eastern mock grama;	52
Lotus	corniculatus	non-native	birds foot trefoil	54
Desmodium	canedense	native	canadian tick clover	58
Oxlis	stricta	native	yellow woodsorrell	59
Symphyotrichum	sericeus	native	silky aster	64
Bidens	vulgata		tall beggar ticks	64
Rudbeckia	subtomentosa	native	sweet coneflower	65
Pycnanthemum	tenuifolium	native	slender mountain mint	66
Salvia	azurea	native	blue sage	66
Rudbeckia	hirta	native	black-eyed susan	70
Eragrostis	curvula	non-native	weeping lovegrass	74
Baptisia	bracteata	native	cream wild indigo	77
Verbena	stricta	native	hoary vervain	78
Astragalus	crassicarpus	native	large ground plum	79
Desmodium	sessillfolium	native	sessile leaf tickclover	81
Buchloe	dactyloides	native	buffalo grass	82
Baptisia	australis	native	blue wild indigo	84
Allium	canadensis	native	canadian onion	85
Liatris	pycnostachya	native	prairie blazing star	85
Yucca	glauca	native	small soapweed	89
Abutilon	theophrasti	non-native	velvetleaf	89
Chenopodium	alba	native	lamb's quarts goosefoot	90
Chamaecrista	fasciculata	native	partridge pea	91
Oenothera	speciosa	native	showy evening primrose	92
Conyza	canadensis	native	tall horseweed	95
Ambrosia	artemisiifolia	native	annual ragweed	96
Echinacea	angustifolia	native	black-sampson coneflower	99
Desmodium	illinoense	native	illinois tick trefoil	102
Sporobolus	airoides	native	alkali sacaton	105
Parthenium	integrifolium	native	wild quinine	106
Senecio	plattensis	native	prairie ragwort	106
Baptisia	leucophaea	native	cream wild indigo	108
Cacalia	atriplicifolia	native	pale indian plantain	112
Zizia	aurea	native	golden alexanders	115
Helianthus	maximilianii	native	maximilian sunflower	115
Solidago	nemoralis	native	old-field goldenrod	116
Panicum	virgatum	native	switch grass	119
Cirsium	vulgare	non-native	bull thistle	119
Solidago	riddllii	native	riddells goldenrod	120
Aster	novae	native	new england aster	121
Baptisia	lactea			123
Solidago	canadensis	native	canada goldenrod	123
Melilotus	officinalis	non-native	yellow sweet clover	123

Genus	Species	Native Status	Common Name	Myc Resp
Helianthus	grosseseratus	native	saw-tooth sunflower	126
Baptisia	leucantha	native	white wild indigo	126
Veronicastrum	virginicum	native	culvers root	133
Heliopsis	helianthoides	native	false sunflower	134
Euphorbia	corollata	native	flowering spurge	134
Eupatorium	altissimum	native	tall boneset	136
Asclepias	verticillata	native	whorled milkweed	142
Dalia	candida	native	white prairie clover	142
Dalea	purpurea	native	purple prairie clover	144
Koeleria	cristata	native	june grass	146
Asclepias	viridis	native	spider milkweed	150
Bouteloua	curtipendula	native	side-oats grama	150
Aster	laevis	native	smooth blue aster	153
Hieracium	longipilum	native	long-bearded hawkweed	155
Rumex	crispus	non-native	curly dock	161
Tridens	flavus	native	false redtop	164
Asclepias	tuberosa	native	butterfly milkweed	167
Vernonia	altissima	native	smooth tall ironweed	170
Allium	stellatum	native	summer pink onion	171
Coreopsis	palmata	native	prairie coreopsis	176
Ratibita	columnifera	native	gray-head prairie-coneflower	179
Lespedeza	cuneata	non-native	silky bush clover	188
Andropogon	virginicus	native	broom-sedge; broom sedge	190
Liatris	spicata	native	marsh blazing star	196
Pycnanthemum	virginianum	native	common mountain mint	200
Helianthus	occidentalis	native	western sunflower	201
Lespedeza	capitata	native	round-headed bush clover	203
Bothriochloa	bladhii	non-native	caucasian bluestem	205
Desmanthus	illinoensis	native	Illinois bundle-flower	211
Andropogon	scoparius	native	little bluestem grass	222
Silphium	terebinthinaceum	native	prairie dock	226
Asclepias	speciosa	native	showy milkweed	230
Sporobolus	heterolepis	native	prairie dropseed	249
Silphium	integrifolium	native	entire-leaf rosinweed; rosin weed	250
Schizachyrium	scoparium	native	little bluestem grass	266
Asclepias	meadeii	native	Mead's milkweed	276
Ratibia	pinnata	native	upright prairie-coneflower	335
Lobelia	cardinalis	native	cardinal flower	336
Echinacea	purpurea	native	purple coneflower	369
Sorgastrum	nutans	native	yellow indian grass	447
Asclepias	asperula	native	antelope-horn milkweed	556
Andropogon	gerardii	native	big bluestem	584
Echinacea	pallida	native	pale purple coneflower	648
Eryngium	yuccifolium	native	rattlesnake master	745
Amorpha	canescens	native	lead plant	1232
Allium	cernuum	native	nodding wild onion	1334
Coreopsis	tripteris	native	tall coreopsis	1637

