THESIS

EVALUATING *BOUTELOUA GRACILIS* CULTIVARS' PERFORMANCE AFTER DROUGHT; THE ROLE OF THE SOIL MICROBIOME

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ABSTRACT

EVALUATING *BOUTELOUA GRACILIS* CULTIVARS' PERFORMANCE AFTER DROUGHT; THE ROLE OF THE SOIL MICROBIOME

Drought has affected the Great Plains throughout history, most notably during the Dust Bowl of the 1930's. While most drought events are not as severe as the Dust Bowl, they still cause significant agricultural losses every year. As research has begun to uncover the mechanisms and responses of drought, there are still unanswered questions. For instance, the mechanisms of ecosystem recovery after drought ends remain relatively unexplored. It is possible that intervention methods such as reseeding will need to be done to help restore ecosystem structure and function after drought. After the Dust Bowl, it was a common practice to reseed native grasses, such as Blue Grama (*Bouteloua gracilis),* in sites severely impacted by the drought. Given forecasts of droughts on par or even more severe than the Dust Bowl, reseeding may need to be employed more frequently in the future to enhance post-drought recovery. However, with reseeding efforts, it is imperative to understand the adaptability of cultivars to the environmental conditions in which they are planted. One aspect of environmental conditions that has rarely been examined the soil microbiome. Here, I used a common garden experiment that included two cultivars of *B. gracilis* that were planted with soil microbial inocula extracted from either previously droughted or non-droughted soils. These soils were collected from a recently ended four-year drought experiment in the shortgrass steppe of northeastern Colorado, which caused the widespread loss of *B. gracilis*. The goal of the greenhouse experiment I conducted was to examine whether the post-drought legacy of altered soil microbial communities affected the growth and performance of two common cultivars of *B. gracilis*. I assessed plant performance by measuring weekly height to estimate relative growth rate and at the end of the experiment, I measured plant above- and belowground biomass. I found no significant differences in relative growth rate or plant biomass, and minimal differences in the bacterial community composition between the two cultivars. These results suggest that the post-drought legacy of altered soil bacterial communities did not differentially affect growth and performance of the two common *B. gracilis* cultivars evaluated in this study, and that the growth of these cultivars did not differ in their effects on the soil bacterial communities found under ambient vs. previously droughted conditions. Overall, both cultivars may be suitable for reseeding in the shortgrass steppe grassland after extreme drought, yet further studies are needed to examine a broader range of *B. gracilis* cultivars and whether soil bacterial communities previously exposed to extreme drought would allow for improved growth and performance of different cultivars to future drought conditions.

ACKNOWLEDGEMENTS

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EVALUATING *BOUTELOUA GRACILIS* CULTIVARS' PERFORMANCE AFTER DROUGHT; THE ROLE OF THE SOIL MICROBIOME

INTRODUCTION

Drought is one of the most prevalent abiotic stressors on plants, imposing a wide range of negative impacts (Felton & Smith 2016) especially when extreme (Smith 2011). Research on plant responses to drought has become more prominent, but most of this research focuses on short-term drought and does not include studying plant responses after drought ends (Vilonen et al., 2022b). This lack of focus on the 'post-drought period' (*sensu* Vilonen et al. 2022b) is particularly troubling given that impacts of drought can extend well beyond the event itself (Vilonen et al., 2022b). These lingering effects are referred to as legacy effects, defined as any alteration caused by drought that persists after drought has ended (Vilonen et al., 2022b). Legacy effects, such as altered soil properties, reduced plant growth due to depleted carbohydrate reserves (Lozano et al., 2022; Vilonen et al., 2022a), or mortality of individuals, can delay recovery of ecosystem structure and function (Yahdjian & Sala 2006). As such, it is important to study this post-drought period to help inform restoration efforts aimed at hastening ecosystem recovery.

A historically important example of an effort to restore drought-ravaged ecosystems is from the Dust Bowl of the 1930's. The Soil Conservation Service began projects in the Great Plains and Southwest to help restore grasslands that suffered from widespread loss due to the combined effects of extreme drought, overgrazing and soil erosion (Fults 1936; Albertson & Weaver 1942; Albertson & Weaver 1944; Albertson et al., 1957; Knapp et al., 2020). These projects included reestablishing native, highly abundant (or dominant) C_4 grasses, particularly *B. gracilis* (blue grama), through methods of sodding and seeding (Fults 1936). *B. gracilis* was favored for re-establishment efforts as it is adapted to many habitats, has high drought tolerance, prevents soil erosion, and is tolerant to grazing (Fults 1936; Montana State University n.d.). Given forecasts for intensified drought events on par with the Dust Bowl for the Great Plains and Southwest in the future (Cook et al. 2015), it is critical that we improve understanding of how different restoration efforts can hasten recovery during the post-drought period. Here, my focus will be on the oft-utilized approach of adding seeds after a disturbance has occurred, hereafter referred to as reseeding. In particular, I consider how reseeding may interact with legacy effects to influence post-drought recovery of a semi-arid, shortgrass steppe grassland located in northeastern CO.

When considering reseeding, a crucial factor in the success of this restoration approach is the genetic diversity of the seeds being used. For reseeding to be successful, it is imperative that the seeds used are from cultivars that are adapted to the local habitat so that species can thrive in the changing climate (Tso & Allan 2018; Hoffman et al., 2020). While this is not a novel concept, it is commonly overlooked in its ability to impact restoration. For example, Riegel (1940) observed that *B. gracilis* grown from seeds from the central, northern and southern regions of the Great Plains displayed great variation in shoot and root growth, and therefore suggested that seeds should be harvested close to the location that is being restored. Currently, there are five main cultivars of *B. gracilis* available for use in restoration efforts (Tso & Allan 2018). While none are sourced from shortgrass steppe grasslands in CO, two of these cultivars are classified by the USDA as drought-tolerant. Therefore, I selected these cultivars for this study.

In addition to consideration of the genetic diversity of seeds utilized in a post-drought reseeding effort, it is important to consider how different cultivars may interact with legacy effects, such as those associated with soil properties. While changes in soil structure and nutrients can be important legacy effects, one that is often overlooked is how changes in the soil microbiome – all the microorganisms found in the soil (Fierer 2017) – may persist post-drought and influence seedling establishment, growth, and performance. Soil microbiota are tightly intertwined with plants leading to plant-microbe interactions that can alter plant performance and fitness (Fierer 2017; Trivedi et al., 2020). Plants have been shown to recruit a specific rhizosphere (the soil compartment in direct contact with plant roots) microbiome, and plant genotype can be an important driver at this interface (Berendsen et al., 2012; Xiong et al., 2021). The rhizosphere microbiome has been shown to have a major effect on plant health, particularly stress tolerance (e.g. drought) (Berendsen et al., 2012; Xiong et al., 2021). These host-microbiome interactions are still poorly understood but a deeper understanding of how these interactions can shape *B. gracilis'* establishment and growth during post-drought reseeding efforts is necessary for maintaining the function of shortgrass steppe grasslands.

