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PLANT MICROBE INTERACTIONS

Divergence in Diversity and Composition of Root-Associated Fungi Between Greenhouse and Field Studies in a Semiarid Grassland

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Abstract

Investigations of plant-soil feedbacks (PSF) and plant-microbe interactions often rely exclusively on greenhouse experiments, yet we have little understanding of how, and when, results can be extrapolated to explain phenomena in nature. A systematic comparison of microbial communities using the same host species across study environments can inform the generalizability of such experiments. We used Illumina MiSeq sequencing to characterize the root-associated fungi of two foundation grasses from a greenhouse PSF experiment, a field PSF experiment, field monoculture stands, and naturally occurring resident plants in the field. A core community consisting < 10% of total fungal OTU richness but > 50% of total sequence abundance occurred in plants from all study types, demonstrating the ability of field and greenhouse experiments to capture the dominant component of natural communities. Fungal communities were plant species-specific across the study types, with the core community showing stronger host specificity than peripheral taxa. Roots from the greenhouse and field PSF experiments had lower among sample variability in community composition and higher diversity than those from naturally occurring, or planted monoculture plants from the field. Core and total fungal composition differed substantially across study types, and dissimilarity between fungal communities did not predict plant-soil feedbacks measured in experiments. These results suggest that rhizobiome assembly mechanisms in nature differ from the dynamics of short-term, inoculation studies. Our results validate the efficacy of common PSF experiment designs to test soil inoculum effects, and highlight the challenges of scaling the underlying microbial mechanisms of plant responses from whole-community inoculation experiments to natural ecosystems.

Keywords Rhizobiome · Semiarid grassland · Plant-soil feedback · Mycobiome · Mycorrhiza · Community composition

Introduction

Interactions among plants, the soil, and root-associated microbes have gained recognition as important drivers of aboveground plant population and community dynamics [1, 2]. In particular, plant-soil feedbacks (PSF), in which plants cultivate species-specific soil microbiota that then differentially alter the population growth of co-occurring plant species [3], have been shown to promote coexistence, alter succession, and correlate with plant species' relative abundances [1, 4–8]. These advances have largely been driven by greenhouse experiments, which cultivate plants in soils with microbial communities that are conditioned by the same plant species (conspecific) or different plant species (heterospecific) [9]. However, while ample evidence of PSFs comes from greenhouse experiments, and less from field mesocosms [10, 11], few studies have tested PSF under natural field conditions [12]. The studies to date that investigated PSFs for the same plants in the greenhouse and under field conditions have yielded idiosyncratic results [6, 13–15]. For example, a study of three grass species in a temperate grassland revealed that PSFs in the field were often opposite in direction to PSF in the greenhouse [15], whereas greenhouse and field-measured PSFs matched in a study of six tree species from a tropical rainforest [6]. Regardless of whether plant responses are the

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same between greenhouse and field experiments, the key to generalizing greenhouse results is to identify the underlying microbial drivers and compare microbial community composition in different study conditions.

Study conditions may drive divergent microbial community assembly on plant hosts, and thus result in alternative PSF outcomes in several ways. First, different abiotic filters between the greenhouse and field environments could alter starting microbial species pools. For example, different precipitation regimes are associated with the proliferation of alternate subsets of the soil fungal community [16], which would result in diverging fungal communities under different water conditions. Other studies have shown that the same host plant species can associate with distinct arbuscular mycorrhizal fungal communities in the greenhouse versus the field, even given the same starting microbial species pool [17–20]. Second, field and greenhouse PSF studies likely differ in their methods to manipulate soil microbes, for logistical reasons. For example, experiments may grow target plants for two phases to generate species-specific soils, or collect soils from host plants in the field as inoculum [21]. Different methods of "inoculation" may vary in efficacy and produce different starting microbial communities [22]. Finally, plant individuals in the field are likely to encompass wider among individual genetic and morphological variation than plants in greenhouse experiments. For example, naturally occurring plants could differ in age and reflect age-related shifts in microbial community composition [23] or in their ability to withstand pathogens or reward mutualists [24, 25]. Therefore, knowledge of the abiotic and biotic contexts in which PSFs are investigated and their effects on plant-microbe associations can help us understand potentially different study outcomes.

To address these pitfalls requires a systematic comparison of similarities and differences in the root-associated rhizosphere microbial communities on the same plant species across study methods and experiment durations. Our prior work investigating PSFs between two desert grasses provides a unique opportunity to conduct such a comparison. Here, we used Illumina MiSeq sequencing of the ITS2 region to characterize the root-associated fungi of *Bouteloua gracilis* (blue grama) and *B. eriopoda* (black grama). These are two dominant grasses that co-occur across large regions of the Southwestern United States, whose dynamics are crucial to understanding dry grassland communities. We compared root samples from a series of studies including a greenhouse PSF experiment, a field PSF experiment, field monocultures planted 7 years earlier, and naturally occurring plants of indeterminate age. We asked the following: (1) How does root-associated fungal diversity, composition, and host-specificity compare among studies? We expected that plants from greenhouse and field PSF experiments would have fungal consortia that were subsets of communities used in inoculation, and that plants in the field would have greater variation among individuals in their root fungal composition. (2) How does soil inoculation alter fungal composition in greenhouse versus field PSF experiments? The most common PSF experiments inoculate plants with soils conditioned by the same species (conspecifics) or a different species (heterospecifics); thus, we replicated these commonly used treatments under both greenhouse and field conditions. We expected soil inoculations to alter fungal composition more strongly in the greenhouse than in the field, due to shorter duration experiments and more controlled conditions, which reduce stochasticity. (3) Does the diversity or composition of root-associated fungi vary with long-term stability of the dominant plant species? PSF experiments typically capture one snapshot in time of the soil microbial community, usually an early life history stage of the plant. Long-term host plant dynamics, such as variation in the stability of plant community composition, provide an important context for inferring plant age structure. We expected that plants in patches with low community stability/high plant species turnover have younger plants, more diverse fungal communities, and greater variability in composition among individual plants than sites with low plant species turnover, where resident plants linger for long time periods. Finally, (4) How well do differences in microbial community composition predict the outcome of plant-soil feedbacks for plants? We predicted that plant host individuals associated with the most unique microbial communities would show stronger feedbacks, owing to a higher probability of escaping specialist pathogens or losing specialist mutualists in heterospecific soils.

Methods

Study Sites and Experiments

All studies were affiliated with northern Chihuahuan Desert grassland communities at the Sevilleta National Wildlife Refuge (SNWR; 34.3591, − 106.688), NM, USA. The mean annual temperature is 13.2 °C and mean annual precipitation is ~ 250 mm. Past and ongoing studies as a part of the Sevilleta Long-Term Ecological Research (LTER) program provided us with a range of temporal and spatial scales to investigate the root-associated fungal communities of two congeneric grasses, *Bouteloua gracilis* (blue grama) and *B. eriopoda* (black grama), that dominate the plant community (accounting for > 60% of total aboveground net primary production). Blue grama is a caespitose grass that forms rings [26], whereas black grama vegetatively expands via stolons and occasionally establishes by seed [27]. Here, we utilized four study types, briefly described below. Figure 1 and Table S1 summarize the relationships among the four study types, their experimental design, and sample sizes.

