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Soil fungal communities are compositionally resistant to drought manipulations – Evidence from culture-dependent and culture-independent analyses

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ABSTRACT

Current environmental change predictions forecast intensified drought conditions. It is becoming increasingly evident that plant communities are sensitive to drought and that soil-inhabiting microbial communities vary along precipitation gradients. However, the drought sensitivity of microbial communities in general and that of soil fungi in particular remains unclear, even though understanding their responses to adverse environmental conditions is vital for better understanding of ecosystem service provisioning. We sampled soils at two sites with established experiments that imposed extreme, chronic drought to assess fungal community responses. We analyzed fungal communities using both culture-dependent and -independent tools and MiSeq-sequenced communities from colony forming units (CFU-PCR) on a drought simulating medium and from environmental DNA (ePCR), to compare the conclusions derived from these two methods. Our data from the two approaches consistently indicate that the composition of fungal communities is not affected by the drought treatment, whereas – based on the CFU-PCR but not ePCR data – their richness and diversity increased under drought conditions at the more mesic of the two sites. Further, based on the direct comparisons of CFU-PCR and ePCR, we estimate that more than 10% of the fungal community and more than 20% of the ascomycetes were culturable. We conclude that although recent research indicates that plant and bacterial communities respond to drought, fungal community responses are more variable, particularly in experiments that impose chronic drought under field conditions.

1. Introduction

The Intergovernmental Panel on Climate Change forecasts changes in climatic means, greater climatic variability, and more frequent extreme weather events including floods, droughts, and heat waves (IPCC 2012). Aboveground plant productivity is sensitive to the predicted shifts in precipitation (Shi et al., 2014; Wu et al., 2018; Felton et al., 2019). Further, the more arid the system, the more sensitive its productivity may be to reduced precipitation and prolonged drought (Huxman et al., 2004; Knapp et al., 2015). Drought events, generally conceptualized by ecologists as a "prolonged absence or marked deficiency of precipitation" (Slette et al., 2019), may have severe and long-lasting effects on terrestrial ecosystems (Weaver and Albertson 1936; Breshears et al., 2005) and the ecosystem services that they provide (McLaughlin 2014). However, such effects vary in magnitude, take place on different timescales, and may be affected by an array of mechanisms (Shi et al., 2014).

Overall, while substantial insights have been gained in the ecosystem process and plant community responses to alterations in precipitation, soil community data are sparse and few empirical studies have focused on the long-term impacts that altered precipitation regimes have on soil microbes (Ochoa-Hueso et al., 2018). Such long-term manipulations are essential to unveil lagging or

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delayed responses to changes in precipitation (e.g., Deveautour et al., 2019). Diverse soil communities are essential providers of ecosystem services (Delgado-Baquerizo et al., 2016), including facilitation of productivity and control of the composition of plant communities (van der Heijden et al., 1998) or regulation of nutrient cycling (Locey and Lennon 2016). Therefore, understanding the effects of predicted shifts in climatic conditions on organismal assembly and community composition demands attention, as such changes may alter the soil community functions and derived ecosystem services (Bellard et al., 2012; Manzoni et al., 2012; McLaughlin 2014).

Thus far, empirical data and meta-analyses conclude that droughts will impact diversity and abundance of soil-inhabiting microbial communities (Wu et al., 2011; Wang et al., 2020), although microbial biomass may be more sensitive to rainfall deficiencies than community composition (Ren et al., 2018). No current consensus exists on the magnitude or direction of soil community responses to drought. Some studies report moisture-related changes in soil microbial community composition (Bernard et al., 2013; McHugh et al., 2014; Maestre et al., 2015) and function (Zeglin et al., 2013; Bach and Hofmockel 2015), whereas others have observed few differences in the microbial communities inhabiting wet and dry soils (Landesman and Dighton 2010; Curiel-Yuste et al., 2014).

Similar to microbial communities in general, evidence is accumulating that soil water availability may affect the abundances and diversity of soil fungi (Toberman et al., 2008; Castro et al., 2010; Hawkes et al., 2011; Curlevski et al., 2014; de Oliveira et al., 2020), whereas the community composition may be more resistant to changes in precipitation or driven by other factors (Zhang et al., 2013; Curlevski et al., 2014; Kaisermann et al., 2015). Although some studies highlight the fungal community resilience to changes in water availability (e.g., Jumpponen and Jones 2014), these observations are far from universal generalities. Ochoa-Hueso et al. (2018), using chronic drought manipulations in North American and Australian grassland systems, reported that bacterial and fungal communities consistently responded to the manipulations, although fungi did so to a lesser extent than bacteria. Interestingly, they reported that the fungal responses were proportional to water availability and that the fungal communities in the more mesic sites were more responsive to drought than those in the arid sites. This is particularly interesting as the fungal responses contrast those observed for plant communities (Grime et al., 2000; Tielbörger et al., 2014). Zhang et al. (2015), in a study attempting to decouple co-limitations and interactive effects of warming, drought and nutrient additions in temperate grasslands in China, predicted that it is indeed water limitations that are the underlying primary control of community responses to interacting environmental change factors. They based this prediction on their observations that detrimental responses to warming only occurred when coinciding with dry conditions and positive responses to nutrients, in contrast, were only observed when water was not severely limiting. Further, Hawkes et al. (2011) reported that over four years of rainfall manipulations in a grassland system in northern California, more abundant, diverse and consistent communities predominated under the dry conditions, whereas the wetter conditions supported distinct, less abundant, less diverse and more dynamic communities. As illustrated by Zhang et al. (2015), the community responses may be context dependent or differ depending on historical legacies. In the study by Hawkes et al. (2011), the community responses to spring rain additions were only observed when the rainfall exceeded a stress threshold and did not do so consistently through the span of the experiment. The inconsistencies in fungal community responses to manipulations of water availability may also lay in the phylogenetic context: some taxa may indeed be drought tolerant or even xerophilic and their responses relative those of drought sensitive taxa likely determine the community dynamics that the researchers observe (de Oliveira et al., 2020).

