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Distinguishing Between the Impacts of Heat and Drought Stress on the Root Microbiome of *Sorghum bicolor*

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ABSTRACT

Water deficit and extreme temperatures regularly and considerably reduce crop productivity. Although several studies have explored the role of the plant microbiome in drought tolerance, it remains to be determined how the often co-occurring factor of heat conjunctly shapes plant–microbe interactions. In order to compare the roles that temperature and drought stress play in plant microbial recruitment, we conducted a growth chamber experiment with a series of temperatures (22, 30, and 38°C) and watering (drought versus watered) conditions in *Sorghum bicolor*, sampling soil mixtures and seedling roots at 7 and 21 days after initial stress initiation. We found that bacterial root communities had the lowest α diversity when under drought and at 38°C, and that temperature influenced the β diversity of soil mixture and root microbiomes to a greater extent than watering treatment. Additionally, we observed that the relative abundances of members of phylum *Actinobacteria* increased both under

drought and, incrementally, with higher temperatures. Furthermore, unique *Actinobacteria* were indicator species of either temperature or watered conditions, and indicators of high temperature in watered conditions were predominantly in the phylum *Actinobacteria*. Together, these data suggest that heat and drought stress differentially affect microbiome assembly in significant ways and unique *Actinobacteria* are recruited under either stress. This work informs our understandings of how abiotic stresses jointly shape crop microbiomes, as well as highlights the need for additional work to describe what mechanisms may be involved in host-mediated microbial recruitment and adaptation under various stresses in field-based studies.

Keywords: agriculture, amplicon sequencing, crop, drought, ecology, endophytes, heat stress, microbiome, sorghum

In the predictions of what the future holds for our global community, numerous questions arise regarding how to fortify food production in light of heightening impacts of climate change and rapid population growth. An important component of solution-oriented efforts for improved food security involves understanding the mechanics and interplay of the microbial world with that of crop plants. Recent work has highlighted the significance of plant–microbe interactions in supporting plant health and yield (Santoyo et al. 2017), and evidence suggests that microbes can promote plant drought tolerance (Xu and Coleman-Derr 2019). Furthermore, microbes in roots (i.e., endophytes) are known to affect an extensive

range of drought traits in host plants (Dastogeer et al. 2018; Dhanyalakshmi et al. 2019; Malinowski and Belesky 2000; Qin et al. 2019). Although several studies have been conducted in the field investigating drought stress impacts on the microbiome, numerous additional factors, including high temperatures, often co-occur with water deficit and may potentially influence microbial recruitment to a significant degree. Indeed, past work indicates that drought and heat waves can restructure soil microbial communities at large to a greater extent when in combination, over soil drying alone (Bérard et al. 2011). The concurrent and disparate effects of heat and drought stress on plant microbiome assembly, however, remain to be elucidated.

In plants, a significant number of studies have noted separate and interactive effects of drought and heat stress on growth and yield. A number of genetic, biochemical, and physiological factors influence stress responses (Lamaoui et al. 2018), and hundreds of transcripts are known to uniquely shift with either heat or drought stress, in addition to when both stresses are combined (Rizhsky et al. 2002, 2004). Furthermore, when alone and, to a greater extent, when combined, both stresses reduce photosynthetic rates, root nutrient uptake, and yield (Hussain et al. 2019; Zandalinas et al. 2018). Root morphological traits can significantly vary across drought, heat, and

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both combined stresses, with increasing reductions in root biomass and length (Wu et al. 2017). Despite this variation, and the fact that root system architecture has been demonstrated to influence root and rhizosphere microbiome establishment (Saleem et al. 2018), we know of no studies that have distinguished between the impacts of heat and drought stress on the sorghum root microbiome. In order to develop a more comprehensive understanding of how the phytobiome functions, particularly with regards to the numerous agricultural challenges we face, investigations that seek to disentangle microbiome responses to coinciding stressors—and the ongoing development of research approaches to do so—are needed.