Field Monocultures

Field monoculture plots were a part of grass competition and diversity experiment at the SNWR initiated by S. L. Collins in

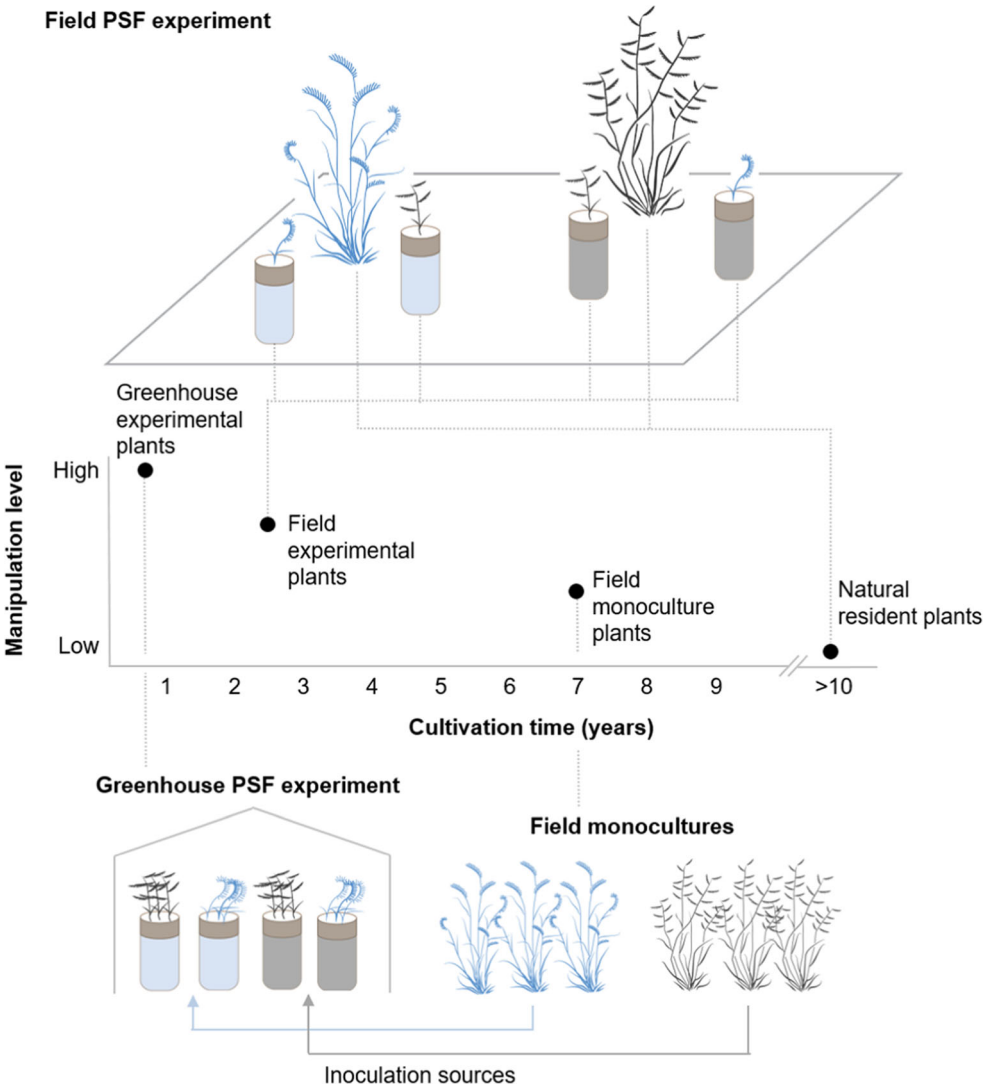

Fig. 1 Sampling design. Root samples came from four study types that differed in their level of manipulation and length of cultivation. In this figure, blue grama plants and soils are colored blue, black grama plants and soils are colored in grayscale

2005. Plants were established via seeding from southwestern US regional seed stock (Curtis and Curtis, Clovis, NM; Western Native Seed, Coaldale, CO) at a rate of 90.1 kg/ha. Monoculture treatments, with five replicate plots per plant species (blue grama, black grama) were maintained by weeding thrice each year (see http://sev.lternet.edu/data/sev-174 for more information). In October 2012, we collected root fragments from one plant growing in each monoculture plot; these were stored at − 80 °C (monoculture plants). Individual root samples were individual plants. At the same time, we also collected soil inocula for the greenhouse PSF experiment (described next).

Greenhouse PSF Experiment

A greenhouse experiment was conducted during 2012–2013 to examine the effects of PSF and the presence of surface biological soil crust (biocrust) on competition and coexistence between blue and black grama (detailed methods in [8]). We used rhizosphere soils from the field monocultures (sampled from the same plants described above), as well as field-collected biocrusts to inoculate plants in the greenhouse in a 2 × 2 × 2 factorial design. Biocrust addition had little effect on root-associated fungi; thus, we focused on rhizosphere soil inoculation and host plant effects. From the greenhouse, we collected roots from the highest density monoculture pots to mimic field monoculture conditions in May 2013, and stored them at − 80 °C until processing (greenhouse plants). Root samples were homogenized from four to six plants per 8 cm × 8 cm × 20 cm deep pot because it was not possible to separate roots of different individuals within a pot.

Field Residents in High- and Low-Stability Plant Communities

Sevilleta LTER has monitored vegetation dynamics biannually along line-intercept transects since 1989 (see http://sev.lternet.edu/data/sev-4, [28] for more information). Using

these long-term data, we identified 20 patches (4 m long) with persistent differences in historical plant community stability (pattern described in [28]). Briefly, "dynamic" patches (n = 10) showed frequent changes in species dominance along with large magnitudes of changes in blue grama and black grama relative abundances in the 25 year record, whereas "static" patches showed little change in species relative abundances through time (n = 10). In August 2015, roots were collected from one naturally occurring resident plant of each *Bouteloua* species within each patch type (static or dynamic), and stored at − 80 °C until processing (field residents, Fig. 1). Individual root samples were individual plants.

Field PSF Experiment in High- and Low-Stability Plant Communities

A field experiment was conducted at the Sevilleta LTER transect site in 2014–2016 to examine the relationship between temporal plant community stability and PSF (Chung et al. in prep). In this field PSF experiment, we experimentally transplanted seedlings around naturally occurring, resident blue or black grama plants (the same individuals as "field residents" above), planting into areas adjacent to the dynamic/static sections of the transect (Fig. 1). Transplant seedlings were grown in the greenhouse in sterilized sand for 3 months, then planted into the field in July 2014, using 30-μm mesh cylinders to prevent root competition. To test PSF, transplant seedlings were established near resident plants of the same plant species (conspecific) or the other species (heterospecific) to facilitate microbial colonization from conspecific or heterospecific rhizospheres. In July 2016, surviving transplants were harvested (total 30 plants), and root samples stored at − 80 °C until processing (field transplants). Individual root samples were individual plants.