Here, I used a greenhouse experiment to evaluate host-microbiome interactions between two *B. gracilis* cultivars and field soil microbes during drought recovery. I utilized an International Drought Experiment site (Smith et al. 2024), located at the Central Plains Experimental Range (CPER) in northeastern Colorado, that imposed four full years of year-round extreme drought. At the end of the drought, the rainfall exclusion shelters were removed, and recovery commenced. I collected soil from these study plots at the start of the recovery period. This allowed me to investigate two main questions: 1) How does the soil microbiome affect growth and performance of *B. gracilis* seed cultivars, and 2) How do different cultivars of *B. gracilis* affect the soil microbiome? The goal of this study is to provide deeper insight into factors potentially affecting the success of post-drought reseeding efforts, and whether certain *B. gracilis* seed cultivars are more suitable for restoration of semi-arid grassland after disturbance events, such as extreme drought, that can lead to the large-scale loss of ecologically and economically important grasses, such as *B. gracilis*.

MATERIALS AND METHODS

Experimental Design

The common garden greenhouse study took place at Colorado State University's Plant Growth Facility over a 16-week period (17 July to 6 November) in 2023. The experimental design consisted of two factors, each with two levels: 1) soil inoculum sources: ambient and previously droughted (described below), and 2) seed cultivars: Hachita and Bad River (described below). These four treatment combinations were replicated 15 times (n = 60 total). In addition, there were three control treatments: 1) soil control treatment - sterilized soil only (n = 3) 2) Hachita controls – sterilized soil plus seeds (n=3), and 3) Bad River controls - sterilized soil plus seeds (n=3). Soil controls were used as a baseline for the soil microbial analyses. The plant controls were intended to be used as a baseline for plant growth but were not included in the analyses because the Hachita control replicates did not germinate.

The potting soil for all treatments consisted of a mixture of homogenized 50% Green's Grade Fritted Clay (Profile Products LLC, Buffalo Grove, IL, USA) and BX potting mix (ProMix, Quakertown, PA, USA). This was sterilized through two rounds of autoclaving. To establish the replicates of each treatment combination, pots (each 10.16 cm x 33.02 cm tree pots) were filled with the sterilized growth media and 25 mL of soil slurry was added. Then, two seeds of a cultivar were planted to a depth of 1 cm in each pot. A randomized complete block design comprising 5 blocks was used to arrange pots on the greenhouse bench. Each block, representing a large container (each 40.64 cm x 40.64 cm) was used to hold 16 individual pots. Three of the blocks contained four replicates of each soil inoculum x cultivar treatment while the fourth block contained three replicates of each treatment combination and one replicate of each control treatment (Soil, Hachita and Bad River). The fifth block contained two replicates of each control treatment. Water was supplied once a week to each treatment pot to reach the treatment target of 20 % Volumetric Water Content (VWC). Water addition volumes were determined using a HydroSense II Water Content Sensor (Campbell Scientific, Logan, UT, USA) with 20 cm probes. Pots were rotated within the blocks weekly on the greenhouse bench to minimize microclimate effects. Throughout the experiment, greenhouse conditions were maintained at an average of 21.1° C, with supplemental lighting for a 16 h photoperiod duration.

Soil Inocula

Live inoculum was obtained from soil collected from the International Drought Experiment (IDE) at the Central Plains Experimental Range site located in northeastern CO 40.81, -104.71). The IDE study applied experimental drought from June 2018 to Mar 2023 (see Smith et al. 2024 for details). In May of 2023, soil samples were collected at a depth of 10 cm from a total of twenty plots, ten of which received ambient precipitation (referred to as *control*) for the duration of the study, while the remaining ten experienced a 66% reduction in precipitation year-round for all years of the study except 2018 (referred to as *previously droughted*). Soil samples were stored at -20°C until inoculum was extracted in the lab by mixing 30 g of soil with 180 ml of sterilized deionized water. Samples were shaken vigorously for 60 seconds (method adapted from Panke-Buisse et al 2015). The inocula were made the same day as the experiment set up to limit any potential microbial contamination from outside sources.

B. gracilis Seed Cultivars

Present day, there are 5 commercially available cultivars of *B. gracilis* that are utilized for restoration purposes (Tso & Allan 2018). Three cultivars, Alma, Lovington and Hachita are commonly used on the Colorado Plateau, while the two cultivars, Bad River and Bird's Eye, are used across the Great Plains (Tso & Allan 2018). For this study, Hachita, originally sourced from southwestern New Mexico (United Stated Department of Agriculture 1982), and Bad River, originally sourced from central South Dakota (United States Department of Agriculture 1997), were chosen for this study due to their potential drought tolerance (United Stated Department of Agriculture 1982; United States Department of Agriculture 1997). Cultivar seeds were purchased from Granite Seed (Denver, Colorado).

Sampling

At the beginning of the experiment, I reserved samples of the control and previously droughted soil inocula to perform 16S and ITS amplicon sequencing. At weeks one, nine and fifteen, I took rhizosphere soil samples from each pot to perform amplicon sequencing and create a timeline of any changes in the soil microbiome. Soil samples that were taken from pots with plants that did not survive the duration of the experiment were excluded from the analysis. Midway through the experiment (~ week 9), plant leaves were shown to be yellowing from nutrient depletion. To circumvent alleviate this, I applied Osmocote Plus at the recommended low dosage (0.25 g/ 0.092903 m^2) to each pot.

Of the fifteen replicates of each soil inoculum x seed cultivar treatment that were set up at the beginning of the experiment, 9 and 10 of the Bad River replicates of the previously droughted and control soil inoculum treatments survived, respectively, while 8 and 6 of the Hachita replicates survived, respectively.

Throughout the experiment, I collected both height and flowering data from surviving individuals. Height measurements began in the second week when the first seeds germinated and continued until the 14th week. Height was measured to the nearest cm from the soil surface to the tip of the longest leaf (with leaves stretched) on a weekly basis. If two plants were present in a pot, height measures were averaged before analysis. To calculate the relative growth rate, I used the following equation: RGR = [ln(max height measurement) – ln(first recorded measurement)]/[date of max height measurement – date of first recorded measurement]. On the 16th week, I harvested all plant biomass and separated it into aboveground leaf, flower, and belowground root categories. Soil was rinsed from root tissue using a 2 mm mesh screen and low-pressure water nozzle. All biomass was allowed to dry at 55ºC for 3 days before weighing.

DNA Extractions, Amplicon Sequencing and Data Processing

I extracted the DNA from each soil sample using the Qiagen DNeasy PowerSoil Pro Kit (QIAGEN, Germantown, MD, USA) following the manufacturer's instructions. All DNA extractions were done in the Smith lab at Colorado State University. All DNA samples were assessed for quality and quantity using a NanoDrop Lite Spectrophotometer (Fischer Scientific, MA, USA). DNA was stored at -20°C until the samples were sent to the Fierer Lab at University of Colorado (Boulder, CO) for both library preparation and amplicon sequencing. Library preparation was performed by PCR in duplicate using Platinum II Hot Start Master Mix (ThermoFisher) on SimpliAmp thermocyclers (ThermoFisher). No template controls were processed with the samples. Illumina MiSeq 2 x 150 bp paired-end sequencing (Illumina Inc., CA, USA) was used to sequence the V4 region of the bacterial 16S rRNA gene (515F/806R primers). The raw data was returned as multiplexed FASTQ files.