It is now apparent that host recruitment of microbes can significantly affect the extent to which abiotic stress influences plant growth. Seed germination has been shown to improve under heat and drought stress when endophytes are present (Hubbard et al. 2012), and bacterial seed treatment positively affects seedling heat stress tolerance (Abd El-Daim et al. 2014). Past work has also shown that plant biomass can be bolstered under drought stress with the addition of various plant-growth-promoting microbes (Rolli et al. 2015). One pattern of particular interest is that plants under drought enrich for monoderm bacteria, particularly members of phylum *Actinobacteria* (Fitzpatrick et al. 2018; Santos-Medellín et al. 2017; Xu et al. 2018), which have been shown to be more prevalent across a variety of drought-treated soils and rhizospheres (Bouskill et al. 2016, 2013; Nessner Kavamura et al. 2013). Interestingly, *Actinobacteria* have been shown to promote plant growth via a variety of mechanisms (Sathya et al. 2017) and are also thought to be highly tolerant of arid environments (Cherif et al. 2015; Stevenson and Hallsworth 2014). Although this robustness is due, in part, to their ability to sporulate, recent work suggests that *Actinobacterial* enrichment under drought in the plant microbiome is not correlated with this ability (Naylor et al. 2017).

Although the size of *Actinobacterial* enrichment under drought is known to vary across different host systems and studies, as well as study sites, the factors explaining this variation remain to be fully characterized. One study reported slight increases—of 3 to 20%—in the relative abundance of the phylum *Actinobacteria* in a variety of grass species roots under drought, in a field site with temperatures ranging between 6 and 25°C (Naylor et al. 2017). Conversely, in a field site that reached high daytime temperatures of 32 to 38°C, the relative abundance of *Actinobacteria* in droughted plant roots varied between 35 and 80% (Xu et al. 2018). Although the effects of drought on plants have been extensively reported on, it can be difficult to tease apart impacts of drought and co-occurring heat in field studies; this includes the degree of involvement of either in helping explain differences in the magnitude of *Actinobacteria* enrichment. Because temperature is challenging to manipulate in the field, a lab-based, experimental design that can control for both factors is important for achieving a more detailed understanding of how plant microbiomes function. Other edaphic factors—including variation in soil aggregate size and pore space, as well as chemical composition—in addition to differences in drought inducement may also contribute to these reported enrichment differences, further highlighting a need to better characterize the impacts of heat stress, both in combination with and isolated from drought stress, on microbial assembly in plant roots.

In this study, we sought to disentangle temperature and drought impacts on plant-associated microbial communities by using a controlled environmental system to better manipulate and monitor temperature within the system. We hypothesized that *Actinobacteria* would be enriched to the greatest extent in roots when both heat and drought stress were applied, versus either alone. In growth chambers, we utilized three temperature conditions (22, 30, and 38°C) and a

drought and control watering regime to induce a combination of heat and drought stress on sorghum seedlings. We sampled at two time points (TPs)—16 days old and 7 days stressed, and 30 days old, 21 days stressed—in order to characterize the associated microbiome over a period of prolonged stress exposure. Our results demonstrate that temperature and watering treatments distinctly affect the diversity and composition of sorghum root bacterial communities, particularly in relation to degree of *Actinobacterial* enrichment. Additionally, we show that TP influences the relative impact of heat versus drought on the amount and type of indicator species present. These findings contribute to our understandings of how abiotic stress factors coordinate host recruitment of microbial communities, specifically in a drought-tolerant crop, and inform efforts geared toward utilizing the microbiome for improved plant growth in adverse growing conditions. Furthermore, our findings highlight the importance of supplementing field-based studies with investigations under controlled environmental conditions, in order to advance system-level understandings of the phytobiome and how it operates to promote plant productivity.

MATERIALS AND METHODS

Experimental set-up. To test the individual and combined impacts of drought and temperature stress on the root bacterial microbiome of sorghum, we conducted a set of growth chambers experiments in fall 2019 to modulate temperature and other growing conditions. Relative humidity was set at 30% and a period of 16 h of light and 8 h of darkness was employed for the duration of the experiment, where photosynthetic photon flux density ranged between 80 and 120 $\mu\text{mol}/\text{m}^2/\text{s}$. In order to ensure greater ease in extracting roots from the growth substrate, as well as to reduce fungal contamination and increase water infiltration, a mixture of field soil collected from the University of California Gill Tract, vermiculite, and calcine clay (1:2:17) was utilized. At the start of the experiment, samples of the field soil and soil mixture batches were collected for DNA extraction and 16S ribosomal RNA (rRNA) gene sequencing and stored at -80°C until processing.