The empirical data on microbial responses to precipitation in general and fungal responses in particular appear contradictory and conflicting, highlighting the urgent need for greater research emphasis to elucidate soil community responses to environmental fluctuations. In this study, we used a drought manipulation experiment at two sites to specifically examine whether fungal communities respond to experimental drought. We hypothesized that fungal communities would shift their composition and richness in response to chronic drought. These predictions are consistent with previous studies that report fungal community changes in response to variability in drought and precipitation (Clark et al., 2009; Hawkes et al., 2011; Ochoa-Hueso et al., 2018). We tested these hypotheses using culture-dependent and culture-independent approaches. We then compared the results from our two approaches to estimate the proportion of culturable fungi in our samples.

2. Methods

2.1. Field sites and soil sampling

We utilized an established field experiment, the Extreme Drought in Grassland Experiment (EDGE), which spans six grassland sites in the central and southwestern United States (Knapp et al., 2015). Established in 2014, each EDGE site consists of replicate 6 × 6 m^2 plots designed to evaluate ecosystem responses to chronic drought imposed using 10 × 10 m^2 rainout shelters (Stuppy Inc., Kansas City, MO) designed to passively reduce each rainfall event by 66% during the growing season − April−September in our sites − resulting in an approximate 40% reduction in the precipitation overall (Griffin-Nolan et al., 2019). The experiment also includes an ambient precipitation control without a rainout shelter.

For this project, we used two of the six EDGE grassland sites: Hays Agricultural Research Center (HAR) located in western KS (39°05′N 99°09′W) and the Konza Prairie Biological Station (KPBS) located in northeastern KS (39°05′ N, 96°35′ W). The HAR site is a mixed grass prairie with MAP of ~577 mm, whereas KPBS is a tallgrass prairie and receives nearly 50% greater MAP, ~860 mm. Each site contains both chronic drought and ambient control treatments, with ten replicate plots for each. Each plot is divided into four 2 × 2 m sampling subplots. In the 4th year of the chronic drought (June 2018), we collected three soil cores (10 cm depth; 1.8 cm diameter) within a subplot dedicated to destructive sampling in each experimental unit. We sampled the topmost profile of the soil as it represents the highest microbial biomass and activity. The three soil cores were composited into one sample per experimental unit for each of the two sites (N = 40 samples), sealed in Ziploc bags, transferred on ice to the laboratory, and stored at −20 °C within 12 h of harvesting to await further processing.

2.2. Soil dilution series and isolation of soil-borne fungi

The composited soil samples were manually homogenized and roots and rocks removed by hand. A 5g subsample was oven-dried for 48 h at 55 °C to estimate gravimetric water content, and the dry to fresh weight (dw:fw) ratio was recorded. Given that the fungal colony count was unknown, we established a 10-fold dilution series to isolate soil-borne fungi (Seeley and Vandemark, 1981). A total of 1g of soil (fw) thawed from −20 °C storage was diluted in 9 mL sterile, deionized water for a 1:10 dilution, and serially diluted up to 1:10,000. Two 1 ml aliquots of each dilution were stored in −20 °C

for further processing and a 200 μl aliquot of each dilution level of each sample was plated on two different pure culture media. We chose Dichloran Glycerol Agar (DG-18; ThermoScientific, Pittsburg, PA, USA) which controls water availability (a_w = 0.95) and is commonly used as a selective medium for xerotolerant fungi from food stocks, and Potato Dextrose Agar (PDA; Difco Laboratories, Detroit, MI USA), which is a rich general medium with a comparable nutrient availability. To suppress bacterial growth and to establish defined fungal colonies, each medium was amended with 6 mL/L chloramphenicol and 50 mg/L Rose Bengal (Smith and Dawson 1944). Each dilution level of each sample was plated on both media types, resulting in a total of 240 plates. The establishing colonies were counted every two days for a total of ten days. After adjusting for dilution and dry weight, the number of fungal colony forming units (CFUs) was calculated for each of the forty experimental units.

2.3. DNA extraction from environmental and CFU samples

We compared environmental (ePCR) and pure-cultured (CFU-PCR) communities from the 1:100 dilution because this had a large – yet countable – number of fungal colonies. Although we expected the xerotolerant selective medium to inhibit fungal colonies, the CFU numbers on the drought-selective, DG-18 medium were greater than those on PDA (see also Copetti et al., 2009). As a result, we only used DG-18 for the ePCR and CFU-PCR comparisons. A 200 μl aliquot – equal to that plated for CFU estimation – was collected from the preserved 1:100 dilution sample and transferred to a DNA Isolation Kit PowerBead Tube (PowerSoil DNA Isolation Kit; MoBio Laboratories, Carlsbad, CA USA). Tissue from colonies established from the 1:100 dilution on the DG-18 medium was obtained by adding 2.5 mL of 0.1% Triton-X onto the plate. The colonies with the added surfactant were scrubbed vigorously using a flame-sterilized cell spreader, the liquid transferred into a 2 mL microcentrifuge tube, and centrifuged for 3 min at 10,000 g to pellet the extracted fungal tissue. The supernatant was removed without disturbing the pellet, replaced with 750 μl of the Bead Solution from the PowerSoil DNA Isolation kit, and the pellets vortexed until dissolved. The supernatant was transferred into the PowerSoil DNA Isolation Kit Bead tube for CFU-PCR DNA extraction. The forty soil aliquots for ePCR and forty colony extracts for CFU-PCR were processed as per the manufacturer's protocol, except that we reduced the homogenization time on vortex from 10 min to 1 min to avoid excessive DNA shearing.

