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RESEARCH

Abiotic Stress Reorganizes Rhizosphere and Endosphere Network Structure of *Sorghum bicolor*

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ABSTRACT

Sorghum bicolor is a promising bioenergy feedstock with high biomass production and unusual tolerance for stresses, such as water and nutrient limitation. Although the membership of the sorghum microbiome in response to stress has been explored, relatively little is known about how microbe–microbe networks change under water- or nutrient-limited conditions. This is important because network changes can indicate impacts on the functionality and stability of microbial communities. We performed network-based analysis on the core bacterial and archaeal community of an agronomically promising high biomass bioenergy genotype, Grassl, grown under nitrogen and water stress. Stress caused relatively minor changes in bacterial abundances within soil, rhizosphere, and endosphere communities but led to significant changes in bacterial network structure and modularity. We found a complete reorganization of network roles in all plant compartments, as well as an increase in the modularity and proportion of positive associations, which potentially could represent coexistence and cooperation in the sorghum bacterial/archaeal community under stress. Although stressors are often believed to be destabilizing, we found stressed networks were as or more stable than non-stressed networks, likely due to their redundancy and compartmentalization. Together, these findings support the idea that both sorghum and its bacterial/archaeal community can be resilient to future environmental stressors.

Keywords: abiotic stress, bioenergy, drought, microbe–microbe associations, nitrogen limitation, plant microbiome

Plants live in close association with microbes, many of which can expand the functional capabilities of their plant hosts and aid in their growth (Backer et al. 2018; Berendsen et al. 2012; Goh et al. 2013; Haney et al. 2015; Trivedi et al. 2020). For these reasons, plant-microbe symbioses are thought to be particularly important under conditions of abiotic stress, such as nutrient deficiency (Alori et al. 2017; Majeed et al. 2018) and water limitation (Armada et al. 2018; Chandra et al. 2021; Gontia-Mishra et al. 2016; Mayak et al. 2004). Stressful conditions are likely to increase due to climate change (Cavicchioli et al. 2019; Classen et al. 2015; Compant et al. 2010) and will particularly affect bioenergy crops as marginal land is pressed into production to meet energy demands without affecting food supply (Fazio and Monti 2011; Kang et al. 2013; Langholtz et al. 2016).

Sorghum (*Sorghum bicolor* [L.] Moench) is a promising cereal grain feedstock for biofuel production on marginal lands due to its high biomass yield, nitrogen-use efficiency, and drought tolerance (Boyles et al. 2019; Chai et al. 2021; Gelli et al. 2014; Mace et al. 2013; Rooney et al. 2007). Previous studies have demonstrated that sorghum responses to environmental stresses can influence the assembly of microbial taxa on their roots (Chai et al. 2021; Lopes et al. 2021; Sheflin et al. 2019; P. Wang et al. 2021), but none has characterized network relationships. While some microbial taxa associated with cereal crops such as sorghum exhibit plant growth-promoting effects (Carvalho et al. 2014; Hara et al. 2019;

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e-Xtra: Supplementary material is available online.

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Kochar and Singh 2016; Xu et al. 2018), others potentially lead to growth reductions (Chai et al. 2021; van der Heijden et al. 2008), and most have no obvious phenotypic effects when added individually. Recent evidence suggests that the effect of microbial communities, which can include thousands of individual strains, on plant growth may be different than the sum of the effects of each individual microbe due to the complexity of microbe–microbe interactions (Sanchez-Gorostiaga et al. 2019). Thus, a better understanding of the interactions between the host, environment, and microbiome will be important for the expansion of biofuel production on marginal lands.

To date, most plant microbiome studies have focused on microbial diversity (the number of species and their abundances) and composition (the taxonomic or phylogenetic makeup of those communities) as contributors to plant performance (Abdul Rahman et al. 2021; Banerjee et al. 2018; Edwards et al. 2018; Jones et al. 2019). These findings have been useful in cases where shifts in microbial composition are large or phenotypes can be attributed to a narrow set of bacteria. However, it is also likely that differences in plant performance can be caused by changes in large-scale root-associated microbial interspecies associations, which become difficult to parse from high-dimensional datasets such as microbiome profiles. In these cases, network analysis can be a useful approach to exploring the complex relationships among rhizosphere and endosphere microbes. Although caution should be used when interpreting microbial networks (Faust 2021) (e.g., experimental validation is often needed to confirm true interactions between microbes), global parameters such as connectivity and modularity (i.e., clustering) can provide valuable insights into community stability under abiotic stresses (Lv et al. 2019; van der Heijden and Hartmann 2016). For instance, networks inferred from microbial communities under stress might be less clustered and have more positive edges than networks from non-stressed communities (Hernandez et al. 2021). Both of these traits have been linked to network destabilization (Coyte et al. 2015), which can have profound consequences for plant performance when keystone microbes that contribute to plant growth become less abundant or absent.