With a 10% bleach solution, seed of *Sorghum bicolor* (L.) Moench variety RTx430 were surface sterilized and rinsed with autoclaved water, and 3 seeds per 3.8 liter pot were then planted approximately 2 cm below the soil mixture surface. Sixty pots were seeded in total, to allow for five replicates per harvesting TP ($n = 2$) per watering treatment ($n = 2$) per temperature treatment ($n = 3$). For the first 8 days after planting, temperature was set at a constant 30°C and seedlings were thinned to one plant per pot. On day 9, temperature and drought treatments were begun, where plants were randomly selected to be grown under one of the following temperatures: 22°C day and 16°C night (as a cooler temperature control), 30°C day and 24°C night, or 38°C day and 32°C night, with a total of 20 plants in each temperature treatment, 10 of which also were subjected to drought by the cessation of all watering. The remaining plants were checked daily and watered as needed to keep soil mixtures fully saturated, as determined by water draining into the base pan, and this excess water was removed. Twice weekly, plants were rotated within growth chambers to minimize corner and center effects due to lighting and potential differences in water evaporation rates.

Sample collection and processing. In addition to harvesting plant roots and soil, measurements to determine soil moisture and plant height, shoot biomass, and leaf relative water content were collected at a first TP, when plants were 16 days old and 7 days stressed, and a second TP, when plants were 30 days old and 21 days stressed (Supplementary Fig. S1). Five plants were harvested, as replicates, per treatment per TP, along with a sample of

the soil mixture approximately 7 to 8 cm away from plant stems and at depths of 0 to 20 cm below the mixture surface, with a 10% bleach sterilized small hand auger. Whole roots were collected, and roots and soil mixture samples were stored on dry ice until transferred to a -80°C freezer to hold until processing.

Root fractions were cleaned as described in detail previously (Simmons et al. 2018). In brief, whole roots were placed in an epiphyte removal buffer (0.75% KH_2PO_4 , 0.95% K_2HPO_4 , and 1% Triton X-100 in double-distilled H_2O ; filter sterilized at $0.2\ \mu\text{M}$) and then sonicated (pulses at 160 W for 30 s, separated by a 30-s pause for 10 min at 4°C), followed by two subsequent steps of rinsing with sterile water, in order to fully remove the rhizosphere fraction. After drying with absorbent tissue, roots were homogenized and ground to a fine powder using liquid nitrogen and a mortar and pestle, before being returned to -80°C .

DNA extraction and library preparation. Soil mixture and powdered root DNA were isolated using extraction kits (DNeasy PowerSoil Kit; Qiagen Inc., Carlsbad, CA, U.S.A.) following the manufacturer's protocol. We then amplified the V3-V4 region of the 16S ribosomal gene using a dual-indexed 16S rRNA Illumina iTags primer (341 F [5'-CCTACGGGNGBCASCAG-3'] and 785 R [5'-GACTACNVGGGTATCTAATCC-3']), as described previously (Takahashi et al. 2014), using 5-Prime Hot Master Mix (catalog number 2200410). After DNA extraction, DNA was diluted to $5\ \text{ng}/\mu\text{l}$ and randomized in 96-well plates. Water blanks were included on each 96-well plate as negative controls. Peptide nucleic acid (PNA) clamps were used to minimize host-derived amplicons from both chloroplast and mitochondrial 16S rRNA gene sequences (Lundberg et al. 2013). Reactions included $11.12\ \mu\text{l}$ of DNase-free sterile H_2O , $0.4\ \mu\text{g}$ of bovine serum albumen, $10.0\ \mu\text{l}$ of 5-Prime Hot Master Mix, $2\ \mu\text{l}$ of template, and $0.75\ \mu\text{M}$ chloroplast and mitochondria PNAs. PCR assays were performed in triplicate in three thermocyclers (to account for possible thermocycler bias) with the following conditions: initial 3-min cycle at 94°C ; then, 30 cycles of 45 s at 94°C , 10 s at 78°C , 1 min at 50°C , and 1.5 min at 72°C ; followed by a final cycle of 10 min at 72°C . Triplicates were then pooled and the DNA concentration of each sample was quantified using a Qubit 3 Fluorometer (Invitrogen, Carlsbad, CA, U.S.A.). Pools of amplicons were constructed using $100\ \text{ng}$ for each PCR product. Before submitting for sequencing, pooled samples were cleaned with $1.0\times$ volume Agencourt AMPureXP beads (Beckman-Coulter, West Sacramento, CA, U.S.A.), according to the manufacturer's directions, except for the modifications of using $1.0\times$ rather than $1.6\times$ volume of beads per sample, dispensing $1,500\ \mu\text{l}$ of 70% EtOH to each well rather than $200\ \mu\text{l}$, and eluting in $100\ \mu\text{l}$ of DNase-free H_2O rather than $40\ \mu\text{l}$. An aliquot of the pooled amplicons was diluted to $10\ \text{nM}$ in a $30\text{-}\mu\text{l}$ total volume before submitting to the QB3 Vincent J. Coates Genomics Sequencing Laboratory facility at the University of California, Berkeley for sequencing using Illumina Miseq. 300-bp paired end with v3 chemistry. Sequences were returned demultiplexed and with adaptors removed.