The raw data FASTQ files were imported into Qiime2 version 2023.5 (Bolyen et al., 2019) using EMPPairedEndsequences file types. Each file was demultiplexed using the demux plugin and assessed for quality filtering (Hamady et al., 20018; Hamady & Knight 2009). The dada2 plugin was used for joining forward and reverse reads and denoising with a left trim of 13 and right trim of 150 (Callahan et al., 2016). Taxonomy was assigned to the OTUS using the GreenGenes2 database (v 2022.10) for the 16S V4 region (McDonald et al., 2023). I also removed any taxa assigned to chloroplast or mitochondria from the dataset using the taxa filter-table Qiime2 command. The data files were exported and imported into R software v 4.3.2 for downstream analysis.

Statistical Analysis

Mixed models were used to evaluate whether seed cultivar and soil inoculum affected max height, relative growth rate and plant biomass (above- and belowground). Seed cultivar and soil inoculum were fixed factors in the model, while block was a random factor. For all models, seed cultivar x soil inoculum interactions were tested. For flower biomass, a one-way ANOVA was used to test the two factors individually as the previously droughted treatment for the Hachita cultivar did not flower. All analyses were performed in R v. 4.3.2, R packages *lmerTest* v 3.1.3 and *car* v 3.1.2.

For the microbial analyses, I used mctoolsr version 0.1.1.9 (Leff, 2022) which was created to handle large microbial community datasets. I rarefied the data to 9000 sequences per sample using the single_rarefy function for accurate comparison between samples as reads per sample ranged from 9,426 to 76,916. I measured alpha diversity by calculating richness (# of

OTUs) with the mctoolrs calc_diversity function and Shannon's diversity using the vegan package v 2.6.4 diversity function (Oksanen et al., 2022). I measured beta diversity first using mctoolrs calc_ordination function to create Principal Coordination Analysis (PCoA) plots. I ran permutational multivariate analysis of variance (PERMANOVAs) in the vegan package to test for significant differences between the soil inoculum and seed cultivar treatments on bacterial beta diversity. I used *metacoder* version 0.3.6 to create differential abundance heat trees to run comparisons between the starting soil inocula, Bad River and Hachita cultivars in both the control inoculum and previously droughted inoculum. P-values were calculated by a Wilcoxon rank-sum test followed by a Benjamini-Hochberg (FDR) multiple testing correction were calculated to assess significance differences in OTUS for each comparison.

RESULTS

Effect of soil inoculum and seed cultivar on plant performance

There was not a significant effect of either soil inoculum source or seed cultivar on maximum height (Fig 1, Table S1). However, there was a significant effect of soil inoculum on growth rate, with both seed cultivars having a higher, but not different, mean relative growth rate with the previously droughted soil inoculum (*p*=0.041; Fig. 2, Table S2). Both soil inoculum and seed cultivar did not influence aboveground or belowground biomass (Fig. 3 ,Table S3).

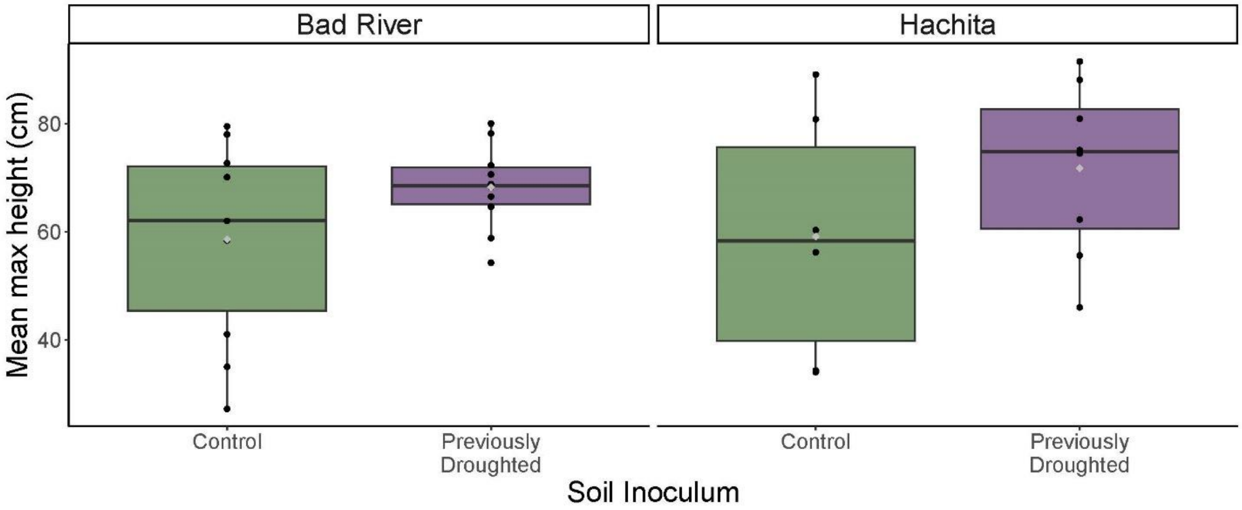

Figure 1. Effects of soil inoculum and seed cultivar treatments on maximum height for the thirteen weeks of height measurements. Boxplots are shown, with the box center line represents the median and the whiskers are 1.5x the interquartile range. The grey dot represents the mean for each treatment. No significant differences were found among the treatments (Table S1).

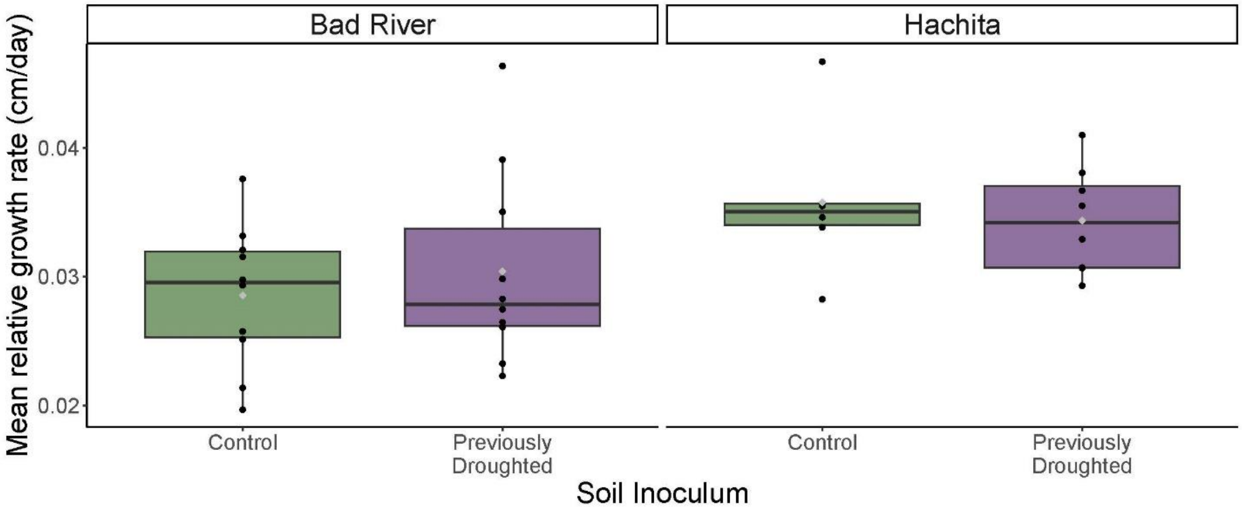

Figure 2. Effects of soil inoculum and seed cultivar treatments on relative growth rate (see Methods for details) for the thirteen weeks of height measurements. Boxplots are shown, with the box center line represents the median and the whiskers are 1.5x the interquartile range. The grey dot represents the mean for each treatment. No significant differences were found among the treatments (Table S2).