DNA Extraction and Sequencing

Genomic DNA was extracted from 0.2-g ground root tissue (or less when limited by sample size) from all samples in September 2016 using MOBIO power soil DNA extraction kits following manufacturer's protocol. The extracts were quantitated using NanoDrop and standardized to 2 ng/μl for PCR-amplification. We targeted the ITS2 region using forward primer fITS7 [29] and reverse primer ITS4 [30] as it has been shown to better reflect diversity and community composition, and has better representation in databases than the ITS1 region [31]. While ITS2 region primers can under-sample some fungal groups such as the arbuscular mycorrhizal fungi (AMF), past microscopy and AMF-specific work in this ecosystem has shown that AMF are a small component of the rhizobiome [32]. We amplified template DNAs from all samples in three technical primary PCR replicates, using a primary PCR to amplify the targeted region and a secondary PCR to anneal molecular identifiers (MID tags) [33]. Primary PCRs used 10 μl template DNA (20 ng), 5 μl dNTPs (0.2 mM final concentration), 5 μl each of forward and reverse primers (1 μM final concentration), 10 μl 5× Phusion Green HF Buffer containing 7.5 mM $MgCl_2$, 14.5 μl molecular grade water, and 0.5 μl Phusion Green Hot Start II High-Fidelity DNA polymerase in 50 μl reactions. We included one positive (*Saccharomyces cerevisiae*) and negative (molecular grade water) control that were processed similarly to the genomic DNA samples. PCR cycles included an initial denaturing at 98 °C for 30 s, 30 cycles of 94 °C for 10 s, 52 °C for 10 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min.

For each sample, 40 μl of amplicons from each technical replicate of the primary PCRs were pooled and purified using the Agencourt AmPure XP magnetic 96-well SPRIplate system (Beckman Coulter, Indianapolis, IN, USA) following the manufacturer's protocol with 1:1 AmPure XP solution to amplicon ratio, and one additional ethanol cleaning step. A secondary PCR was performed on the purified amplicons (10 μl as template) using fITS7 and ITS4 primers with sample-specific 12 bp molecular identifier (MID) tags for 5 cycles identical to the primary PCR. Secondary PCR products were purified again as described above, and DNA quantitated using NanoDrop. The cleaned amplicons were pooled into a single library at equal DNA amounts, and the library sequenced at the Integrated Genomics Facility at Kansas State University (Manhattan, KS, USA) as 2 × 300 bp reads on 2/3 of a flow cell using Illumina MiSeq v3. Raw sequence data are archived at the Genome Short Read Archive (SRA) at the National Center for Biotechnology Information (NCBI) (BioProject PRJNA434727 and BioSample SAMN08568347).

Bioinformatics

Sequence data were contiged, quality-filtered, trimmed to equal length, de-replicated, checked for chimeric sequences, and clustered into operational taxonomic units (OTUs) using the mothur pipeline [34]. In brief, contigs that were shorter than 250 bp, contained any ambiguous bases, or homopolymers longer than six bases were discarded. This removed ~ 40% of total sequences. Remaining high-quality sequences were then trimmed to the minimum length of 250 bp to facilitate clustering without alignment. Sequences were preclustered [35] and putatively chimeric sequences were identified using chimera.uchime function with default settings [36], and removed. Sequences were clustered into OTUs using the nearest neighbor method at 97% similarity. OTUs with < 10 sequences were discarded [37], resulting in a data matrix with 2.85 million sequences and 1559 OTUs. OTUs were classified against the UNITE fungal ITS database (November 2016 release [38]). We then kept only

OTUs with taxonomy assigned to fungal phyla with ≥97% confidence (1123 OTUs). Contaminant OTUs in negative controls (15 OTUs) were removed from the dataset by subtracting the number of sequences in the negative controls from all samples. Control samples were not otherwise included in analyses. The final fungal OTU dataset included 2.55 million sequences and 1121 OTUs dominated by the Ascomycota (57% of OTUs), followed by Basidiomycota (21%) and Glomeromycota (18%), and some taxa in Chytridiomycota (2%) (see Table S2 for most common taxa). Rarefaction curves, constructed using the R < vegan > package, were saturating for the majority of samples (Fig. S1), and Good's coverage was >99% for all samples, suggesting adequate sampling of the target communities.

Statistical Analyses

For each subset of the entire fungal OTU table used to answer each research question, we transformed relative abundances of fungal OTUs using the variance-stabilization method in package DESeq2 in R [39]. To investigate root-associated fungal composition, we calculated Bray-Curtis distances using the transformed matrices to assess similarity among samples in community composition, and then used PERMANOVA to test for significant differences among groups (9999 permutations of residuals under a reduced model with Type III sums of squares). For a list of PERMANOVA models, the data examined, and their corresponding ecological questions, see Table 1. Differences among study types in fungal composition were visualized with Nonmetric Multi-Dimensional Scaling (NMDS) using 500 random starting configurations. Tests for differences in community dispersion, or within-group sample variability, were performed using PERMDISP, including all pairwise comparisons when appropriate. To further understand whether community composition differences were driven by rare or abundant taxa, and if known obligately mycorrhizal taxa (Glomeromycota) showed different patterns from other fungi, we repeated compositional analyses for each question using the following: (1) the full fungal dataset included in the samples, (2) the core community subset (102 OTUs present in all four study types), and (3) the Glomeromycota subset (197 OTUs). For question 1, we also performed separate analyses on the peripheral community subset (OTUs not in core community). All compositional analyses and visualizations were conducted in PRIMER v6 [40]. Contrasts from compositional analyses were further investigated using indicator species analyses to determine which OTUs were strong indicators of pairwise differences between groups; these analyses were conducted using the labdsv package in R 3.3.2 [41]. We also visualized the rank abundances of core OTUs in each study to observe whether community structure was preserved among inocula source and inoculated communities.

We also evaluated the differences among sample groups in fungal richness and diversity using general linear-mixed effect models (lme; Table 1). We assessed fungal community richness using OTUs observed and the Chao1 estimator, and diversity using the Shannon and Simpson diversity indices. We calculated diversity and richness indices using the R < vegan > package. Richness and diversity analyses were conducted in R 3.3.2 [41]. Because all richness/diversity metrics were consistent, we presented OTUs observed as a representation in the main text, and provided results for other richness and diversity metrics in Supplement Table S4. Model specification differed slightly for question 3 (Does the diversity or composition of root-associated fungi vary with long-term stability of the dominant plant species?). We considered it useful to code inoculum source not according to plant species provenance (blue grama or black grama), but instead according to whether it matched that of the host plant species (conspecific or heterospecific). This approach has also been commonly used in other PSF studies (reviewed in [9]).