Here, we performed a detailed network-based analysis on the core bacterial community of the agronomically promising high biomass bioenergy sorghum genotype, Grassl, under water and nutrient limitation. Given the large role that plant compartment plays in microbial diversity, we hypothesized that the core soil network would be more complex (e.g., have more nodes and edges) than the rhizosphere and root. Similarly, we hypothesized that core rhizosphere and root networks would have denser connections and increased modularity due to the selective pressure of a plant-associated habitat. Finally, we hypothesized that stress (i.e., low nitrogen and water-stressed conditions) would lead to a change in network hubs, as well as lower modularity and more negative associations than seen in non-stressed networks, leading to a decrease in their stability.

MATERIALS AND METHODS

Study system and experimental design. At two field sites in Nebraska in 2017, we grew the sorghum genotype Grassl under conditions of full and low nitrogen (at Central City, NE), as well as under well-watered and water-stressed conditions (at Scottsbluff, NE). Details describing the experimental design and generation of this dataset can be found in related manuscripts (Chai et al. 2024; Qi et al. 2022). In short, the fields were "naturally" low N or low water and were supplemented according to standard agricultural practice for the local region. The level of organic matter was similar and relatively low at both locations, and both locations were irrigated with low levels of nitrate in the water. Both soils were sandy, with the rest of the major chemical constituents of the soil being similar but not identical between both locations. We sampled the soil, rhizosphere, and endosphere at four time points in the nitrogen experiment and three timepoints in the water experiment using a split-plot design with eight replicate blocks for each treatment, resulting in a total of 336 samples.

DNA extraction, sequencing, and bioinformatics. After excavation of the root ball, approximately 200 g of excess soil (bulk soil) was shaken off and collected, and a representative sample of roots was cut from the root ball. Roots were placed in 50-ml tubes with phosphate buffer (6.3 g liter^{-1} NaH_2PO_4, 8.5 g liter^{-1} Na_2HPO_4 anhydrous) and vigorously shaken to release soil from the roots (rhizosphere). The rhizosphere was further processed by filtering with 100-μm mesh followed by pelleting at 3,000 × *g* for 10 min. Roots were surface sterilized for 30 s with sodium hypochlorite (5.25%) and then ethanol (70%), followed by three washes with ultrapure water. Liquid N was then used to grind the roots to access the endosphere microbial community.

Soil and rhizosphere DNA were extracted using the PowerSoil-318 htp 96-well Soil DNA Isolation Kit (MoBio, Carlsbad, CA), whereas endosphere DNA was extracted using the Applied Biosystems MagMax Plant DNA isolation kit (Thermo Fisher Scientific, Waltham, MA). All samples were quantified with the QuantiFluor dsDNA reagent (Promega, Fitchburg, WI) following the manufacturers' protocols. The V4 region of the 16S rRNA gene was amplified following the Joint Genome Institute iTag amplicon sequencing protocol (available at https://jgi.doe.gov/user-programs/pmo-overview/protocols-sample-preparation-information/) using the 515F-806R primer pair and including chloroplast and mitochondrial peptide nucleic acid blockers (Lundberg et al. 2013). Amplified samples were multiplexed at 184 samples per run and sequenced on an Illumina MiSeq (paired end 2 × 300 bp) at the DOE Joint Genome Institute (Berkeley, CA). Data are available at NCBI under BioProject PRJNA945936.

Raw sequences (mean = 32,851 reads) from separate MiSeq runs were demultiplexed and processed in QIIME2 v. 2020.2 (Bolyen et al. 2019). Primers and adaptors were trimmed using cutadapt, and low-quality reads were removed. Reads were denoised and assigned to amplicon sequence variants (ASVs) at 100% sequence similarity in DADA2 (Callahan et al. 2016). Taxonomy was assigned using the SILVA database release 132 (Quast et al. 2013). Data were filtered for ASVs identified as chloroplast, mitochondria, or those lacking phylum assignment. After removing samples with poor extraction or amplification (i.e., low-quality or very few reads), we were left with our final dataset of 276 samples. All further analyses were conducted in R v. 4.0.2 (R Core Team 2020). ASV and taxonomic tables were merged using the *phyloseq* package (McMurdie and Holmes 2013).