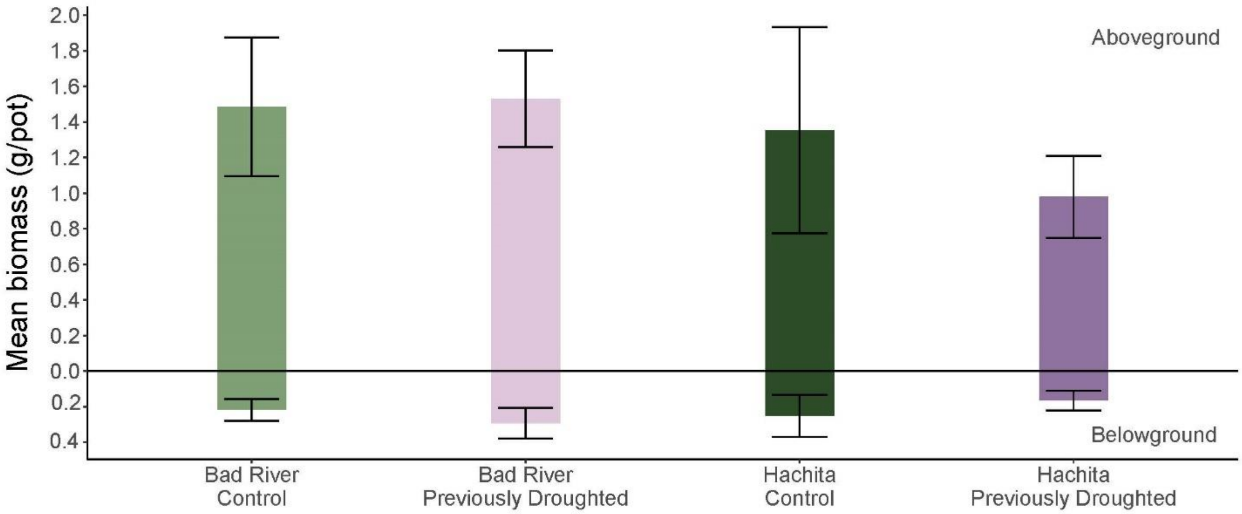

Figure 3. Effects of soil inoculum and seed cultivar treatments on aboveground (top) and belowground (bottom; mean ± SE for both) for the thirteen weeks of height measurements. No significant differences were found among the treatments (Table S3)

<u>Effect of seed cultivar on soil microbiome</u>

Alpha Diversity

For the starting soil inoculum that were created from the field soil and then applied to each treatment pot at the beginning of the experiment (week 0), there was not a significant difference in the bacterial communities between the control and previously droughted for Shannon's diversity (Fig. S1, Table S7). However, there was a trend of lower Shannon's diversity in the control inoculum compared to the previously droughted inoculum. There was significant difference in richness between the starting soil inocula (p=0.0229; Fig. S1, Table S8); a similar trend to Shannon's diversity was observed with lower richness in the control inoculum compared to the previously droughted inoculum.

There was a significant effect of soil inoculum on Shannon diversity, but not richness, for bacterial communities associated with the Bad River cultivar (p=0.00495; Fig. 4, Table S9). Bad

River bacterial communities had higher Shannon's diversity and a trend towards higher richness with the control soil inoculum compared to the previously droughted inoculum. For Hachita, there was no significant effect of soil inoculum source on Shannon diversity (Fig. 4c, Table S11) or richness (Fig. 4 ,Table S12). In contrast to the Bad River cultivar, there was a trend of lower Shannon's diversity in the control soil inoculum but not for richness for the Hachita cultivar.

Beta diversity

In the starting soil inocula, there was not a significant difference between the control and previously droughted soil inocula on bacteria diversity (Fig. S2, Table S13). However, this may be due to a small sample size (n=3 of each inoculum) as visually there is no overlap between the control soil inoculum and previously droughted soil inoculum.

Overall, there was a significant effect of soil inoculum source on bacterial beta diversity (p=0.001, Table S14) but no significant effect of seed cultivar (Table S14). When separated for each time point, soil inoculum beta diversity was different for each week (Fig. 5; Week 1 soil inoculum ($p<0.001$; Table S15), Week 9 soil inoculum ($p<0.001$; Table S15), Week 16 soil inoculum ($p<0.001$; Table S15). There was not a significant effect of seed cultivar on beta diversity in each time point (Fig. 4; Table S15).

Bacterial phylum and class differences

In the starting soil inocula, bacterial classes Vampirovibrionia and Ktedonobacteria were more abundant in the control soil inoculum, whereas classes Fimbriimonadia, Deinococci, Ignavibacteria and Dormibacteria were more abundant in the previously droughted soil inoculum (Fig. S3). When comparing the beginning (week 1) bacterial communities vs. the ending bacterial communities (week 15) associated with the cultivars, there were no significant differences between Bad River and Hachita for the control soil inoculum (Fig. 6). However, class Nitrospiria was more abundant for the Bad River rhizosphere soil, while Fusobacteriota was more abundant in Hachita rhizosphere in week 1. In week 15, classes Nitrospiria, and Saccharimonadia were more abundant in Bad River, while Myxococcia and Kapabacteria were more abundant in Hachita.

For comparisons of the bacterial communities associated with the seed cultivars for the beginning (Week 1) communities vs. the ending communities (Week 15) in the previously droughted soil inoculum, there also were no significant differences (Fig. 7). However, bacterial class Nitrospiria was more abundant in Bad River, while Fusobacteriota, Fibrobacteria, Rubrobacteria, and Kapabacteria were more abundant in Hachita in Week 1. By the end of the experiment, in week 15, class Xenobia was more abundant in Bad River.