To answer question 4, we used data from our greenhouse and field plant-soil feedback experiments to determine if differences in microbial community composition help predict the size and direction of plant-soil feedbacks. First, we calculated all plant-soil feedbacks for experimental plants that were sequenced in this study. Within each experiment (greenhouse and field), we calculated feedbacks as ln(biomass in conspecific soil/biomass in heterospecific soil) [21] for all possible blue and black grama plant pairs. For each pair, we then extracted the corresponding fungal community dissimilarity (Bray-Curtis distance) from the community composition analysis (question 2). We evaluated if fungal community dissimilarity between paired conspecific/heterospecific treatments could predict plant-soil feedbacks for those same treatments using a linear model with additional fixed effects of plant species (blue or black grama), study type (greenhouse or field), and all interactions. Additionally, we used DESeq2 to identify OTUs that were differentially abundant between blue and black grama hosts (host-specific taxa, following [42]), and present in >6 sample pairs (25 OTUs). To explore the potential for each of these taxa to predict plant-soil feedback in experiments, we first fit global linear models with plant-soil feedback as the response, the effect of log twofold change in OTU sequence abundance as a predictor, and corrected for multiple comparisons using Benjamini-Hochberg false discovery rate procedure (FDR). We then further explored taxa that significantly predicted PSF after FDR correction by including the effects of plant host species, study type, and their interactions in addition to the global model when appropriate and sample sizes were sufficient.

Table 1 Research questions and analysis models

Research question	Effects tested	Plant samples included			
		Greenhouse	Monoculture	Field resident	Field transplant
(1) How does root-associated fungal diversity, composition, and host-specificity compare among studies?	Study type Host plant identity Interaction	Conspecific-inoculated plants only	All	All	Plants in conspecific microbial environments only
(2) How does soil inoculation alter fungal composition in greenhouse versus field PSF experiments?	Study type Inoculum provenance Host plant identity All two-way interactions	All			All
(3) Does the diversity or composition of root-associated fungi vary with long-term stability of the dominant plant species?	Study type Host plant identity Community stability All two-way interactions Random factor: Spatial block			All	Plants in conspecific microbial environments only
(4) How well do differences in microbial community composition predict the outcome of plant-soil feedbacks for plants?	Community dissimilarity Study type Host plant identity All interactions	All			All

For questions 1–3, we performed analyses using the full fungal dataset included in the samples, the core OTU subset, and the Glomeromycota OTU subset. For question 1, we additionally performed analyses with the peripheral (non-core) OTU subset. For question 4, we used the full fungal dataset to calculate community dissimilarity

Results

How Does Root-Associated Fungal Diversity, Composition, and Host-Specificity Compare Among Studies?

We found a shared fungal consortium present in all four study types, which comprised 9.1% of OTUs but accounted for > 50% of raw total sequence abundance (Fig. 2). Root-associated fungal communities significantly differed among studies and between blue and black grama plants (study type: *Pseudo-*$F_{3,67} = 5.15$, $P < 0.001$, $R^2 = 0.18$; plant identity: *Pseudo-*$F_{1,67} = 1.58$, $P = 0.003$, $R^2 = 0.02$; Fig. 3a). However, study type accounted for 18% of the total variation in fungal community composition and plant species for only a small fraction (< 2%). The interaction between host plant identity and study type was not significant (*Pseudo-*$F_{3,67} = 1.06$, $P = 0.28$), suggesting consistent host-specificity among study types. Visualization of community composition using NMDS showed high stress (2D stress = 0.26), likely driven by the strong divergence in composition among study types and the large abundance of a shared core community (Figs. 2 and 3a). Therefore, we additionally analyzed fungal composition with only the core community, and different study types still resulted in distinct fungal communities (study type: *Pseudo-*$F_{3,67} = 4.42$, $P = 0.001$, $R^2 = 0.16$; plant identity: *Pseudo-*$F_{1,67} = 1.50$, $P = 0.053$, $R^2 = 0.02$; Fig. S3A). Analysis with peripheral taxa only also showed significantly different composition among studies, but no distinction between plant species (study type: *Pseudo-*$F_{3,67} = 5.44$, $P = 0.001$, $R^2 = 0.19$; plant identity: *Pseudo-*$F_{1,67} = 1.31$, $P = 0.089$ $R^2 = 0.01$). We found similar results when analyzing only the Glomeromycota (study type: *Pseudo-*$F_{3,62} = 3.75$, $P = 0.001$, $R^2 = 0.18$; plant identity: *Pseudo-*$F_{1,62} = 1.62$, $P = 0.080$ $R^2 = 0.03$).

Plants that were more highly manipulated in studies with shorter durations hosted more similar root fungal communities across replicate plants than long-term field residents. Greenhouse plants had the least variation (dispersion) in microbial composition among replicate samples, followed by field transplants, then field monoculture plants and finally, field resident plants (Fig. 3a). All study types differed from each other in community dispersion (pairwise tests, all $P < 0.001$) except for field monoculture plants versus field residents, for which pairwise dispersion did not differ ($t = 0.30$, $P = 0.95$). There were no differences in fungal community dispersion between black grama and blue grama ($t = 0.27$, $P = 0.78$).

A few taxa drove composition differences among study types. At the phylum level, greenhouse plants had higher relative abundances of Glomeromycota (arbuscular mycorrhizal fungi) and lower abundances of Basidiomycota than the other study types (Fig. S2A). This was reflected in the indicator taxon analyses, where two of the top five indicator OTUs for greenhouse study plants were assigned to *Paraglomus*

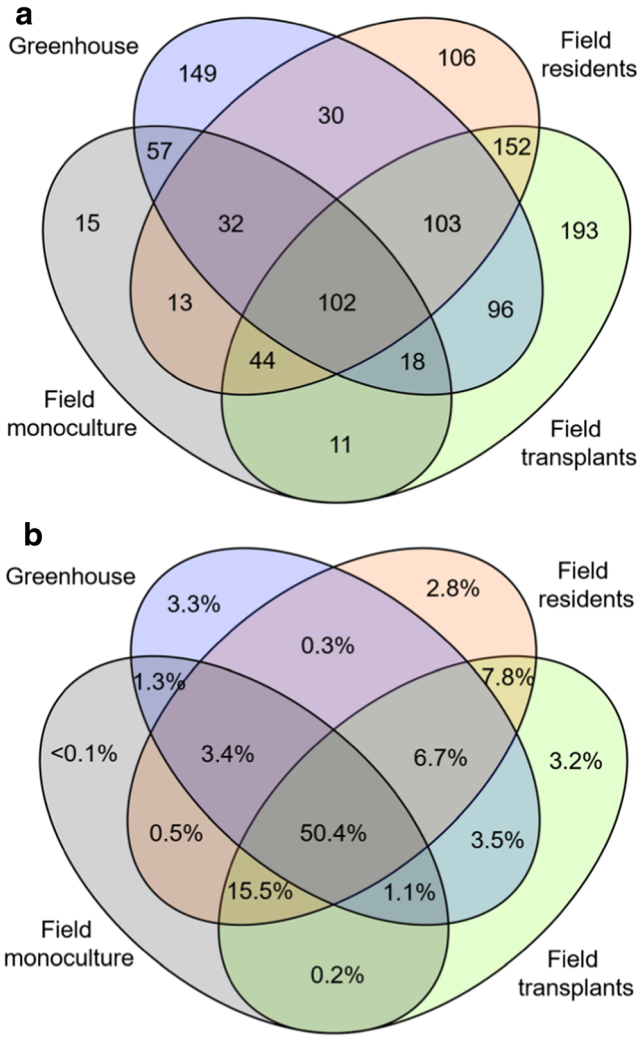

Fig. 2 Shared fungal OTU **a** occurrences and **b** relative sequence abundances (% of total) across study types. Values are summed across all samples. The 102 OTUs shared among all study types are the "core" taxa in subsequent analyses

brasilianum (Glomeromycota, Table S3). Interestingly, in field resident plants, the top 5 indicator OTUs were all assigned to *Moniliophthora*, a Basidiomycete genus known to include phytopathogens [43] (Table S3).