Amplicon sequence data processing, operational taxonomic unit classification, and taxonomic assignment. Our sequencing data were analyzed using the iTagger pipeline developed by the U.S. Department of Energy's Joint Genome Institute (Tremblay et al. 2015). This pipeline wraps several packages for filtering, merging, clustering, and taxonomy assignment, including CUTADAPT, FLASH, USEARCH, and RDP (Alloui et al. 2015; Magoc and Salzberg 2011; Martin 2011; Wang et al. 2007). In brief, after filtering 16S rRNA raw reads for known contaminants (Illumina adapter sequence and PhiX), primer sequences were trimmed from the 5' ends of both forward and reverse reads. Low-

quality bases were trimmed from the 3' ends prior to assembly of forward and reverse reads with FLASH (Magoc and Salzberg 2011). The remaining merged reads were clustered with simultaneous chimera removal using UPARSE (Edgar 2013). After clustering, 3,080,184 read counts mapped to 4,369 operational taxonomic units (OTUs) at 97% identity. The resulting reads produced, on average, approximately 998,475 and 1,431,892 reads per sample for soil mixtures and roots, respectively. Taxonomies were assigned to each using the RDP Naïve Bayesian Classifier with custom reference databases (Wang et al. 2007). For the 16S rRNA V3-V4 data, this database was compiled from the May 2013 version of the GreenGenes 16S database (DeSantis et al. 2006), the Silva 16S database (Quast et al. 2013), and additional manually curated 16S rRNA sequences, trimmed to the V3-V4 region. After taxonomies were assigned to each OTU, we discarded all OTUs that were not assigned a kingdom level RDP classification score of at least 0.5. To remove low-abundance OTUs that, in many cases, are artifacts generated through the sequencing process, we removed OTUs without at least two reads in at least two samples. We also removed samples that had less than 10,000 reads, which yielded 1,997 high-abundance OTUs for downstream analyses. These thresholds were found to be suitable using technical replicates in a dataset published previously (Coleman-Derr et al. 2016). To account for differences in sequencing read depth across samples, all samples were rarefied to 10,000 reads per sample for specific analyses to yield 1,140,000 measurable, rarefied reads for downstream analysis. The raw sequencing reads for this project will be deposited in the NCBI Short Read Archive and can be accessed through BioProject SUB7734635.

Statistical analyses. RStudio (version 1.0.136; RStudio Team) was utilized for all statistical analyses with the packages phyloseq (McMurdie and Holmes 2013) and vegan (Dixon 2003). For plant phenotype data, scatter plots were generated using ggplot2, and analysis of variance (ANOVA) was performed with function aov. For the α diversity measurement, Shannon Index of diversity and observed OTUs were calculated with the estimate_richness function in the R package phyloseq. ANOVAs were performed with function aov for sample type, temperature treatment, watering treatment, and TP. A Tukey's post hoc test was performed using the function TukeyHSD in the stats package and with HSD.test in the package agricolae to test which levels were significantly different from one another. β Diversity was measured using Bray-Curtis distances and UniFrac distance with function ordinate in the R package phyloseq. Trees were built with default parameters using FastTree (Price et al. 2010) with an alignment constructed in Muscle (Edgar 2004), and figures were produced with the Integrative Tree of Life (Letunic and Bork 2007). Permutational multivariate analyses of variance (PERMANOVAs) were performed with the Adonis function in the R package Vegan using 999 permutations and the Bray-Curtis distances as inputs. Canonical analysis of principal coordinates (CAP) was performed for subsets of the data with each sample type and TP to determine the percent variance explained by treatment, TP, and replicate, or treatment, sample type, and replicate, respectively, using the capscale function in the R package vegan (Dixon 2003). The nonparametric Kruskal-Wallis test in R was used to compare Shannon indices and class-level relative abundances between treated and untreated within each TP and sample type. Indicator species analyses run on root samples to determine genera that were enriched for either control or amendment treatments were performed using R package indicspecies (De Cáceres and Legendre 2009), with P values < 0.05 based on permutation tests run with 999 permutations. All scripts used can be found at a public repository on Github.