Appendix B. List of Scientific/Technical Publications

Avolio, M.L., S. E. Koerner, K.J. La Pierre, K.R. Wilcox, G.W.T. Wilson, M.D. Smith and S.L. Collins. 2014. Changes in plant community composition, not diversity, during a decade of nitrogen and phosphorus additions drive aboveground productivity in a tallgrass prairie. Journal of Ecology. 102: 1649-1660.

Bauer, JD, E Koziol, JD Bever. 2018. Late successional plant species as conservation priorities. *AoB Plants*. In press.

Bauer, JT, KML Mack, and JD Bever. 2015. Plant-soil feedbacks as drivers of succession: Evidence from remnant and restored tallgrass prairies. *Ecosphere. (9):158.* *http://dx.doi.org/10.1890/ES14-00480.1*

Duell, E.B., G.W.T. Wilson, and K.R. Hickman. 2016. Above- and belowground responses of native and invasive prairie grasses to future climate scenarios. *Botany*. 94:471-479.

Greer, M.J. and Gail W. T. Wilson. 2014. Restoration ecology: Introduction in a "Timely" manner. *Ecology 101: Bulletin of the Ecological Society of America* 95:274–280.

Greer, M.J., G.W.T. Wilson, K.R. Hickman, and S. Wilson. 2014. Experimental evidence that invasive grasses use allelopathic biochemicals as a potential mechanism for invasion: Chemical warfare in nature. *Plant and Soil*. 385:165-179.

House, Geoffrey L. and J. D. Bever. 2018. Biochar soil amendments in prairie restorations do not interfere with the benefits provided by arbuscular mycorrhizal fungi. *Restoration Ecology*. Submitted.

House, Geoffrey L. and J. D. Bever. 2018. Patterns of arbuscular mycorrhizal fungal community composition in grasslands across a precipitation gradient and their sensitivity to disturbance. *Ecological Applications*. In Press.

House, Geoffrey L., Saliya Ekanayake, Yang Ruan, Ursel Schütte, Wittaya Kaonongbua, Geoffrey Fox, Yuzhen Ye, James D. Bever. 2016. Sequence variation in the nuclear ribosomal RNA gene within isolates of arbuscular mycorrhizal fungi: Tests of phylogeny and clustering methodologies. *Applied and Environmental Microbiology*. 82:16 4921-4930.

Johnson, N.C., G.W.T. Wilson, J.A. Wilson, R.M. Miller, and M. Bowker. 2015. Mycorrhizal phenotypes and the law of the minimum. *New Phytologist*. 205: 1473-1484.

Johnson, N.C., R.M. Miller, and G.W.T. Wilson. 2017. Mycorrhizal interactions with climate, soil parent material, and topography. Pp 47-61. *In*: Johnson, N.C., C. Gehring, and J. Jansa (eds.). *Mycorrhizal Mediation of Soil*. Elsevier Inc. Cambridge, MA, USA.

Koziol, E and JD Bever. 2015. Mycorrhizal response trades off with plant growth rate and increases with plant successional status. *Ecology*. 96:1478–1484.

Koziol, L and JD Bever. 2016. The missing link in grassland restoration: arbuscular mycorrhizal fungi inoculation increases plant diversity and accelerates succession. *J Applied Ecology*.

10.1111/1365-2664.12843

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