Surprisingly, plants in the greenhouse experiment hosted the most diverse fungal communities ($F_{3,67} = 27.92$, $P < 0.001$, Table S4). On average, greenhouse plants harbored 18% richer fungal communities than field transplants and more than twofold greater richness than field monoculture plants or naturally-occurring field residents (Fig. 4a). There were no differences in fungal richness between blue grama and black grama ($F_{1,67} = 0.19$, $P = 0.67$, Table S4).

Between black and blue grama, root fungal communities differed most in their relative abundances of Basidiomycota versus Glomeromycota. Blue grama fungal communities included a greater proportion of Glomeromycota and lesser of Basidiomycota than black grama (Fig. S2B). Of the fungal OTUs in samples inoculated with conspecific soils or grown directly in the field, 23% were unique to blue grama, and 17% were unique to black grama plants. While differences in fungal composition between blue and black grama were subtle, a few OTUs were significant indicators for each plant species (Table S3). The best indicator for black grama, a Glomeromycota species (OTU 279), was nearly twice as abundant in black grama as in blue grama samples. Two OTUs in the genus *Phaeosphaeria* (OTU 224 and OTU 240) were strong indicators for both black grama plants and black grama rhizosphere inoculum. For example, OTU 240 only occurred in plants inoculated with black grama rhizosphere soils in the greenhouse experiment, and 89% of all OTU 224 sequences occurred in black grama plants inoculated with black grama rhizosphere soil. The best indicator of blue grama rhizobiomes was *Macrodiplodiopsis desmazieri* (OTU 69), a potentially saprotrophic ascomycete [44] that was 132% more abundant in blue grama samples than black grama samples. *Claroideoglomus drummondii* (OTU 88), an arbuscular mycorrhizal fungus, was a strong indicator for blue grama host and for its inoculum; 47% of its total sequences occurred in blue grama plants inoculated with blue grama rhizosphere soil.

How Does Soil Inoculation Alter Fungal Composition in Greenhouse Versus Field PSF Experiments?

The efficacy of experimental inoculation or "soil training" in PSF experiments is often assumed based on differential plant biomass responses. Using plants from greenhouse and field PSF experiments, we confirmed that inoculation of rhizosphere soils from blue grama vs. black grama altered the root-associated fungal communities of experimental plants (*Pseudo-*$F_{1,40} = 2.24$, $P < 0.001$, $R^2 = 0.04$; Fig. 3b). We expected soil inoculations to alter fungal composition more strongly in the greenhouse than in the field, due to shorter duration experiments and more controlled conditions. In line with our expectations, the shift in ordination centroid due to inoculation was 3.9 × larger in greenhouse plants compared to field transplants, demonstrating stronger soil inoculation effects in the greenhouse (interaction *Pseudo-*$F_{1,40} = 2.10$, $P < 0.001$, $R^2 = 0.03$; Fig. 3b). In addition, the provenance of soil inoculum had a larger effect on fungal composition than the identity of the planted, living host plant. In both field and greenhouse PSF experiments where plants were inoculated from either provenance, host species identity was not a significant predictor of root fungal composition (*Pseudo-*$F_{1,40} = 1.18$, $P = 0.16$ $R^2 = 0.02$; Fig. 3b). Estimated variation from PERMANOVA for inoculation provenance was 6.7 × larger than variation explained by host plant species identity. As in results from question 1, fungal compositions differed between greenhouse and field PSF experiments (*Pseudo-*$F_{1,40} = 12.79$, $P < 0.001$ $R^2 = 0.21$; Fig. 3b). Results were qualitatively

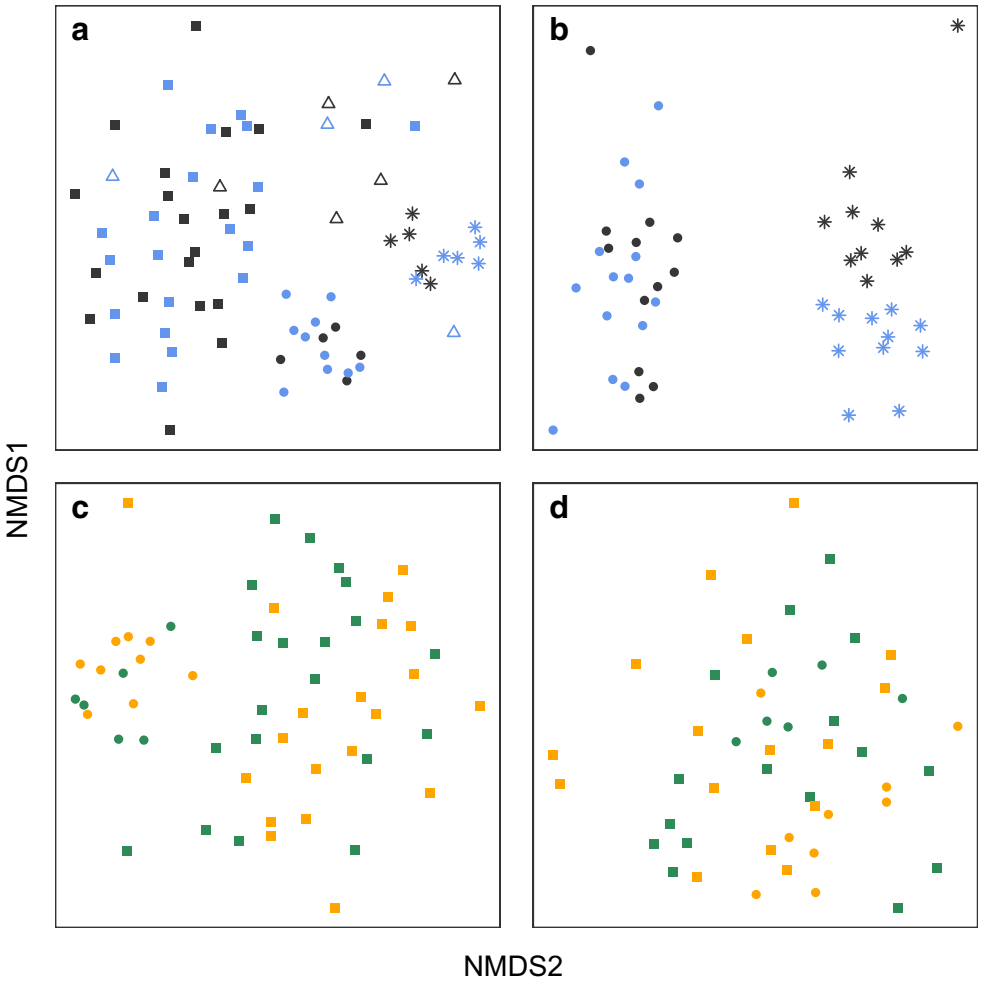

Fig. 3 NMDS visualization of the difference in root-associated fungal composition among samples. Shapes correspond to study types: Greenhouse (∗), field resident (■), field monoculture (△), and field transplant (●). **a** Question 1: Total fungal composition differed among study types and host plants. Blue grama plants are in blue and black grama plants are in black. **b** Question 2: Total fungal composition differed between experimental inocula. Plants inoculated with blue grama source microbes are in blue and black grama source microbes in black. **c** Question 3: Total fungal composition did not differ between dynamic and static patches in the field. Plants from dynamic patches are in green and static patches in orange. **d** Question 3: Glomeromycota composition differed between dynamic and static patches. Colors follow those in **c**

similar (significant effects of study type, inoculation source, and their interaction) when we repeated analyses on core community or Glomeromycota subsets. Fungal diversity was similar among study types, plant species, and inoculum provenances, on average 113 taxa per sample (Table S4; Fig. 4b).