2.4. ePCR, CFU-PCR, and MiSeq library preparation

We chose the Internal Transcribed Spacer 2 (ITS2) region of the ribosomal RNA gene – the proposed universal fungal barcode (Schoch et al., 2012) – for our analyses. We used the fITS7 (Ihrmark et al., 2012) and ITS4 (White et al., 1990) primers with unique 12bp barcodes in each 5′-end in 50 μl PCR reactions. The volumes and final concentrations of reagents were as follows: 10 μl forward and reverse primer (1 μM), 10 μL template DNA (1 ng/μl), 5 μl dNTP (200 μM), and 0.25 μL (1/2 unit) Phusion Green Hot Start II DNA polymerase (ThermoScientific, Pittsburg USA), 10 μL of Phusion 5X HF Buffer with 7.5 mM $MgCl_2$, and 14.5 μL molecular grade water. The PCR reaction began with an initial denaturing step for 30 s (98 °C) and was followed by 30 cycles with 10 s of denaturing (98 °C); 30 s of annealing (54 °C); 1 min of extension (72 °C); and, concluding with a 10 min final extension (72 °C). Amplification of PCR contaminants was determined by a negative PCR control in which templates were replaced with ddH_2O. Each sample was PCR-amplified in triplicate and 20 μL of each amplicon was combined into one per experimental unit. The pooled 60 μl amplicons were purified using the Agencourt AMPure XP Bead Clean-up solution (Backman Coulter, Indianapolis, IN) following a modified manufacturer protocol with a 1:1 ratio of PCR product to AMPure solution and two rinse steps with 80% ethanol. Following cleanup, a total of 250 ng of amplified DNA per experimental unit was pooled into one. Because the negative controls yielded DNA quantity measurements similar to the elution buffer alone, the entire elution volume from the cleanup (40 μl) was included. Illumina adapters and indices were added using four PCR cycles, KAPA Hyper Prep Kit (Roche, Pleasenton, CA USA), and 0.5 μg starting DNA. The library was sequenced (2 x 300 cycles) using the Illumina MiSeq Personal Sequencing System at the Integrated Genomics Facility (Kansas State University, Manhattan KS USA). The sequence data are available through the Sequence Read Archive under BioProject PRJNA702439; BioSamples SAMN17948325- SAMN17948404.

2.5. Sequence data processing

The sequence data were processed using the mothur pipeline (v. 1.38.0; Schloss et al., 2009) following the MiSeq standard operating protocol to generate OTUs (Kozich et al., 2013). In brief, the sequence data were extracted from the paired-end fastq files and contiged. Sequences with more than 1 bp difference with the primers, without an exact match to the sample-specific identifiers, or with long homopolymers (maxhomop = 9) were omitted. The sequences were truncated to the length equal of the shortest high-quality read (236 bp excluding primers and sample-specific identifiers), >99% similar sequences pre-clustered (Huse et al., 2008), and potential chimeras identified (UCHIME; Edgar et al., 2011) and removed. The quality-screened sequences were clustered using vsearch (Rognes et al., 2016) and assigned to Operational Taxonomic Units (OTUs) at 97% similarity. Rare OTUs (2340 OTUs or ~49% of OTUs representing a total of 12,711 sequences and less than 0.7% of the retained sequence data) represented by fewer than ten sequences in the entire dataset were removed as potential artifacts (Brown et al., 2015; Oliver et al., 2015). Additionally, all OTUs (total of 72 OTUs, less than 3% of all retained OTUs, representing 5057 sequences or less than 0.3% of all retained sequence data) that were detected in the negative controls were removed from the dataset as potential contaminants. The remaining OTUs were assigned to taxon affinities using the Naïve Bayesian Classifier (Wang et al., 2007) and the UNITE taxonomy reference (http://unite.ut.ee/repository.php; Köljalg et al., 2013). Non-target OTUs (those with no match in the UNITE-curated INSD, or assigned to Protista and Plantae in this dataset) were removed from further analyses.

2.6. Statistical analyses

We compared $\log_{10}$-transformed CFU counts on DG-18 and PDA across the two sites and across the treatments within sites using Analysis of Variance (ANOVA) in a nested design wherein the treatment was nested within site. For the CFU-PCR and ePCR communities, we calculated diversity and richness estimators in mothur (v. 1.38.0; Schloss et al., 2009). We estimated Good's coverage (complement of the ratio between local singleton OTUs and the total sequence count) for each experimental unit to evaluate the representativeness of our sampling. To estimate richness and diversity, we iteratively (100 iterations) calculated observed (S_{Obs}) and extrapolated (Chao1) OTU richness, diversity (Shannon's diversity; $H' = \sum p_i \ln p_i$), and evenness (Shannon's equitability; $EH = H'/\ln S_{Obs}$) with the data subsampled to 2297 sequences per experimental unit, as recommended in Gihring et al. (2011) to avoid biased comparisons of diversity and richness estimators in samples with unequal sequence yields. The richness and diversity estimators between the CFU-PCR and ePCR datasets were compared using the paired, non-parametric Wilcoxon tests that are based on simple

signed rank scores; in case of ties, average ranks were used. The p-values were based on Student's t approximations (Iman 1974). Because the two assay methods substantially differed in the richness and diversity metrics, the site and treatment effects were tested separately for the CFU-PCR and ePCR communities. In these analyses, the $\log_{10}$-transformed richness and diversity estimators across the sites and treatments were compared using Analysis of Variance (ANOVA) in a nested design wherein the drought treatment was nested within site. The "within site" treatment effects were further tested using slice effects that test for differences with each level of a treatment within each level of another; in our case, control vs. drought within each of HAR and KPBS.

To visualize and infer compositional differences in the fungal community composition, we estimated pairwise Bray-Curtis distances and analyzed these data with Non-metric Multidimensional Scaling (NMDS; Mather 1976) in mothur (v. 1.38.0; Schloss et al., 2009). The optimal number of dimensions (k) was selected based on stabilizing stress less than 0.20 using 100 runs with empirical data and a random seed starting value. The entire dataset comparing CFU-PCR and ePCR communities across all samples was optimally resolved in four axes (k = 4). Similar to the richness and diversity analyses, the CFU-PCR and ePCR communities were compositionally distinct and we consequently analyzed these communities separately as well. The CFU-PCR and ePCR communities were optimally resolved with three (k = 3) and four (k = 4) dimensions, respectively. The community data across the sites and treatments were compared using a nonparametric permutational analog of traditional analysis of variance (Analysis of Molecular Variance – AMOVA; Excoffier et al., 1992; Anderson 2001). We also tested whether the variability in the fungal communities inferred from the ePCR and CFU-PCR or among the treatment combinations was homogeneous using Homogeneity of Molecular Variance (HOMOVA). HOMOVA is a nonparametric analog of Bartlett's test for homogeneity of variance, which has been used to test whether or not pairwise distances within two or more populations are homogeneous (Stewart and Excoffier 1996). To identify differentially abundant OTUs among those that commonly occurred in our data, we identified the core communities – those present in at least 25% of all samples – which included a total of 102 OTUs. In the case of compositionally distinct communities, the core communities were further queried for differentially abundant OTUs across treatment conditions using the metastat function and 100,000 iterations in mothur (v. 1.38.0; Schloss et al., 2009). To account for multiple comparisons, the pairwise error rate was conservatively Bonferroni-corrected.