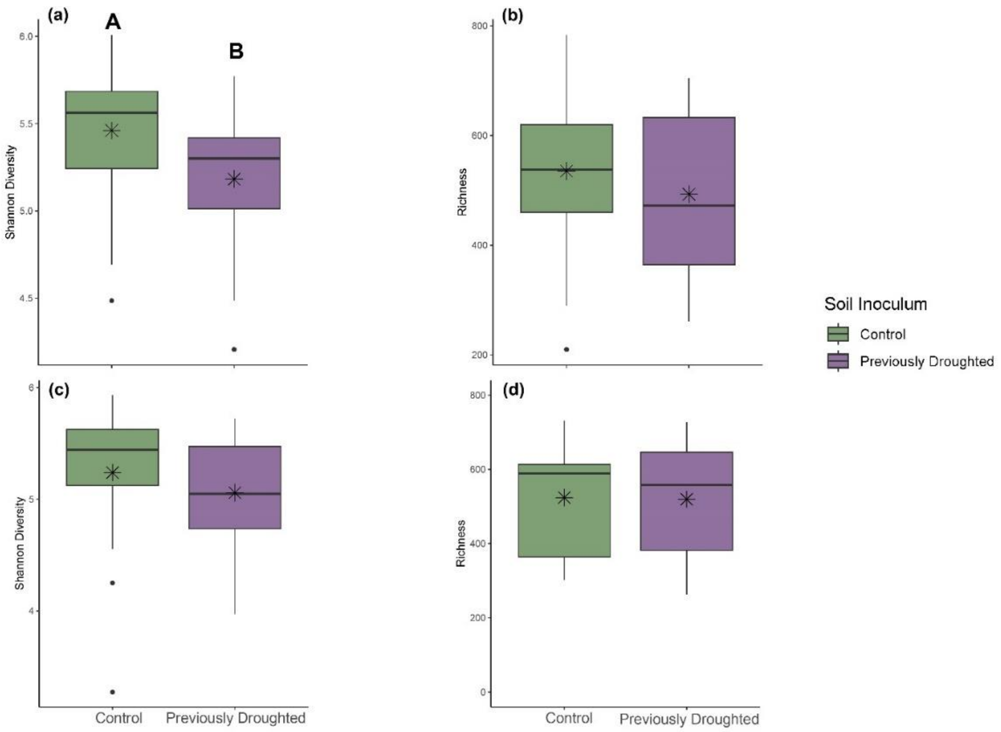

Figure 4. Bacterial Shannon diversity (left) and richness (right) in Bad River (a, b) and Hachita (c,d) soil inocula from all soil sampling points. The box center line represents the median and the whiskers are 1.5x the interquartile range. The black star represents the mean for each treatment. Significant differences were only found in Bad River Shannon diversity and are indicated by different letters ($p < 0.05$).

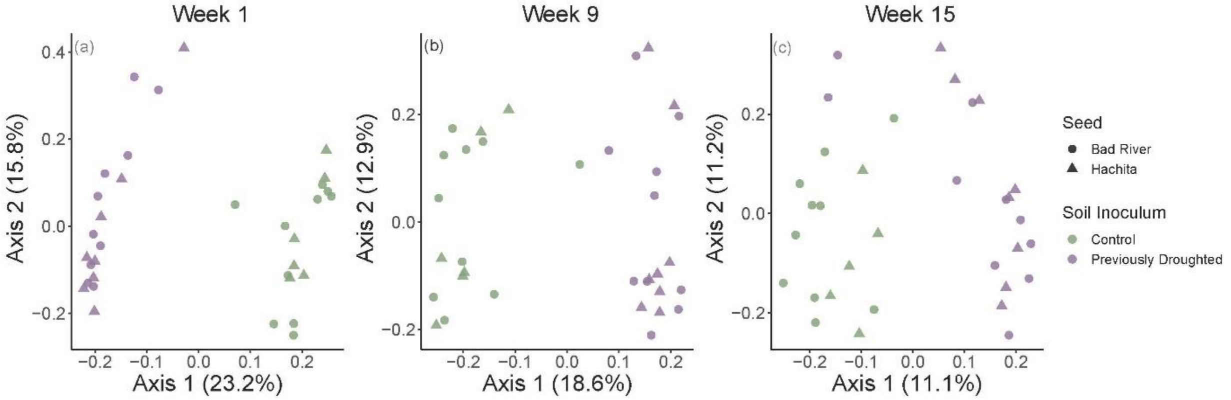

Figure 5. Bacterial beta diversity (principal coordinate analysis with Bray-Curtis dissimilarity metric) of soil inoculum and seed cultivar across soil sampling time points: Week 1 (a), Week 9 (b) and Week 15 (c). Significant differences on beta diversity between the soil inocula were found for each time point (Week 1 *p*<0.001, Week 9 *p*<0.001,Week 15 *p*<0.001).

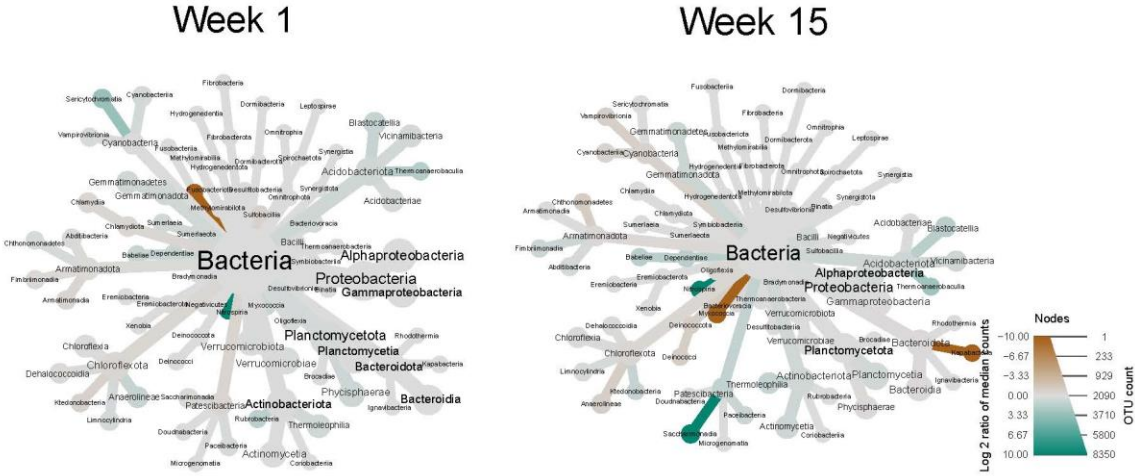

Figure 6. Comparison of taxa (down to class level) between the seed cultivars, Bad River and Hachita, in the control soil inoculum for Week 1 and Week 15. The size of the nodes is proportional to the abundance of the taxon, while the color indicates the log-2 ratio of median proportions of OTUs observed in each sample. Teal corresponds to greater abundance in Bad River while brown corresponds to greater abundance in Hachita. Adjusted p-values were

calculated by the Wilcoxon rank-sum test followed by a Benjamini-Hochberg (FDR) correction for multiple testing. There were no significant differences.

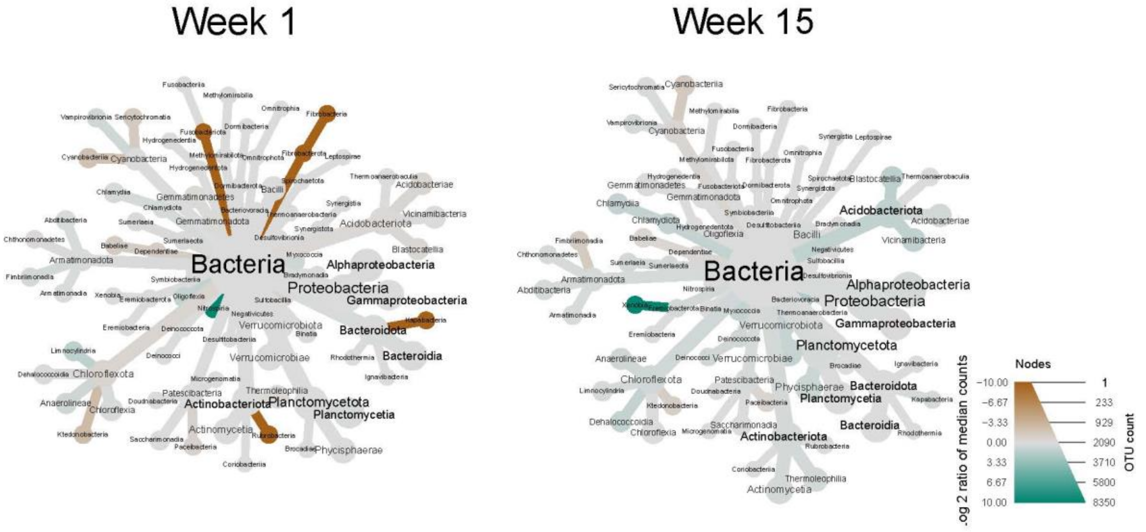

Figure 7. Comparison of taxa (down to class level) between the seed cultivars, Bad River and Hachita, in the previously droughted soil inoculum for Week 1 and Week 15. The size of the nodes is proportional to the abundance of the taxon, while the color indicates the log-2 ratio of median proportions of OTUs observed in each sample. Teal corresponds to greater abundance in Bad River while brown corresponds to greater abundance in Hachita. Adjusted p-values were calculated by the Wilcoxon rank-sum test followed by a Benjamini-Hochberg (FDR) correction for multiple testing. There were no significant differences.