While experimental inoculation successfully altered fungal composition, the root fungal communities of plants in greenhouse and field PSF experiments were not direct subsets of the source communities from which the inocula were collected. This discrepancy was strongest in the greenhouse experiment,

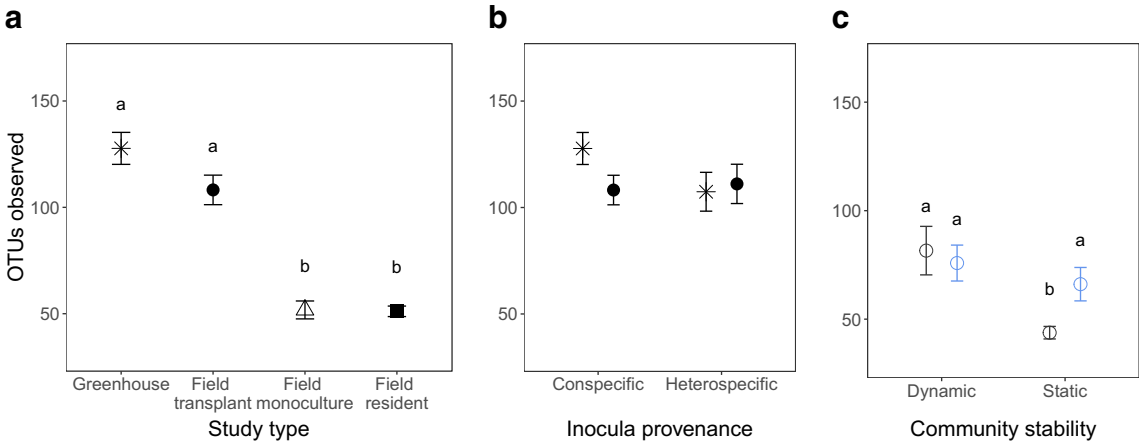

Fig. 4 Richness (OTUs observed) of root-associated fungal communities among **a** study types, **b** inocula provenances in the greenhouse and field PSF experiments, and **c** between different community stability types. Symbol shapes in **b** correspond to study types in **a**. Colors in **c** indicate blue grama (in blue) or black grama (in black); residents and transplants are considered together. Significantly different pairwise comparisons in each panel are labeled with different letters

where only 36% of the OTUs present (representing 80% of total sequence abundance) were shared with the originating field monoculture communities. In comparison, transplant seedlings in the field PSF experiment shared 56% of their fungal OTUs (consisting 91% total sequence abundance) with field resident plants (Fig. 2), showing considerably higher overlap with inoculum source. Rank abundances of core community taxa in the experimental plants versus plants that provided the inoculum source differed (Fig. 5), suggesting that the inoculated communities at the time of harvest no longer reflected source community structure.

Does the Diversity or Composition of Root-Associated Fungi Vary with Long-Term Stability of the Dominant Plant Species?

We expected that plants in dynamic patches with high plant species turnover (thus, low community stability) would have more diverse fungal communities with higher dispersion than patches with low turnover (static patches). Plants in dynamic patches indeed had higher fungal richness than plants in static patches (main effect $F_{1,54} = 4.46$, $P = 0.04$; interaction $F_{1,54} = 5.37$, $P = 0.03$; Fig. 4c, Table S4). This effect was stronger for the stoloniferous black grama, which had 86% higher richness in dynamic patches, whereas blue grama plants had only a 15% increase. However, within each study type, fungal community dispersion among individual samples did not differ between plants in dynamic vs. static patches ($P = 0.52$ and $P = 0.58$ for transplants and residents respectively, Table S4).

Both analysis of Glomeromycota alone and indicator taxon analysis detected differences in fungal composition between dynamic and static patches. While our choice of primer can under-sample this group, we found that Glomeromycota composition significantly diverged between dynamic and static patches (*Pseudo*-$F_{1,34} = 2.33$, $P = 0.012$, $R^2 = 0.04$), as well as between resident and transplants (*Pseudo*-$F_{1,34} = 1.96$, $P = 0.043$, $R^2 = 0.04$; Fig. 3d). Of the significant indicator taxa, *Glomus* sp. BEG104 (OTU-258) was the only one in Glomeromycota and occurred 1.8 × more frequently in dynamic than static patches (Table S3). The best indicator of dynamic patches was *Fusarium redolens* (OTU-17), which was more than seven times more abundant and occurred with 12% greater frequency in dynamic patches (Table S3). This fungus is a known pathogen in agricultural settings [45, 46], but also occurs as an endophyte in natural settings. The most abundant indicator of static patches was *Monosporascus cannonballus* (OTU 20), which occurred in 20% of static patch residents, but rarely in other plants (Table S3). This fungus is a known pathogen on melons and a common grass endophyte in this ecosystem [47, 48].

Despite these indicators and the diversity differences between static and dynamic patches, total fungal composition in plant roots only differed between the resident plant and transplants (*Pseudo*-$F_{1,39} = 5.15$, $P < 0.001$, $R^2 = 0.08$) and not between static and dynamic patches (*Pseudo*-$F_{1,39} = 1.09$, $P = 0.31$, $R^2 = 0.02$; Fig. 3c). We found similar results when we repeated analyses using the core community subset.

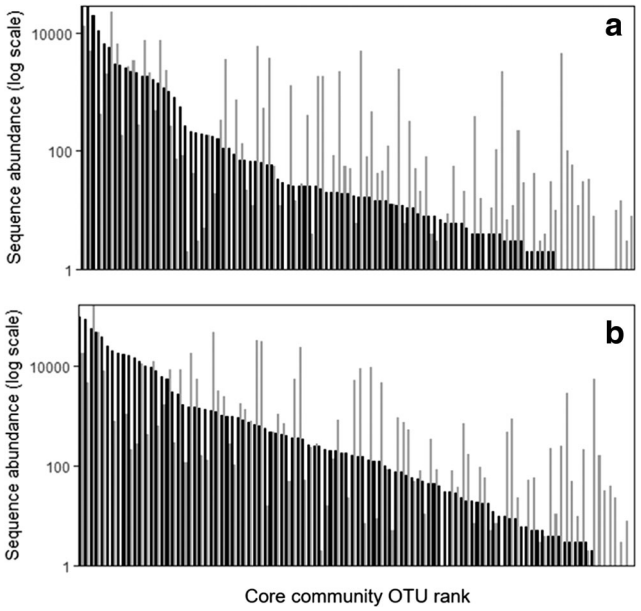

Fig. 5 Experimental communities (gray) do not recapitulate core fungal taxa rank abundance in originating communities (black). **a** Rank abundance of core community taxa in field monoculture plants (black) compared to greenhouse experiment plants (gray). **b** Rank abundance of core community taxa in field resident plants (black) compared to field experiment transplants (gray)

How Well Do Differences in Microbial Community Composition Predict the Outcome of Plant-Soil Feedbacks for Plants?