3. Results

3.1. General description of the sequence data

We retained a total of 1,814,334 sequences after quality control and removal of rare or potential contaminant OTUs. The sequencing yields ranged from 2297 to 48,170 per sample with a mean yield of 22,679 ± 10,443 (st. dev.). The taxon affinities of OTUs and their observed frequencies are listed in Supplemental Table S1. In brief, the sequence data were overwhelmingly dominated by ascomycetes (87.3% of all sequences and 56.4% of all OTUs), but included many basidiomycetes (7.0% of all sequences and 20.7% of all OTUs) plus some glomeromycetes (0.2% of all sequences and 3.0% of all OTUs) and basal taxa (Chytridiomycota, Zygomycota, and Rozellomycota; 3.5% of all sequences and 5.0% of all OTUs). A small proportion of the sequences could not be assigned to a phylum and remained unclassified (2.0% of all sequences and 14.9% of all OTUs). The total of 2455 OTUs were assigned to 309 genera, dominated by *Penicillium* (40 OTUs), *Mortierella* (25 OTUs) and *Hypocrea* (18 OTUs).

3.2. Fungal community responses to chronic drought

The CFU count data alone provided no evidence for differences between the sites or treatments (Table 1). The CFU counts (adjusted for g^{-1} dw and dilution) on DG-18 neither differed between the sites (ANOVA: $F_{1,38} = 0.17$; $P = 0.6863$) nor the treatments within sites (ANOVA: $F_{1,18} = 1.57$; $P = 0.2220$) (Table 1). This was also true for the PDA CFU comparisons between the sites (ANOVA: $F_{1,38} = 0.40$; $P = 0.5304$) and treatments (ANOVA: $F_{1,18} = 2.12$; $P = 0.1350$). Analyses of CFU-PCR- or ePCR-inferred richness and diversity estimators provided no evidence for site effects but suggested some responses to drought manipulation (Table 1). CFU-PCR-based observed richness (S_{Obs}) differed among treatments (ANOVA: $F_{2,36} = 3.58$; $P = 0.0383$) but not between the two sites (ANOVA: $F_{1,36} = 0.58$; $P = 0.4522$). This tended to be true also for the extrapolative richness (Chao I) that did not differ among sites (ANOVA: $F_{1,36} = 0.33$; $P = 0.5672$) but tended to differ among treatments (ANOVA: $F_{2,36} = 2.49$; $P = 0.0968$). Dissection of the nested effects using "slice effects" determined that the observed differences were exclusively attributable to treatment effects at the more mesic KPBS site, where richness was higher in the drought treatments (S_{Obs}: $F_{1,36} = 6.95$; $P = 0.0123$; Chao I: $F_{1,36} = 4.67$; $P = 0.0374$) (Table1). Consistent with the richness responses, diversity (Shannon's H′) tended to differ among treatments (ANOVA: $F_{2,36} = 2.61$; $P = 0.0878$) but not among sites (ANOVA: $F_{1,36} = 0.04$; $P = 0.8406$). These effects also were attributable to higher diversity in the drought treatment than in the control at the more mesic KPBS site. No similar effects were obvious for the evenness estimator (Shannon's equitability E_H). The ePCR-based estimators provided a contrasting view. The ePCR richness and diversity estimators differed neither among the sites (ANOVA: $F_{1,36} < 1.08$; $P > 0.3046$) nor the treatments within sites (ANOVA: $F_{2,36} < 2.06$; $P > 0.1423$). However, in contrast to the CFU-PCR data, evenness ($E_{H'}$) tended to differ between the drought and ambient treatments (ANOVA: $F_{2,36} = 2.83$; $P = 0.0720$) but not between the sites (ANOVA: $F_{1,36} = 0.01$; $P = 0.9334$). The dissection of the nested effect indicated that this response was attributable to the lower evenness in the drought treatment at the more mesic KPBS site (Table 1).

In contrast to analyses of fungal richness and diversity, the CFU-PCR and ePCR data compositionally distinguished the communities at the two experimental grassland sites but provided no strong evidence for fungal community responses to chronic drought (Fig. 1A and B). Our analyses strongly suggested that the CFU-PCR- (AMOVA: $F_{1,38} = 5.23$; $P < 0.001$) and ePCR-inferred (AMOVA: $F_{1,38} = 9.74$; $P < 0.001$) communities were distinct between the two grassland sites (Fig. 1A and B). However, there was no evidence that the chronic drought and ambient treatments differed either at HAR or KPBS sites based on the CFU-PCR (AMOVA: $F_{1,19} < 1.36$; $P > 0.218$) or ePCR data (AMOVA: $F_{1,19} < 1.39$; $P > 0.118$). In sum, the compositional variability in the CFU and ePCR was mainly captured within the site differences. Furthermore, there was no strong evidence that the community variability differed between the sites or drought treatments (HOMOVA: $P > 0.099$).