RESULTS

Phenotypic analysis of heat and drought stress. To determine how heat stress may contribute to an enrichment of *Actinobacteria*, as characterized prior in roots under drought stress (Naylor et al. 2017; Simmons et al. 2020; Xu et al. 2018), we utilized a set of growth chambers to grow sorghum in a mixture of field soil, calcine clay, and vermiculite and initiated temperature and drought stresses when seedlings were 9 days old. To establish treatment effect sizes, we measured percent moisture in soil mixtures, plant height, fresh shoot biomass, and leaf relative water content at both times of sampling the soil and root microbiomes, with seedlings that were grown under control and stress conditions for 1 week (TP1) and 3 weeks (TP2) (Supplementary Fig. S1).

Within each temperature treatment, we found that drought markedly affected moisture content of soil mixtures, as was most apparent by the second TP (P value < 0.001) (Fig. 1). Importantly, however, within the watered treatments, no significant effect of temperature was observed for mixture moisture levels, as well as leaf relative water content (Supplementary Fig. S2a), suggesting that downstream effects on microbiome composition can be primarily attributed to temperature alone. We also observed that plant height and shoot biomass were generally reduced with increasing temperature and drought (Supplementary Fig. S2b to d). Conducting ANOVA for all attributes measured, temperature and watering treatment were statistically significant factors in all tests (Supplementary Table S1). These results demonstrate that our temperature and drought treatments induced physiological changes in the plant host and soil mix moisture levels.

Bacterial diversity shifts most significantly in response to combined temperature and drought stress. In order to characterize

the separate and combined impacts of heat and drought stress on the sorghum microbiome, we performed amplicon sequencing of the V3-V4 region of the 16S rRNA gene for soil mixture and root bacterial microbiomes across both TPs. Using Shannon diversity indices, we determined that α diversity at large decreased under drought, with the only exception being roots from the 22°C temperature treatment (Fig. 2). We also found that higher temperatures (30 and 38 versus 22°C) corresponded to higher α diversity levels in both soil mixture and root fractions (Fig. 2). Distinctions based on TP, particularly in roots, were apparent, where diversity was highest in the watered 30°C treatment in the first round of sampling and, by TP2, was highest in the watered 38°C treatment (Fig. 2).

With ANOVA, we determined that watering treatment was a significant factor across all TPs and sample types (P values < 0.05), while temperature treatment was a statistically significant factor in explaining α diversity variation only for TP1 roots (P value < 0.01) and TP2 soil mixtures (P value < 0.001) (Supplementary Table S2).

To further explore the impacts of temperature and watering treatment on bacterial composition, as compared with more well-characterized factors of sample type and TP, we utilized principle coordinate analysis and CAP of Bray-Curtis dissimilarity. As expected, we found that samples clustered primarily by sample type (Supplementary Fig. S3), and variation in β diversity was explained to a greater degree by temperature ($F = 9.3$, P value < 0.001) than by watering treatment ($F = 6.6$, P value < 0.001), as determined by PERMANOVA (Supplementary Table S2). Furthermore, these factors combined explained between 36 and 39.5% of the variation in roots and 29.4 and 42.8% in the soil mixture (Fig. 3). Additionally, the variation in β diversity attributed to temperature treatment was greater in roots (29.7 to 31.4%) than soil mixture (21 to 30.9%) but the opposite pattern occurred with watering treatment (6.3 to 8.1% for roots versus 8.4 to 11.9% for soil mixture) (Fig. 3). Unsurprisingly, the later TP also showed an accentuation of the variation attributed to both factors. Our analyses further indicated that temperature and watering treatments, separately, were statistically significant factors explaining β diversity variation within all sample types and TPs (P value < 0.01), with the important exception of watering treatment in TP1 roots (Supplementary Table S3). These results showed that the bacterial diversity associated with both plant roots and the surrounding substrate shifted with heat and drought stress, and greater variation was attributed to temperature treatment than drought.