DISCUSSION

The goal of this study was to assess if *B. gracilis* seed cultivars differentially performed when exposed to soil microbial communities that were or were not exposed to extreme drought, and if the coupled effects of seed cultivar and soil microbes would modify plant growth and performance. As both seed cultivars used in the present study, Bad River and Hachita, are classified as drought-tolerant (United Stated Department of Agriculture 1982; United States Department of Agriculture 1997), I expected that both cultivars would perform moderately well. However, Bad River was originally sourced from South Dakota where annual precipitation is

406.4 mm while Hachita is from New Mexico where annual precipitation is 250.0 mm (United Stated Department of Agriculture 1982; United States Department of Agriculture 1997). Taking this into consideration, I expected that Hachita may have an advantage compared to Bad River, as Hachita cultivars are experience drier soils. As neither cultivar was originally sourced from the shortgrass steppe grassland in northeastern CO, it is uncertain how they would be affected by the native soil microbial communities. Hence, the shortgrass steppe soil microbiota may interact differently with the cultivars and cause unexpected phenotypic responses. Soil microbiota have been shown to play a vital role in grassland restoration, including facilitating seed establishment (Duell et al., 2022; Koziol et al., 2023). Understanding how drought legacy effects in the soil microbiome interact with these seed cultivars could provide deeper insight into how to improve restoration practices in the semi-arid grasslands of the western US.

While my findings revealed no statistical significant differences between any soil inoculum x seed cultivar treatments in maximum height, relative growth rate, above- or belowground biomass, or flower count and biomass (Figs. 1, 2,3, Table S5, S6), there were trends observed. Both seed cultivars had a higher maximum height and relative growth rates when grown with the previously droughted inoculum (Figs. 1,2). Similar greenhouse experiments (e.g., Ulrich et al., 2019) observed trends in higher growth in *B. gracilis* shoots when inoculated with soil microbes under well-watered conditions, providing more evidence for *B. gracilis* opportunistic growth strategy (Turner & Klipple 1952). It is possible when exposed to the previously droughted soil microbial communities, Bad River cultivars express drought avoidance strategies that could explain the patterns of higher relative growth rate.

Soil microbes can modify flowering time and even accelerate it (Lau & Lennon, 2011; Fitzpatrick et al., 2019). Here, soil inoculum did not have an effect on flower count or biomass

(Table S5, S6) and flowering time was not assessed. In future studies, it will be critical to analyze flowering time to investigate this phenomenon.

How the seed cultivars interact with the native soil microbiome is a crucial component to consider in restoration practices. It is well-known that drought events cause shifts in the microbial community and composition, with general trends of drought events leading to enrichments of Gram-positive bacteria and depletion Gram-negative bacteria (Naylor & Coleman-Derr 2018). It also has been demonstrated that genotypes under drought events can associate with specific soil microbes that may be beneficial to plant drought tolerance (Santos-Medellín et al., 2017). In the present study, growing these two drought-tolerant seed cultivars with the native shortgrass steppe grassland microbiome allowed me to assess if the cultivars have different impacts on the soil microbiota.

Before the soil inocula were applied to each treatment pot, I assessed whether there were differences in microbial community composition between the control and previously droughted soil bacterial communities. While there was not a significant difference in the bacterial beta diversity, there were stark visual differences in the starting soil inocula bacterial composition, providing evidence that these soil inocula have different bacterial communities (Fig S3) that can be attributed to the past exposure to different climate conditions. These differences between the soil inoculum treatments were maintained throughout the whole experiment and were statistically different (Fig. 5, Table S15). Interestingly, there was no effect of the seed cultivars on bacterial beta diversity (Fig. 5, S15). I compared taxa at the class-level between seed cultivars in both the control and previously droughted soil inocula at the beginning (Week 1) and end (Week 15) of the experiment to assess whether the seed cultivars began to associate with any specific bacteria. While none of the comparisons yielded statistically significant differences, by the end of the experiment in the control inoculum, Bad River and Hachita associated with different bacteria-- Bad River with gram-negative bacteria, Nitrospiria, and Saccharimonadia, Hachita with gram-negative bacteria, Myxococcia and Kapabacteria. In the previously droughted soil inoculum, Bad River pots were initially enriched with Nitrospiria, then later with Xenobia bacteria.

The genus Nitrospiria is known for its ability to perform complete oxidation of ammonia to nitrate (termed as comammox) and is widespread in terrestrial ecosystems (Li et al., 2023). Nitrospiria was previously thought to solely live in oligotrophic environments (low nutrients, high oxygen), which is the type of environment that is found in droughted soils, but recent work has shown that Nitrospiria can thrive under copiotrophic environments as well (nutrient rich) (Li et al., 2023; Naylor & Coleman-Derr 2018). The copiotroph–oligotroph trends found for Nitrospiria help explain the pattern observed here with Nitrospiria being found in both soil inocula. Explaining why Bad River associates with Nitrospiria more than Hachita is more difficult, as research on Nitrospiria is still in its infancy. It could be due to niche differences driven by plants that have been observed with well-known ammonia-oxidizing bacteria and archaea communities (Trivedi et al., 2019), but more research will need to be done to fully explain these results.

Conclusions

In the restoration field, there has been debates of where and how seeds should be sourced for reseeding efforts particularly for grasslands (Bucharova et al., 2019; Lyons et al., 2023). A growing body of research has focused on addressing knowledge gaps with recent studies focused on either the seed cultivar genetics (e.g Tso & Allan 2018) or the impact soil microbes on grassland restoration (e.g Kozio et al., 2021; Duell et al., 2022). However, understanding the complex interactions between dominant grassland species and the soil microbiome needs to be further investigated to ensure that the seed cultivars will thrive in the native soil microbial communities present at the restoration site.

Overall, my results of no significant differences in plant growth and performance between these two seed cultivars with control vs. previously droughted soil microbial communities is surprising. I expected Hachita cultivars to perform better than Bad River due to their origin from a dry climate similar to Colorado. However, finding no significant differences in the rhizosphere bacterial communities between seed cultivars for both the control soil and previously droughted inocula could explain this lack of difference in plant growth and performance. Overall, these results suggest that both the seed cultivars would interact positively with the native soil microbiota in the shortgrass steppe after extreme drought.