To identify those community members that were differentially abundant across the two target sites and drove community distinctions on the site level, we aimed to identify differentially abundant OTUs. In the core CFU-PCR dataset that included only those OTUs that occurred at least in 25% of the samples (69 OTUs in total), none of the OTUs remained differentially abundant after our conservative Bonferroni correction for multiple comparisons ($P > 0.05$) (Supplemental Table S2). In contrast, the core communities in the ePCR analyses (100 OTUs in total) included four OTUs that remained differentially abundant after the conservative correction. All four differentially abundant OTUs were more

Table 1
Coverage, richness (S_{Obs}), extrapolated richness (Chao I), diversity (Shannon's H′) and evenness (E_H') of the ambient and drought-exposed communities in more arid (Hays Agricultural Research Station (HAR)) and more mesic (Konza Prairie Biological Station (KPBS)) sites inferred from culturable (CFU-PCR) and direct environmental (ePCR) samples. The richness and diversity metrics were consistently higher for ePCR than for CFU-PCR, whereas the coverage metric was higher for CFU-PCR. Differing superscript lower case letters (HAR) and upper case letters (KPBS) identify treatment differences when the two sites were analyzed using "slice effects" to dissect significant or nearly significant effects in the ANOVA model that included drought treatment nested within the site.

	Site = HAR		Site = KPBS	
CFU-PCR				
	CFU-PCR Ambient	CFU-PCR Drought	CFU-PCR Ambient	CFU-PCR Drought
coverage (%)	99.72 ± 0.11^{a}	99.7 ± 0.08^{a}	99.73 ± 0.09^{A}	99.68 ± 0.06^{A}
S_{Obs}	23.09 ± 7.38^{a}	24.01 ± 5.45^{a}	19.11 ± 6.19^{A}	25.34 ± 3.95^{B}
Chao I	30.87 ± 10.48^{a}	32.66 ± 7.66^{a}	26.77 ± 8.97^{A}	33.86 ± 5.68^{B}
Shannon (H′)	1.47 ± 0.34^{a}	1.56 ± 0.43^{a}	1.30 ± 0.35^{A}	1.69 ± 0.35^{B}
Evenness (E_H)	0.47 ± 0.07^{a}	0.49 ± 0.11^{a}	0.44 ± 0.09^{A}	0.52 ± 0.10^{A}
ePCR				
	ePCR Ambient	ePCR Drought	ePCR Ambient	ePCR Drought
coverage (%)	97.80 ± 0.92^{a}	97.90 ± 1.03^{a}	97.84 ± 1.14^{A}	97.36 ± 1.41^{A}
S_{Obs}	194.44 ± 51.09^{a}	186.74 ± 53.58^{a}	218.14 ± 62.99^{A}	203.09 ± 59.87^{A}
Chao I	236.31 ± 72.97^{a}	225.81 ± 73.13^{a}	253.29 ± 81.36^{A}	260.74 ± 94.82^{A}
Shannon (H′)	4.02 ± 0.55^{a}	3.98 ± 0.48^{a}	4.33 ± 0.36^{A}	3.84 ± 0.72^{A}
Evenness (E_H)	0.76 ± 0.07^{a}	0.77 ± 0.08^{a}	0.81 ± 0.05^{B}	0.72 ± 0.11^{A}
CFU				
	CFU Ambient	CFU Drought	CFU Ambient	CFU Drought
DG-18	15,743.8 ± 8706.9^{a}	18,751.6 ± 9291.7^{a}	14,925.1 ± 5811.5^{A}	21,664 ± 11,155^{A}
PDA	11,520.9 ± 12,121.6^{a}	9769.1 ± 7005.7^{a}	8208.7 ± 5070.4^{A}	15,823.3 ± 11,647^{A}

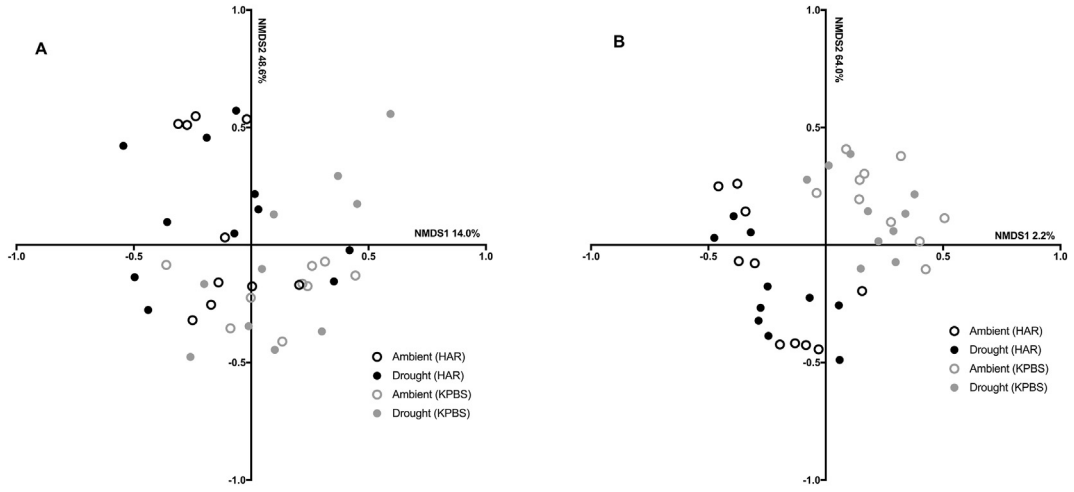

Fig. 1. Non-Metric Multi-Dimensional Scaling (NMDS) ordination of the fungal communities in ambient or chronic drought treatments in arid (HAR) and mesic (KPBS) sites. (A) CFU-PCR inferred communities: the ordination was optimally resolved on three axes (k = 3) for a final stress = 0.18 and R^2 = 77.8%. The two first NMDS axes in the figure represent 62.6% of the variation in the CFU-PCR dataset and distinguish the two sites but not treatments within sites. (B) ePCR inferred communities: the ordination was optimally resolved on four axes (k = 4) for a final stress = 0.15 and R^2 = 79.1%. The two first NMDS axes represent 66.2% of the variation in the ePCR dataset and distinguish the two sites, but not the treatments within the sites.

abundant at the more mesic site (KPBS) and included exclusively members of Hypocreales in either Hypocreaceae (one OTU) or Nectriaceae (three OTUs) (Supplemental Table S3).