Relative abundance of *Actinobacteria* dynamically responds to both heat and drought treatment. To further explore the impacts of temperature and watering treatment on bacterial composition, as compared with more well-studied factors of sample type and TP, we plotted the relative abundances of the top 11 dominant phyla (Fig. 4). For soil mixtures, we observed that several patterns emerged across treatments, including a relatively reduced abundance of members of phyla *Gemmatimonadetes*, *Acidobacteria*, and *Bacteroidetes* and greater abundance of members of phyla *Actinobacteria* and *Firmicutes* with drought (Fig. 4A and B). Additionally, increasing temperature promoted *Verrucomicrobia* abundance, whereas drought repressed it in soil mixtures (Fig. 4A and B). Root fractions showed more distinct changes in composition with treatment than soil mixtures, where we detected decreasing relative abundances of *Proteobacteria* with heat treatment, while members of *Bacteroidetes* increased (Fig. 4C and D). For *Actinobacteria* specifically, increasing temperature and drought corresponded with greater relative abundances in both plant roots and the surrounding soil mixture (Fig. 4). To further characterize shifts in *Actinobacteria* due to treatment, we plotted the top nine most abundant genera in the phylum *Actinobacteria* by sample type

Fig. 1. Boxplot of soil mixture moisture level (percent) across all treatments: C.W = 22°C and watered, C.D = 22°C and drought, H1.W = 30°C and watered, H1.D = 30°C and drought, H2.W = 38°C and watered, and H2.D = 38°C and drought.

(Supplementary Fig. S4). We found greater counts in roots, as compared with the soil mix, and observed that *Streptomyces* spp. largely dominated the root fraction for both TPs, particularly when higher temperature was combined with drought (Supplementary Fig. S4). These results demonstrate that drought and temperature corresponded to changes in relative bacterial community composition, as observable on the phylum level, with greater relative Actinobacterial enrichment observed under a combination of higher temperatures and drought than with cooler temperatures and watered conditions.

Temperature is associated with a greater number of indicator taxa than drought in roots. To identify which bacterial lineages may be differentially enriched relative to temperature and watering conditions, we performed a series of indicator species analyses (P value < 0.05) in TP1 and TP2 root fractions (Fig. 5; Supplementary Figs. S5 and S6). We observed that a majority of indicator taxa corresponded to watered treatment conditions in both TPs (TP1 and TP2 = 9 and 77 watered indicators and 100 and 83% of all indicators, respectively) (Supplementary Fig. S5a; Fig. 5A), and *Actinobacteria* were wholly indicative of drought in TP2 (13 operational taxonomic units [OTUs]) (Fig. 5A and C). In order to determine which indicators uniquely corresponded to temperature, versus drought and temperature combined, we separated our data into watered and drought subsamples for separate analyses. We also eliminated the middle temperature range (30°C) for this analysis, focusing on the 22 and 38°C temperature treatments, in attempts to analyze the greatest distinction between treatments. For TP1 watered roots, there were more indicators for high temperature ($n =$

28) as compared with 22°C ($n = 16$) and, interestingly, a majority of *Actinobacteria* were indicators for 38°C (83%, five of six total) and all indicator species of Cyanobacteria grouped with 38°C (Supplementary Fig. S5b and d). In the watered subset for TP2 roots, we similarly found a greater number of indicators for 38 than 22°C treatments (64 versus 57, respectively), and both types were generally well parsed across major phyla, with the exception of phylum *Actinobacteria*, which primarily comprised high-temperature indicators (82% for 38°C, 9 of 11 total) (Fig. 5B and C). Additionally, phyla Chloroflexi and Cyanobacteria only contained high-temperature indicators in TP2 watered roots (Fig. 5B).

In the drought subset for TP1 roots, there were overall fewer temperature indicators for drought, as compared with the watered subset (21 versus 41, respectively), and a minority of *Actinobacteria* were indicative of 38°C (1 of 3 total) (Supplementary Fig. S5c). Analyzing the drought subset of TP2 roots, 58 of 63 indicators corresponded to 22°C, with approximately one-fourth of *Actinobacteria* (3 of 11 total) being indicators corresponding to high temperatures (Supplementary Fig. S6). Interestingly, of all *Actinobacteria* indicators from these analyses ($n = 32$), only 4 are a shared indicator of both watering treatment and temperature treatment (Supplementary Table S4). Taken together, these results show that not only did temperature alone restructure the bacterial root microbiome but also high temperature corresponded to unique indicator taxa that overlapped little with those corresponding to drought. Furthermore, TP comparisons demonstrated that heat exerted a greater influence than drought on root microbiome assembly earlier on in plant development.

Fig. 2. Boxplots of α diversity, as based on Shannon diversity index, for the **A**, soil mixture and **B**, roots across both time points and all treatments: C.W = 22°C and watered, C.D = 22°C and drought, H1.W = 30°C and watered, H1.D = 30°C and drought, H2.W = 38°C and watered, and H2.D = 38°C and drought.