Total fungal community dissimilarity did not predict measured plant-soil feedback in the greenhouse or field experiment ($F_{1,137} = 1.11$, $P = 0.29$). However, a handful of specific fungal taxa were correlated with PSF outcomes for plants. Of the 25 host-specific taxa identified for further analysis (see "Methods" section), five had abundance patterns that significantly predicted plant-soil feedbacks, after correcting for multiple comparisons with FDR (Fig. S4). Of these, four occurred in both greenhouse and field experiments, and one only in the greenhouse.

A simple prediction is that mutualists are more commonly associated with positive PSF and pathogens with negative PSF, and three candidates were consistent with that prediction. Increases in two Glomeromycota taxa were associated with more positive plant-soil feedback: Glomerales OTU-106 (main effect $F_{1,72} = 82.95$, $P < 0.001$), which had a stronger effect in the field than greenhouse (interaction $F_{1,72} = 4.47$, $P = 0.013$; Fig. S4C), and Glomeromycetes OTU-147 (main effect $F_{1,24} = 41.17$, $P < 0.001$; Fig. S4D). In contrast, an increase in *Fusarium redolens* OTU-17, a known plant pathogen, was associated with more negative plant-soil feedback (main effect $F_{1,54} = 8.83$, $P =$

0.004), but only in the field (interaction $F_{1,54} = 5.19$, $P = 0.027$; Fig. S4A). Interestingly, *Fusarium redolens* was also associated with dynamic patches that frequently altered in dominance between blue grama and black grama, as well as black grama inoculum (Table S2).

Some results diverged from simple expectations. *Paraglomus* OTU-259, a Glomeromycota found only in the greenhouse, was associated with more negative plant-soil feedback for blue grama alone (main effect $F_{1,8} = 0.71$, $P = 0.424$, interaction $F_{1,8} = 7.09$, $P = 0.029$; Fig. S4E). A Dothideomycetes OTU-69 (Ascomycota) was associated with more positive feedback, but only in the field experiment (main effect $F_{1,110} = 8.38$, $P = 0.005$, interaction $F_{1,110} = 4.98$, $P = 0.028$; Fig. S4B).

Discussion

Root Fungal Communities Differed Among Studies

While plants from all studies shared a core community of common taxa, study conditions had a large influence on plant mycobiome composition, which was not simply driven by stochasticity in rare taxa. Plants in the greenhouse experiment and those raised in the greenhouse, then transplanted to the field both maintained more similar fungal communities across samples and had higher fungal diversity than plants naturally occurring or experimentally sowed as seeds in the field (Fig. 4a). Several factors may explain the larger divergence among individual plants under field conditions relative to greenhouse, including longer time since establishment, more variable abiotic conditions that act as environmental filters, and a wider range of plant genotypes sampled [49–51]. In addition, naturally occurring blue and black grama plants live several decades [52] and may selectively filter root-associated fungi over decadal time scales, resulting in lower diversity and greater among plant dispersion than in plants observed in short-term inoculation studies. Taken together, these results suggest a strong contribution of interactions between field microenvironment and microbial species pool in driving plant microbiome composition in the field [42].

There are several explanations for the observation of greater fungal diversity in young, greenhouse-grown plants than in older, field-established plants. First, decreasing diversity and richness with increasing plant age has been reported in studies on rhizosphere bacteria and arbuscular mycorrhizal fungi associated with tropical tree seedlings [23, 53]. Such observations were hypothesized to result from a high number of generalist symbionts early in plant development. A definitive test would require growing plants across a range of ages in both the greenhouse and the field, which has not, to our knowledge, been done. Second, it is possible that plants initially grown in sterilized sand in the greenhouse developed microbial associations best suited to a greenhouse environment, and these persisted even when transplants were moved into the field. A small proportion of OTUS (96 OTUs, 8.6% of total richness) occurred only in samples from the greenhouse plants and field transplants, and not in any other study type. These OTUs were not greenhouse fungal contaminants commonly reported in the literature, such as *Thelephora terrestris*, *Laccaria laccata*, *Mycelium radicis atrovirens*, *Pulvinula convexella*, or *Sphaerosporella brunnea* (Table S5). However, they were comparatively enriched in the relative proportion of taxa in Glomeromycota, which increased from 17.5% of all OTUs to 26% of OTUs that were unique to plants exposed to the greenhouse. Moist, cool greenhouse conditions may have promoted germination of dormant spores of arbuscular mycorrhizal fungi from field inocula, spores that are typically inactive under field conditions. This hypothesis is consistent with two previous field studies in this ecosystem, which reported high or low colonization of blue grama roots by arbuscular mycorrhizal fungi during wet and drought periods respectively [32, 54]. Finally, we suggest that it is unlikely that the number of plants sampled was a cause of higher fungal diversity in greenhouse-grown plants because we found similarly high per sample diversity in both the greenhouse experiment, which had four–six plant individuals growing in the same pot, and in the greenhouse-grown field transplants, which were grown as single individuals.

Congeneric Perennial Grass Species Harbor Distinctive Fungal Communities

Our findings highlight that host-specificity in plant-fungal symbioses can be recovered across multiple time scales, across experimental approaches, and across various methods of plant cultivation and microbial inoculation. While host-specificity in root microbiome composition is common in naturally occurring plants in many ecosystems [55], in our study, host specificity was preserved across all study types, and core taxa showed more host specificity than did rare taxa. Although the differences between blue grama and black grama were relatively small, perhaps because the species are congeneric, this encouraging result suggests that experiments using different study methods can all capture the dominant, core community. The core community may be the most important component for plant-soil feedbacks, given the higher host specificity among core taxa. These findings also complement our results on plant fitness, which similarly preserved the outcome of plant-soil feedbacks between blue and black grama in both the greenhouse [8] and the field (Chung et al. in prep). That we observe strong differences between studies and host plants despite using closely related, congeneric hosts makes this a conservative test of our hypotheses. Future work that includes a larger selection of plant species will help to understand whether the existence of a host-specific, core microbial

community, present in both field and greenhouse, is a general phenomenon in plants.