3.3. Comparison of culturable and ePCR communities

The coverage metric that estimates representativeness of the sampling effort was high for both CFU-PCR (99.7% ± 0.1%) and ePCR (97.7% ± 1.1%) communities (Table 1). Regardless, the sequencing effort more completely captured the CFU-PCR communities than the ePCR communities (Wilcoxon T = −408.0, P < 0.0001). The CFU-PCR and ePCR communities were distinct in richness and diversity (Table 1) as well as in composition (Supplemental Fig. S1). Overall, the ePCR communities included nearly seven times as many OTUs as the CFU-PCR communities; 2383 vs. 341 OTUs. The ePCR communities consistently had greater richness (S_{Obs}: Wilcoxon T = 410.0, P < 0.0001; ChaoI: Wilcoxon T = 410.0, P < 0.0001) and diversity (H': Wilcoxon T = 410.0, P < 0.0001) than the CFU-PCR communities. Similarly, evenness as estimated by Shannon's equitability (E_H: Wilcoxon T = 410.0, P < 0.0001) was greater in the communities determined using ePCR, indicating that CFU-PCR communities had more extreme differences in the OTU rank-abundances as also supported by the recovery of some very abundant families in the CFU-PCR analyses (Fig. 2A and B). Similar to the richness and diversity analyses, the NMDS analyses (Supplemental Fig. S1) and subsequent non-metric ANOVA analogs clearly distinguished the communities inferred by CFU-PCR and ePCR (AMOVA: $F_{1,78}$ = 13.85; P < 0.001). Furthermore, the ePCR communities were more heterogeneous than the CFU-PCR communities (HOMOVA: B = 0.58, P < 0.001).

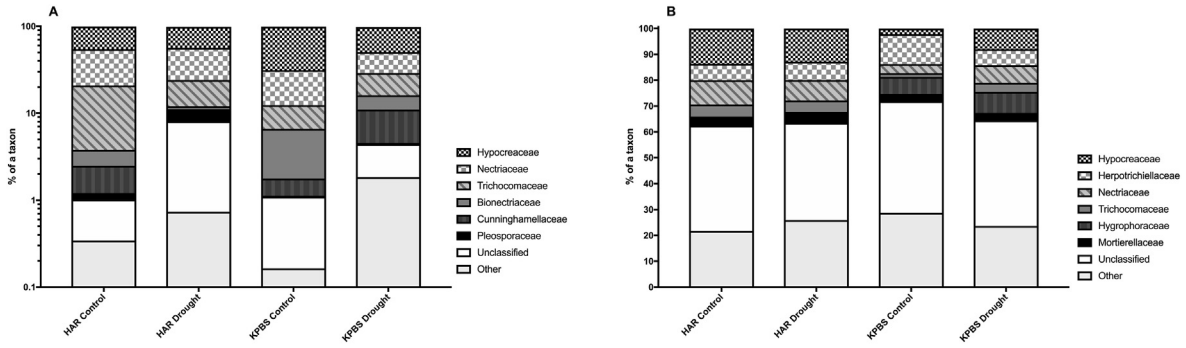

Fig. 2. The most commonly observed families in either (A) culture-dependent CFU-PCR or (B) culture-independent ePCR datasets ranked by their relative abundances within each dataset. "Unclassified" refers to all sequence data that could not be classified to a family, whereas "Other" refers to all families that were not included into the listed family-level groupings. Note that while Fig. 2B has a linear y-axis scale, Fig. 2A has a $\log_{10}$ scale to better illustrate the steep differences in the proportions of the most abundant families.

The NMDS-inferred compositional distinctions were clearly reflected in the taxonomic composition, as well as in the differentially abundant OTUs in core communities identified in the metastat analyses. In general, the CFU-PCR communities included nearly exclusively members of phylum Ascomycota (96.1% of all CFU-PCR sequences and 287 or 84.2% of CFU-PCR OTUs). Other taxa were a minor component (basal clades formerly included in Zygomycota – 2.5% of sequences and 16 OTUs or 4.7% of all CFU-PCR OTUs; Basidiomycota – 1.4% of sequences and 33 or 9.7% of OTUs). Only four CFU-PCR OTUs remained unclassified (1.2% of sequences). In contrast to the CFU-PCR communities and consistent with the other analyses, the ePCR communities were taxonomically more diverse. Although the ePCR communities were also dominated by Ascomycota (74.9% of all sequences and 1312 OTUs and 55.1% of all OTUs), they also included a substantial proportion of other taxa: Basidiomycota (15.0% of sequences and 507 OTUs or 21.3%); Glomeromycota (0.4% of sequences but 75 OTUs or 3.1%); and, basal clades including those formerly assigned to Zygomycota (3.8% of sequences and 53 OTUs or 2.2%) and Chytridiomycota (1.1% of sequences and 66 OTUs or 2.7%). In contrast to the CFU-PCR communities, a large proportion of the ePCR OTUs (364 OTUs or 15.4% of all ePCR OTUs) and a modest proportion of the ePCR sequences (4.8%) remained unclassified beyond a phylum. On a generic level, the CFU-PCR communities were strongly dominated by *Penicillum* (33 OTUs) followed by *Hypocrea* (15 OTUs) and *Trichoderma* (13 OTUs). Although *Penicillium* was also the most OTU-rich genus in the ePCR data, the runners-up were *Mortierella* (25 OTUs), *Spizellomyces* (16 OTUs) and *Entoloma* (16 OTUs) further highlighting the taxonomic breadth of the communities inferred through ePCR.