DISCUSSION

Although past work has shown that environmental stressors individually can significantly affect plant–microbe interactions, much remains unknown regarding how the phytobiome independently coordinates responses to co-occurring and correlated stresses. This study provides one of the first looks into how heat and drought stress, separately and when combined, contribute to assembly patterns in the root bacterial microbiome, particularly with regards to enrichment of the phylum *Actinobacteria*. We hypothesized that heat and drought stress together would correspond to distinct microbial community profiles, including a greater enrichment of *Actinobacteria*, than with either stress alone. Using growth chambers to test how a series of temperatures and drought affected the root microbiome, we found that increasing temperature did, indeed, increase the relative abundance of *Actinobacteria*, independent of

drought, in the root. In heat-stressed soils, *Actinobacteria* have been observed as a dominant taxa in soil communities (Riah-Anglet et al. 2015), as well in sugar beet rhizospheres (van der Voort et al. 2016). *Actinobacterial* metabolism is also known to shift in response to heat stress (Bursy et al. 2008), and the ability of *Actinobacteria* to withstand high temperatures has been used in selective isolation (Guo and Zhang 2014; Hayakawa et al. 2010). Several factors are thought to contribute to these characteristics, including that many *Actinobacterial* taxa are Gram-positive and monoderms, in which a thick peptidoglycan cell wall and ability to accumulate osmolytes may improve desiccation resistance (Hartman and Tringe 2019), can sporulate to enter a stable and quiescent state to better persist through periods of environmental stress (Holmes et al. 2000), and can produce various siderophores and other secondary metabolites that prove advantageous for survival and recruitment by plants hosts (Lewin et al. 2016). There

Fig. 3. Constrained analysis of principal coordinates (CAP) of β diversity, as based on Bray-Curtis distances, with the models of CAP1 attributed to temperature treatment and CAP2 attributed to watering treatment for the **A** and **B**, soil mixture and **C** and **D**, roots for each time point and treatment: C.W = 22°C and watered, blue; C.D = 22°C and drought, light blue; H1.W = 30°C and watered, orange; H1.D = 30°C and drought, yellow; H2.W = 38°C and watered, red; and H2.D = 38°C and drought, light red.

is a need for future work that describes the specific molecular mechanisms involved in differential microbial community assembly for host plants experiencing heat and drought stress, when stressors are in isolation as well when combined. In addition, having utilized a field soil mixture, we recommend that additional studies investigate and compare enrichment patterns across a

variety of soil types. In further characterizing how heat affects bacterial root communities, particularly for the phylum *Actinobacteria*, our knowledge of how the phytobiome functions as a system, in relationship to the environment, will better allow for targeted and improved fortification of crops grown under more extreme climatic conditions.

Fig. 4. Bar graphs of the relative abundances of top 11 phylum across time point and **A and B**, soil mixture and **C and D**, root samples for treatments: C.W = 22°C and watered, C.D = 22°C and drought, H1.W = 30°C and watered, H1.D = 30°C and drought, H2.W = 38°C and watered, and H2.D = 38°C and drought.

In analyzing the distinct impact of heat on root bacterial communities, we found differentiation between drought and water in terms of specific indicator taxa, such that Actinobacterial enrichment predominantly corresponded to higher temperatures in watered

conditions, rather than under drought conditions. This suggests that drought may be driving specific Actinobacterial enrichment that may overpower heat stress-driven responses when together, and a different subset of *Actinobacteria* may be specialized for heat

Fig. 5. Phylogenetic trees of bacterial operational taxonomic units (OTUs) from time point 2 root communities that are indicator species ($P < 0.05$). **A**, Indicators for watered (dark gray) and drought (light gray) conditions, as specified with the outer ring. **B**, When community data are subset to watered conditions, 22°C (blue) and 38°C (red) indicators, as is specified with the outer ring. Outer bar graphs on trees show the log₁₀ abundance, as determined by combining the total read counts across replicates, for each indicator OTU in time point 2 roots. **C**, Bar graphs showing the relative abundance of the top six phyla representative of OTUs in either indicator type (watered versus drought and 22 versus 38°C) for both analyses.