While these results are promising, I note that this study was conducted in a greenhouse under isolation, and therefore results may differ when exposed to other variables such as competition with other species or variation in environmental conditions. Furthermore, all the treatment pots were well-watered (20% Volumetric Water Content) throughout the duration of the experiment which is consistently wetter than conditions found in the field. These well-watered conditions could lead to a difference in plant growth and performance observed here versus out in the field. Despite these caveats, these results provide a good starting point for future studies which should aim to include multiple cultivars of *B. gracilis* in greenhouse studies to examine how the non-drought tolerant cultivars perform when exposed to droughted soil microbial legacies. Additionally, applying recurrent drought events would test if the previously droughted soil microbiota provide resilience to the plants as demonstrated in other studies (Lau & Lennon 2012; Ulrich et al., 2019). Finally, future studies should strive to be conducted both in the greenhouse and field to fully understand the complex interactions between *B. gracilis* cultivars and the soil microbiome.

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APPENDIX 1

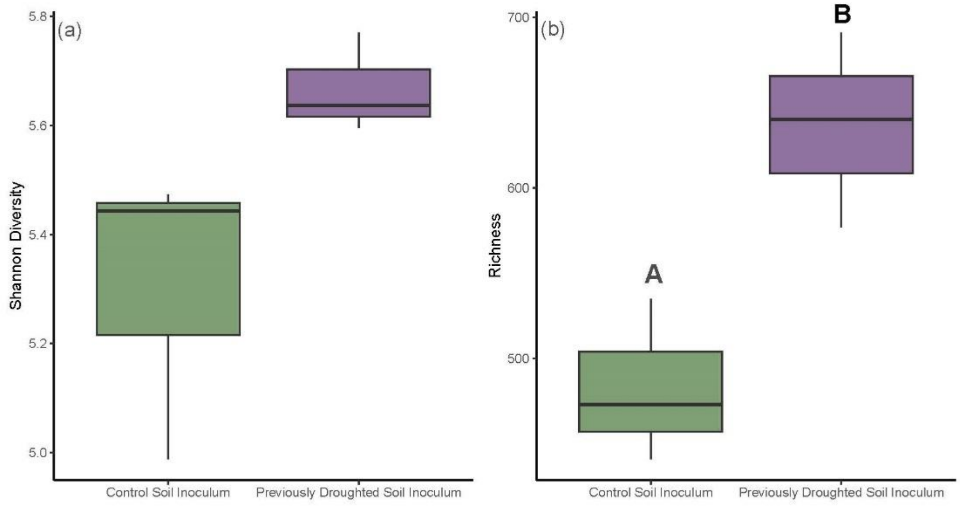

Figure S1. Bacterial Shannon diversity (a) and richness (b) in starting soil inocula. The box center line represents the median and the whiskers are 1.5x the interquartile range. The black star represents the mean for each treatment. Significant differences were only found in richness between the two soil inocula and are indicated by different letters ($p < 0.05$, Table SX)

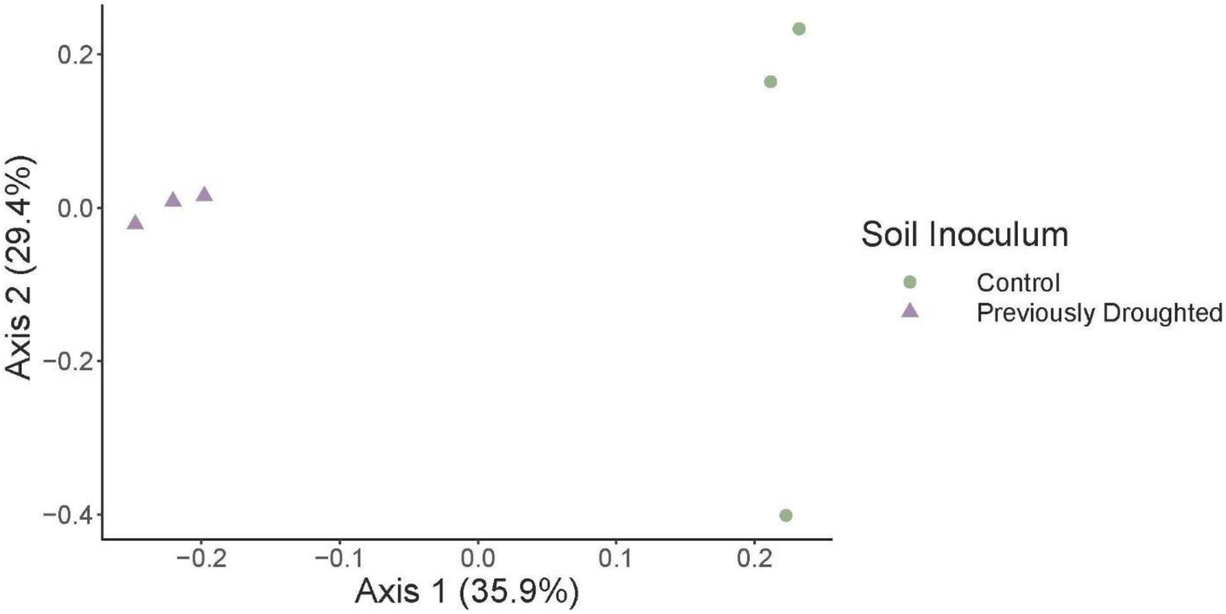

Figure S2. Bacterial beta diversity (principal coordinate analysis with Bray-Curtis metric) of starting soil inocula. Significant differences were only found for beta diversity for Week 1 soil inoculum ($p<0.001$), Week 9 soil inoculum ($p<0.001$),Week 15 soil inoculum ($p<0.001$)