Despite the distinct community attributes, a large proportion of all CFU-PCR OTUs were shared with the ePCR communities (262 OTUs or 76.8% of all CFU-PCR OTUs). All or nearly all basidiomycete (31 of 33), zygomycete (15 of 16) and unclassified (4 of 4) CFU-PCR OTUs were shared with the ePCR communities. However, the proportion of all OTUs that were culturable varied among taxa (Fig. 3). For example, a total of 116 (63%) of the 184 Hypocreales OTUs were observed through CFU-PCR, whereas – in contrast – only seven (3.6%) of the 196 Agaricales OTUs were. The analyses of differentially abundant OTUs corroborated the distinct taxon distribution: we observed a total of 66 differentially abundant OTUs – 60 in the ePCR and 6 in the CFU-PCR communities (Supplemental Table S4). All six differentially abundant OTUs that were enriched in CFU-PCRs were ascomycetes (OTU0001 – *Hypocrea lixii*; OTU0002 – *Haemonectria haematococca*; OTU0315 – Nectriaceae; OTU0408 – *Hypocrea lixii*; OTU601 – Nectriaceae; and, OTU617 – Hypocreaceae). In contrast, the sixty differentially abundant OTUs enriched in the ePCR community represented a greater taxonomic diversity (Supplemental Table S4).

4. Discussion

In the current study, we used comparable culture-dependent and culture-independent approaches to address the effects of chronic drought on soil fungal communities. Our data indicate that while communities did not respond to drought compositionally, their richness and diversity tended to increase as a result of drought when estimated through CFU-PCR and only at the more mesic site. These data provide some support to two current hypotheses of fungal community responses to manipulations of water availability. First, our data suggest that fungal communities may be compositionally resistant to drought, whereas their diversity may be sensitive. Second, our data suggest that fungal diversity and richness may be more sensitive to drought in more mesic environments. Finally, our comparisons of culture-dependent and culture-independent methods strongly suggest that a considerably large proportion of fungi – ascomycetes in particular – may be rather effortlessly culturable.

4.1. Fungal community responses to chronic drought

Drought poses a severe stress to soil fungi in mesic systems (Ochoa-Hueso et al., 2018), possibly leading to compositional and functional community changes (Toberman et al., 2008; Bell et al., 2009; Manzoni et al., 2012; Zeglin et al., 2013; Lagueux et al., 2021). Yet, evidence also exists that fungal communities may be compositionally resistant to changes in precipitation (Zhang et al., 2013; Curlevski et al., 2014; Jumpponen and Jones 2014; Kaisermann et al., 2015), even though their diversity can be impacted (Toberman et al., 2008; Castro et al., 2010; Hawkes et al., 2011; Curlevski et al., 2014; de Oliveira et al., 2020). Some studies highlight this compositional resistance (e.g., Jumpponen and Jones 2014), whereas others have reported distinct changes in fungal communities that inhabit soil (Hawkes et al., 2011; Ochoa-Hueso et al., 2018; Schmidt et al., 2018; Wang et al., 2020) or roots (Deveautour et al. 2018, 2019; Lagueux et al., 2021).

Our data provide support for the compositional resistance to drought regardless of the method (CFU-PCR, ePCR) chosen for analyses. Additionally, our data provide some limited support for increases in soil fungal richness in response to drought, but only when inferred using the CFU-PCR method and only at the more mesic site. The incongruence between the methods set aside, fungal community responses to drought stress remain incompletely understood and empirical data are conflicting. To exemplify, seasonal drought manipulations in a heathland system lowered fungal diversity and abundance in soil (Toberman et al., 2008), whereas similar experimental manipulations in a grassland system resulted in greater diversity and higher fungal abundances (Hawkes

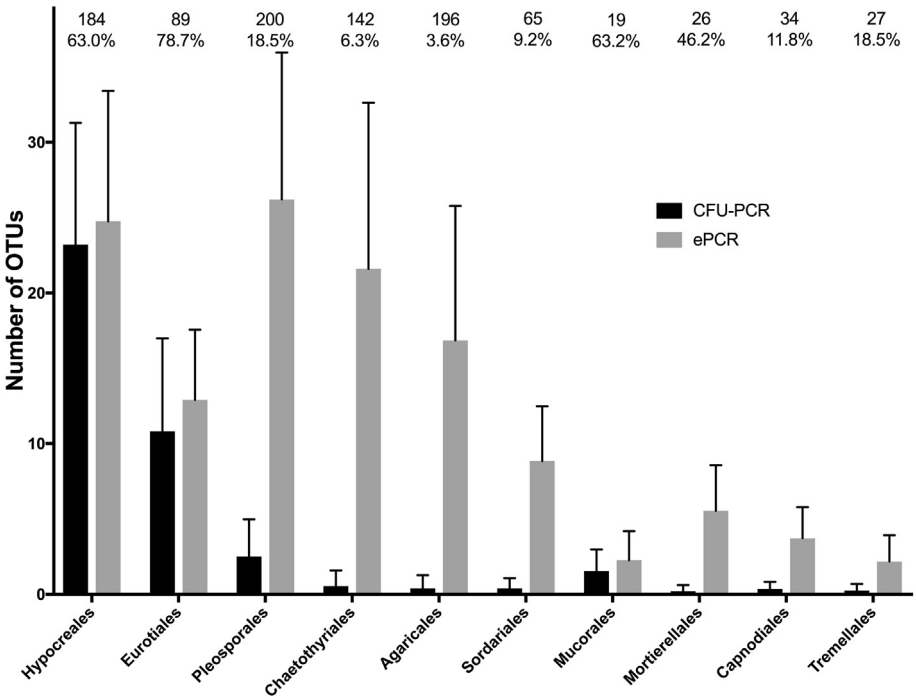

Fig. 3. Number of CFU-PCR (black bars) and ePCR (gray bars) OTUs (Mean; Standard Deviation) within the ten most abundant orders as measured by sequence yield. The numbers and percentages above are the total number of OTUs within the order and the proportion of those OTUs that were observed though the CFU-PCR.

et al., 2011). The discrepancies among studies may emerge from the environmental context: fungal communities seem more sensitive to drought in mesic environments than they are in arid environments. In a broad study that included drought manipulations in Australia and six sites across the North American grasslands, Ochoa-Hueso et al. (2018) observed that the largest fungal community differences were at the most mesic end of the precipitation gradient. These observations contrast predictions based on analyses of plant communities that indicate that above ground productivity is most sensitive in the more arid grasslands (Knapp et al., 2015). Although our community data suggested fungal community resistance to drought, our richness and diversity analyses corroborate observations by Ochoa-Hueso et al. (2018). It remains unclear how well the observations and predictions from plant communities extrapolate to fungal communities that may be characterized by a large proportion of dormant members similar to bacterial communities (Lennon and Jones 2011).