Experimental Inoculations Outranked Plant Species Identity in Shaping Fungal Assemblages in Roots

Our results validate one of the most common methods of PSF research: inoculating plants in the greenhouse with field soil and demonstrating the importance of the initial microbial species pool (i.e., legacy) in the assembly of root-associated fungal communities [56]. While we recovered host-specificity in "control" plants across multiple experiments, the influence of experimental soil inoculation outweighed the influence of plant species identity in both field and greenhouse PSF experiments. Our results dovetail with other work identifying legacy effects of plant species on soil microbiota that persist to affect the growth of subsequent plant species as well as the composition of the developing microbial community [22, 57]. However, although one prior study reported concordance in ectomycorrhizal fungal community structure between the greenhouse and field [58], our results revealed that even for abundant, shared core taxa, fungal-relative abundances were very different between the field-collected inocula and the communities that subsequently assembled on field- or greenhouse-grown plants (Fig. 5). So, despite substantial overlap, greenhouse and field studies did not have a perfect match in microbial community structure. Although our sample sizes were relatively small in this analysis, these discrepancies will be important to consider in studies that aim to identify the microbial mechanisms that underlie the effects of plant-soil feedback on plant fitness.

Experimental inoculation had a stronger influence on fungal composition in the greenhouse than in the field, suggesting that greenhouse experiments will maximize the potential to detect inoculum effects on plant growth. Several differences in the inoculation methods may underlie the stronger influence of inoculation in the greenhouse than in the field. First, plants in the greenhouse were grown in sterile sand that was inoculated once with a small volume of field-collected rhizosphere soil, whereas field transplants were "inoculated" by planting them close to a "donor" resident plant which provided a continuous supply of propagules and colonization by vegetative hyphae throughout the experiment. Field inoculation effects could be weaker because field transplants were in a matrix of mixed species grassland, and adjacent to, rather than in isolated, direct contact with, the inoculum source. The effects of the "donor" plant inoculum were likely diluted by the influence of multiple species' rhizospheres that were further away, but still within < 50 cm radius, as well as colonization of spores from atmospheric deposition. The spatial scale of PSF is potentially important in structuring plant communities [59, 60], yet spatially explicit knowledge of the sphere of influence of a plant on the rhizosphere microbial community is lacking. Our results suggest that knowledge of plant spatial neighborhood may be important in ensuring PSF treatment efficacy in designing field PSF experiments. Second, in the greenhouse experiment, plants were grown exclusively in the greenhouse for 6 months, whereas the field transplants were grown for 2 months in the greenhouse and 25 months in the field. Past work has shown that PSF effect size declined as the study duration increased [61]. While there were few studies that lasted more than 12 months to substantiate this pattern, these observations could result from decreased host vulnerability to pathogens with increasing age for those PSFs that are pathogen-driven [62].

Temporally Dynamic Plant Communities Had Higher Fungal Diversity and Different Indicator Taxa than Static Patches with Slow Species Turnover

Subtle differences in rhizobiome composition distinguished plants in patches of fast (dynamic) turnover from those in slow turnover patches (static). As a group, Glomeromycota fungi were more sensitive to temporal fluctuations in the plant community than other groups of fungal taxa (Fig. 2c, d). This difference among fungal groups in their association with plant temporal dynamics likely reflects differences in fungal life history strategy. Fungi in the Glomeromycota are predominantly obligate plant symbionts, making their fitness directly tied to that of their plant host and likely more sensitive to fluctuations in host plant abundance. In contrast, plant-associated fungi in Ascomycota or Basidiomycota often live multiple lifestyles and persist as endophytes and saprobes that decompose dead plant material [63]. Interestingly, this was the only question for which analysis on the Glomeromycota subset yielded different results from the total or core fungal community (e.g., Glomeromycota taxa did not differ more between hosts or among study types compared to other groups). Our analyses also revealed indicator taxa specific to dynamic and static patches that may be key to driving observed aboveground patterns. *Fusarium redolens* (dynamic patch indicator) and *Monosporascus cannonballus* (static patch indicator) are well-known agricultural pathogens that can also live an endophytic lifestyle. Future inoculation studies are needed to elucidate their effects on plant hosts under non-agricultural contexts. Taken together, these results suggest that considering individual taxa or subsets of entire communities will be key to uncovering the subtleties that drive microbial community assembly in association with their aboveground hosts.

Higher fungal diversity in black grama plants may have correlated with higher rates of plant species turnover for several reasons (Fig. 4c). First, dynamic patches may maintain a larger microbial species pool due to the fluctuating abundances of plant species, each favoring different host-specific microbial associates. Others have found spatial turnover of plant communities to correlate with turnover in species richness of soil microbial communities [64]. Second, our results suggest higher

fungal diversity in younger plants, a pattern that has previously been observed for arbuscular mycorrhizal fungi in tree seedlings [53]. While we do not know the specific age distribution of plants in dynamic versus static patches in our field experiment, it is reasonable to assume that higher rates of plant community compositional change would result in younger plants occupying dynamic patches than static patches, thus potentially explaining higher fungal diversity. In addition to plant turnover driving fungal diversity, causality could also occur in the opposite direction (fungi driving plant turnover) [65], or both (plant-soil feedback). Past work has focused mostly on spatial patterns between plant and microbial diversity. Our results highlight the importance of temporal resolution in understanding how plant-microbe interactions shape above- and belowground community assembly processes.

Individual Fungi, Not Community-Level Metrics, Predicted Microbial Roles in Plant-Soil Feedback Experiments

Contrary to our predictions, differences in the total fungal community composition did not predict plant-soil feedbacks measured in the greenhouse or field experiments. While others have found this relationship in studies comparing phylogenetically diverse plant species [42], our more conservative test with two congeners did not reveal the same pattern. This is not surprising as community dissimilarity is a high-level metric that is insensitive to whether underlying changes are due to abundance or membership in pathogenic/mutualistic taxa, which likely relate more directly to plant-soil feedback outcomes. Our additional analyses uncovered a few taxa whose change in abundances directly predicted PSF in the field and/or greenhouse. This suggests that first, analytical tools make it logistically feasible to pinpoint a few candidate drivers of PSF from > 1000 OTUs in a dataset. Second, it is potentially possible to then generalize results from field or greenhouse experiments to nature using natural history knowledge of these key microbial taxa. For example, *Fusarium redolens* was a strong predictor of negative PSF in the field, and indicators of black grama microbial communities and dynamic patches in this study. Therefore, attempts to predict PSF for blue and black grama under different natural scenarios, such as future climate, could strongly benefit from natural history knowledge of the response of *Fusarium redolens* and its interaction with host plants to those conditions. Our analyses provide a tractable approach to identifying these important taxa from whole-community inoculations that may be the key to generalizability.

Conclusions

Our work highlights the importance of experimental design and approach in driving microbial community composition in plant-soil feedback studies, and sheds light on key differences between field- and greenhouse-based assessments of plant-soil microbial interactions. Our results suggest that greenhouse inoculation experiments provide effective, controlled settings that can directly tease apart the effects of microbial communities on plant performance and plant-soil feedbacks. In addition, different study methods recovered a shared, core mycobiome of dominant taxa that were more host-specific than rare, peripheral taxa. However, divergent microbial community assembly under greenhouse experiment, field experiment, and natural conditions caution against the ability to generalize experimental results. To understand the processes that drive plant-microbe interactions in experiments and nature, it may be more informative to identify specific taxa whose abundances are strongly correlated to plant responses as a key to generalize findings from experimental settings to natural phenomena.

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