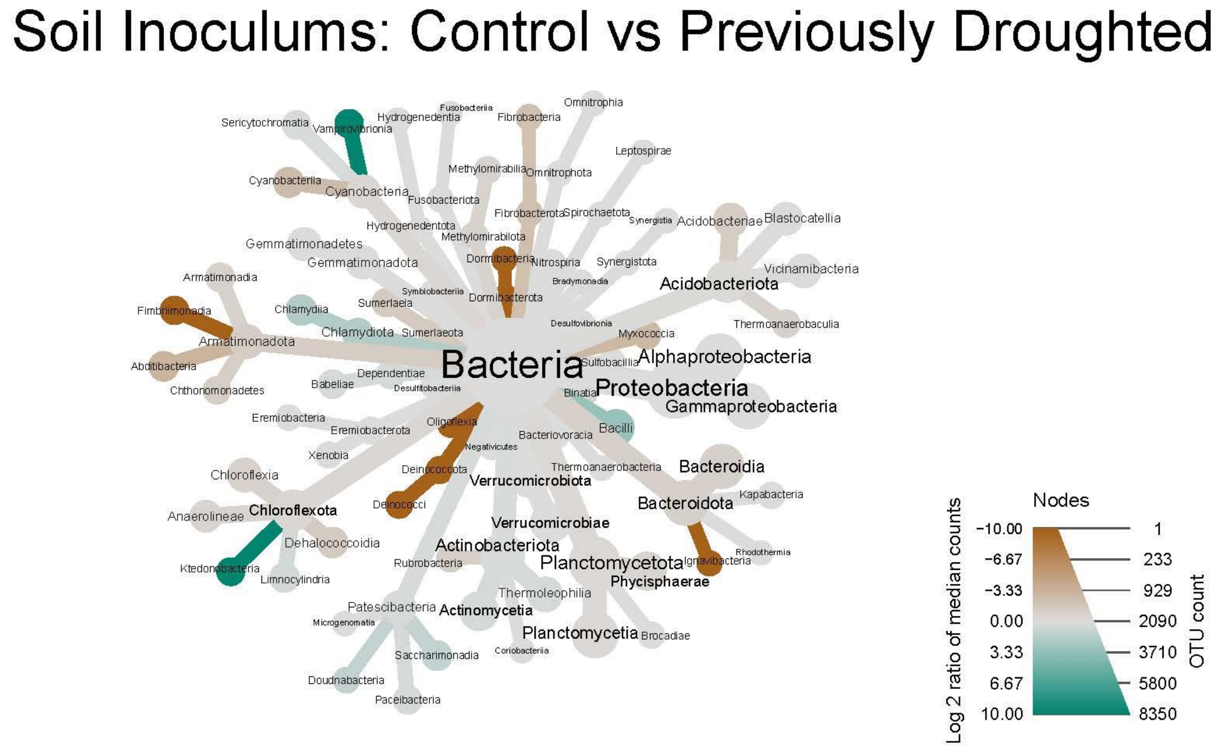

Figure S3. Comparison of taxa (down to class level) between the starting soil inocula. The size of the nodes is proportional to the abundance of the taxon, while the color indicates the log-2 ratio of median proportions of OTUs observed in each sample. Teal corresponds to greater abundance in Control soil inoculum while brown corresponds to greater abundance in Previously Droughted soil inoculum. Adjusted p-values were calculated by the Wilcoxon rank-sum test followed by a Benjamini-Hochberg (FDR) correction for multiple testing. There were no significant differences.

Table S1. Mixed model with Kenward-Rogers method output for Max Height

	Sum of Squares	Mean Squares	Numerator Degrees of Freedom	Denominator Degrees of Freedom	F-value	p-value
Soil Inoculum	621.99	621.99	1	27.424	3.2234	0.08362
Seed	8.30	8.30	1	27.366	0.0430	0.83724
Soil Inoculum: Seed	5.38	5.38	1	27.601	0.0279	0.86858

Table S2. Mixed model with Kenward-Rogers method output for Mean growth Rate

	Sum of Squares	Mean Squares	Numerator Degrees of Freedom	Denominator Degrees of Freedom	F-value	p-value
Soil Inoculum	0.125738	0.125738	1	27.553	4.3768	0.04577
Seed	0.025013	0.025013	1	27.476	0.8707	0.35890
Soil Inoculum: Seed	0.001449	0.001449	1	27.771	0.0504	0.82394

Table S3. Mixed model with Kenward-Rogers method output for Aboveground biomass

	Sum of Squares	Mean Squares	Numerator Degrees of Freedom	Denominator Degrees of Freedom	F-value	p-value
Soil Inoculum	0.56612	0.56612	1	27.556	0.6415	0.4300
Seed	1.11560	1.11560	1	27.478	1.2641	0.2706
Soil Inoculum: Seed	0.54738	0.54738	1	27.774	0.6203	0.4376

Table S4. Mixed model with Kenward-Rogers method output for Belowground biomass

	Sum of Squares	Mean Squares	Numerator Degrees of Freedom	Denominator Degrees of Freedom	F-value	p-value
Soil Inoculum	0.001703	0.001703	1	28.075	0.0343	0.8543
Seed	0.023496	0.023496	1	27.921	0.4737	0.4970
Soil Inoculum: Seed	0.050776	0.050776	1	28.393	1.0236	0.3202

Table S5. Linear model output for Flower count. Analysis was run as a one-way factor test (soil inoculum or seed cultivar) as the Previously droughted x Hachita seed didn't flower

	Degrees of Freedom	Sum of Squares	Mean squares	F-value	p-value
Soil Inoculum	1	0.0005333	0.00053333	0.4244	0.5294
Residuals	10	0.0125667	0.00125667		

Table S6. Linear model output for Flower biomass. Analysis was run as a one-way factor test (soil inoculum or seed cultivar) as the Previously droughted x Hachita seed didn't flower

	Degrees of Freedom	Sum of Squares	Mean squares	F-value	p-value
Seed Cultivar	1	0.00294	0.002940	2.8937	0.1198
Residuals	10	0.01016	0.001016		

Table S7. ANOVA output for bacteria for starting soil inoculum Shannon Diversity

	Degrees of Freedom	Sum of Squares	Mean Squares	F-value	p-value
Soil Inoculum	1	0.2010	0.20104	5.359	0.0816
Residuals	4	0.1501	0.03752		

Table S8. ANOVA output for bacteria for starting soil inoculum Richness

	Degrees of Freedom	Sum of Squares	Mean Squares	F-value	p-value
Soil Inoculum	1	37446	37446	12.91	0.0229
Residuals	4	11600	2900		

Table S9. ANOVA output for bacteria for Bad River Shannon Diversity

	Degrees of Freedom	Sum of Squares	Mean Squares	F-value	p-value
Soil Inoculum	1	1.212	1.2117	8.539	0.00495
Residuals	58	8.230	0.1419		

Table S10. ANOVA output for bacteria for Bad River Richness

	Degrees of Freedom	Sum of Squares	Mean Squares	F-value	p-value
Soil Inoculum	1	27392	27392	1.52	0.223
Residuals	58	1044905	18016		

Table S11. ANOVA output for bacteria for Hachita Shannon Diversity

	Degrees of Freedom	Sum of Squares	Mean Squares	F-value	p-value
Soil Inoculum	1	0.353	0.3527	1.098	0.301
Residuals	39	12.530	0.3213		

Table S12. ANOVA output for bacteria for Hachita Richness

	Degrees of Freedom	Sum of Squares	Mean Squares	F-value	p-value
Soil Inoculum	1	83	83	0.004	0.952
Residuals	39	895651	22965		

Table S13. PERMANOVA output for starting soil inocula

	Degrees of Freedom	Sum of Squares	R2	F-value	p-value
Soil Inoculum	1	0.30071	0.40223	2.6916	0.1
Residual	4	0.44689	0.59777		
Total	5	0.74761	1.00000		

Table S14. PERMANOVA output for bacteria for all soil sampling time points

	Degrees of Freedom	Sum of Squares	R2	F-value	p-value
Soil Inoculum	1	2.3875	0.08828	9.6081	0.001
Seed	1	0.3331	0.01232	1.3404	0.127
Soil Inoculum: Seed	1	0.2202	0.00814	0.8862	0.589
Residual	97	24.1033	0.89126		
Total	100	27.0441	1.00000		

Table S15. PERMANOVA output for bacteria for Week 1, Week 9, and Week 16

Week 1					
	Degrees of Freedom	Sum of Squares	R2	F-value	p-value
Soil Inoculum	1	1.5650	0.25350	10.7174	0.001
Seed	1	0.0997	0.01615	0.6829	0.800
Soil Inoculum: Seed	1	0.1282	0.02076	0.8777	0.521
Residual	30	4.3808	0.70959		
Total	33	6.1737	1.00000		
Week 9					
Soil Inoculum	1	1.1184	0.15077	5.6360	0.001
Seed	1	0.2043	0.02754	1.0296	0.370
Soil Inoculum: Seed	1	0.1420	0.01914	0.7155	0.870
Residual	30	5.9529	0.80255		
Total	33	7.4175	1.00000		
Week 15					
Soil Inoculum	1	0.7090	0.08091	2.7339	0.001
Seed	1	0.3412	0.03894	1.3157	0.092
Soil Inoculum: Seed	1	0.1917	0.02187	0.7390	0.925
Residual	30	7.5212	0.85828		
Total	33	8.7631	1.00000		