In addition to environmental context, targeting different fungal guilds may also lead to contrasting conclusions. Studies that focus on plant-associated fungal communities may be more likely to observe community-level responses to manipulations of water availability (e.g., Li et al., 2015; Gao et al., 2016; Deveautour et al., 2018; 2019; Lagueux et al., 2021) as these fungi experience the direct effects of drought compounded by the indirect effects of changes in plant communities and plant physiology (von Rein et al., 2016). In contrast, studies that more indiscriminately sample soil communities may not detect such responses (Toberman et al., 2008; Jumpponen and Jones 2014). In addition, not all fungi respond to precipitation manipulations, but communities often include an array of members that vary in their tolerance, resistance or sensitivity (Herrera et al., 2011; de Oliviera et al., 2020). These context dependencies that range from system to guild and taxon specificity complicate clear elucidation of soil fungal community responses to drought and consensus on generalities thus remains elusive.

Our study focused on two sites that differ in MAP (577 vs. 860 mm). While the two sites differed in their fungal communities, the experimental manipulations that substantially reduced growing season precipitation had little visible impact. Interestingly, experimental drought increased richness at the more mesic KPBS site (mesic site) but not at the more arid (HAR) site, suggesting more stable communities perhaps as a result of more frequent dry conditions at the arid site. Soil moisture and water availability are pivotal drivers of soil-inhabiting communities (Maestre et al., 2015). However, empirical evidence remains inconsistent: some recent studies have recorded microbial community shifts relating to changes in available soil water or precipitation (Bernard et al., 2013; Ochoa-Hueso et al., 2018; Schmidt et al., 2018; Wang et al., 2020), whereas others have recorded compositionally stable communities (Landesman and Dighton 2010; Curiel-Yuste et al., 2014).

The question remains whether our observed compositional resistance to drought indicate drought resistance and adaptation of the soil-inhabiting fungal communities or if these data simply point to the abundant dormant propagules that can withstand adverse environmental conditions and assume metabolic activity once favorable conditions return (Lennon and Jones 2011). Several lines of evidence highlight the rapid recovery and reliance on dormant propagules. First, Zeglin et al. (2013) recorded rapid metabolic and biomass responses in a mesic tallgrass prairie site within just hours of a precipitation event. Second, Reazin et al. (2016) reported a complete community turnover in response to a fire disturbance and activation of community constituents that were below detection levels before the disturbance. Finally, our current data from culture-dependent and culture-independent approaches were dominated by *Penicilli*, Eurotialean anamorphs that are known for their rapid and abundant production of asexual propagules that likely withstand adverse environmental conditions. Whilst it is certainly important and relevant to address issues about resistance, resilience and adaptation to recurring droughts that characterize the grassland sites sampled in this study, it is also important to

consider whether one would expect community-wide shifts in empirical studies that are limited in temporal (four growing seasons of drought manipulations) and spatial (adjacent 36 m^2 plots covered by 10×10 m^2 shelters) scales (Griffin-Nolan et al., 2019). It remains unclear how important a role short distance dispersal plays in homogenizing the composition of communities in plots that represent the experimental manipulations. Although fungal communities, like those of macroscopic eukaryotes, may be limited by dispersal (Peay et al., 2010), such limitations are unlikely on the within-site scale of the current experiment potentially resulting in stochastic homogenization of the communities and masking of drought responses if such existed.

4.2. Comparison of culturable and ePCR communities

In addition to our focus on drought effects on soil fungi, we aimed to determine the proportion of the fungal community that can be brought into pure culture. To do so, we dissected fungal communities using next-generation sequencing that is commonplace in environmental microbiology. We compared those estimates with fungal communities detected in the pure cultures using methods not very different from those deployed for CFU-PLFAs to provide fingerprints of the culturable proportion in environmental communities (see Pennanen et al., 1998; Kozdrój and van Elsas 2001; Söderberg et al., 2004). Our CFU-PCR and ePCR methods provided incongruent community views as has been reported previously (Allen et al., 2003; Walker et al., 2011) and confirmed earlier observations that culture-based approaches tend to be strongly biased towards ascomycetes (Viaud et al., 2000). However, our approach permitted estimation of the culturable component on the chosen medium and suggests that, on average, ~12% (23.09 ± 7.38 in CFU-PCR vs. 194.44 ± 51.09 in ePCR) of OTUs in a given sample, or ~14% (341 OTUs in CFU-PCR vs. 2383 in ePCR) of all OTUs in the entire experiment could be cultured on this single, rich medium. Remarkably, of the ascomycetes that were observed in the ePCR communities, 21.9% were also observed using the culture-dependent methods. These estimates are surprisingly high and contrast those for soil micro-organisms in general (~1% of micro-organisms – Davis et al., 2005) and fungi specifically. Although many studies have combined both direct environmental sequencing and pure-culturing approaches (e.g., Pitkäranta et al., 2008; Daghino et al., 2012; Man et al., 2015), consistent and comparable approaches – such as simultaneous MiSeq analyses of ITS2 amplicons described here – have been lacking.

The culture-dependent methods have become increasingly unfashionable as a result of their labor intensiveness and requirement for substantial taxonomic expertise for morphological identification, although the increasing availability of environmental sequences representing potentially novel taxa has paved way for sequence-based taxonomy (Hibbett et al., 2011). Boone and Castenholz (2001) briefly summarized the advantages of acquiring individual isolates including elucidating their physiological properties and their metabolic interactions with other microorganisms and the surrounding environment. Our current data suggest that the proportion of culturable fungi maybe as high as one in five ascomycetes and more than one in ten fungi overall. It is of note that our pure-culturing effort was far from comprehensive and likely a gross underestimate of culturability. In this experiment, we merely exemplify a reasonably reliable estimate for the culturable community.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2021.101062.

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