responses. This may be partially explained by how plants respond to these two different abiotic stressors, which has been shown to influence microbial recruitment (Fitzpatrick et al. 2018). Past work indicates that heat stress may be more difficult to buffer against than drought, because plants can employ a number of physiological changes to conserve water and help bolster turgidity but high temperatures can damage protein synthesis, structures, and membranes, and inactivate major enzymes (Fahad et al. 2017). Furthermore, plant growth is reduced during drought (Anjum et al. 2011), which corresponds with a slowing of root respiration (Bryla et al. 1997); as a result, drought may affect the extent to which roots, and their associated microbial communities, can respond to heat stress (Huang et al. 2012). Past work has also found that drought indicators wholly comprise (Naylor et al. 2017) or are largely dominated by the phylum *Actinobacteria* (Xu et al. 2018), whereas members of *Proteobacteria* are dominant within watered indicators (Naylor et al. 2017; Xu et al. 2018). Notably, several studies have also demonstrated that some strains in the phyla *Proteobacteria* and *Firmicutes* promote plant growth under drought or heat stress (Abd El-Daim et al. 2014; Ali et al. 2009; Khan et al. 2020; Majeed et al. 2018). We recommend that further studies characterize what genomic and metabolic differences may exist in indicators of heat versus drought stress in plant roots, in order to better understand how each abiotic stress contributes to microbial recruitment, as well as the implications of those individual taxa for plant growth.

Our results also demonstrate a difference in timing of root microbiome responses to watering treatment versus temperature that included the number of indicator taxa. We found that heat rapidly promotes Actinobacterial enrichment, and this can occur in the absence of drought, suggesting that heat can rapidly restructure bacterial communities and drive recruitment of heat-specific indicators, whereas drought stress effects—particularly with regards to Actinobacterial enrichment—are more apparent after more sustained stress exposure. Indeed, drought has been shown to delay the early development of the sorghum microbiome (Xu et al. 2018) as compared with watered controls, and we recommend more studies to determine whether high temperatures prompt a more rapid development of the microbiome. In addition, not only have various phyla been shown to respond to distinct patterns of root exudation across plant growth (Chaparro et al. 2014; Vives-Peris et al. 2018) but also both stressors are also known to influence root exudation rates and composition, where a higher proportion of carbon is allocated to exudation and greater numbers of secondary metabolites can be present (Gargallo-Garriga et al. 2018; Mainali et al. 2014; Vives-Peris et al. 2018; Williams and de Vries 2020). Some evidence also suggests that increased temperature can increase exudation rates (Uselman et al. 2000), whereas drought reduces overall exudation (Williams and de Vries 2020). To better understand how bacterial communities may uniquely collaborate with plants to survive environmental challenges, we recommend that future studies look at long-term impacts of both stresses alone and combined, where some evidence suggests that crops are more sensitive to stress in reproductive stages of development (Hussain et al. 2019) and developmental stage is known to affect microbial community dynamics (Chaparro et al. 2014).

Whereas past work has shown that abiotic and biotic stress affect the microbiome of crop plants, there are several limitations to field-based approaches in developing a comprehensive understanding of how phytobiomes function. Associations between plants and microbes are highly dynamic and complex, and evidence thus far suggests that the surrounding environment is one of the strongest modulators of these interactions (Coleman-Derr et al. 2016; Naylor et al. 2017; Peiffer et al. 2013; Siciliano et al. 2001). Some environmental conditions can be manipulated in the field with relative

ease, including watering, nutrients, agricultural practices, and disease, in order to determine how the plant microbiome is affected by abiotic and biotic stress. Other conditions are more challenging to modulate; for example, the role of atmospheric conditions of temperature, light, and carbon dioxide levels. Therefore, additional approaches are needed to more rigorously describe how phytobiomes assemble and function across systems characterized by varying degrees of abiotic and biotic stress, including supplementing field studies with more controlled, lab-based studies.

Conclusion. The findings presented here further our knowledge of how abiotic stresses differentially modulate plant–microbe interactions. Specifically, we demonstrated that elevated temperature is sufficient to significantly affect root bacterial communities and has impacts that are distinct from those of drought alone. We further showed that the degree of Actinobacterial enrichment with drought incrementally increases with higher temperatures. We recommend future work to consider the unique roles that these separate abiotic factors play in microbiome assembly and activity, particularly in relationship to plant growth and yield. Such efforts inform the aim of using endophytes for targeted manipulation of specific traits in crop plants such as heat and drought tolerance and increased yield. This research also demonstrates the value of disentangling the contributions of various environmental factors experienced in the field via lab-based approaches and deepens our understanding of how phytobiomes may coordinate individual stress responses.

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