Diversity analyses. We adapted a persistence method developed by Shade and Handelsman (2012) to identify core soil, rhizosphere, and root bacterial communities for the genotype Grassl. Samples from the nitrogen and water experiments were analyzed separately due to differences in location and sampling dates. For each compartment (soil, rhizosphere, or root) and location, we have several subsets of data, where each subset corresponds to a combination of dates and growth conditions. Within each subset (i.e., location × compartment × time point × treatment), an ASV was counted toward the core if it was present (with a positive count) in more than 80% of samples in that subset (or 50% for the root to obtain a roughly equal number of core ASVs while accounting for the higher dissimilarity between samples; Supplementary Figure S1a). By taking the union across all subsets encompassing various dates and growth conditions, we obtained the core bacterial and archaeal community for each of the six combinations of compartment and

location (e.g., the Central City soil core is the union of all subsets from both the full N and low N treatments for each sampling date), resulting in six cores. With this approach, even if an ASV is present in 80% of full-nitrogen rhizosphere samples at the first time point but not in 80% of low-nitrogen rhizosphere samples at that time point, it is still counted as part of the overall "nitrogen rhizosphere core." Unlike other core microbiome methods that only consider the intersection of subsets, our method includes ASVs that may only show up on some combinations of dates and treatments but not on others, so the overall core (i.e., union of subsets) reflects ASVs that appeared and then disappeared over time or across treatments but not those that stochastically appear in only some replicate samples (Supplementary Figure S1b and c). We chose this definition of the core because it allowed us to determine the influence of stress on network structure while holding species richness (number of nodes) constant for each separate combination of compartment and location. ASVs in each of these datasets were then identified as putatively plant growth promoting through a Web of Science literature search, in which the family or genus name was searched alongside the terms "plant-growth promot*," "nitrogen," and "drought," and papers were screened for empirical evidence of plant growth promoting traits. Although this was not an exhaustive search, it provided information for more than 33% of taxa in all core communities (Supplementary Data File S1).

All subsequent analyses were carried out on the cores. Relative abundance plots were conducted in *phyloseq*. Comparisons of community composition were calculated using Bray-Curtis dissimilarities, as well as unweighted and weighted UniFrac distances (Lozupone and Knight 2005). All metrics gave similar results, and thus, only the results of analyses using weighted UniFrac distances are presented. Comparisons of homogeneity of group dispersions (with betadispers) and permutational multivariate analyses of variance were conducted using the *vegan* package (Oksanen et al. 2020), and compositional patterns were visualized with principal coordinate analysis. Finally, we performed differential abundance analysis on ASVs using DESeq2 (Love et al. 2014) with false discovery rate correction using the Benjamini-Hochberg procedure. Data visualizations were created using *ggplot2* (Wickham 2016).

Network analysis. Networks were generated in SPIEC-EASI ($n = 24$ for nitrogen experiment and 20 for water experiment) with the Meinshausen Bühlmann neighborhood selection method (Kurtz et al. 2015). Final networks and their covariance matrices representing the most stable edges were selected using StARS by assessing 20 values of lambda (lambda ratio = 0.01) for 50 sub-sampled generations. We chose to use SPIEC-EASI over direct correlation-based network methods because its use of inverse covariances makes it more robust to the compositionality and sparsity inherent to microbiome datasets—two issues that other inference methods fail to address leading to spurious edges (Gloor et al. 2017). Randomized benchmark networks ($n = 999$) were generated using the rewire function in the *igraph* package (Csárdi and Nepusz 2006), which randomizes the edges between nodes of the empirical networks while preserving their degree distribution. Attributes from our empirical networks were compared with those of the randomized benchmark networks via Wilcoxon rank sum tests to confirm that our networks significantly differ from those formed at random.

Network attributes (for definitions, see Supplementary Table S1), such as clustering coefficient, path length, global efficiency, betweenness centrality, and node degree, were calculated in *igraph* and compared via Kruskal-Wallis test to assess the impact of abiotic stress on microbial network clustering and stability. The *rnetcarto* package (Doulcier and Stouffer 2023) was used to identify modules/clusters via simulated annealing and to calculate (i) network modularity (i.e., a global measure of how clustered or compartmentalized a network is); (ii) each node's within-module degree (z), which measures how connected each node is to the others within its module; and (iii) participation coefficient (P), which measures how distributed a node's associations are across all modules. The latter two attributes were used to estimate the role of nodes in each network following the procedure outlined by Guimerà and Nunes Amaral (2005). In brief, nodes were defined by the region of the z-P parameter space they occupied: hubs are nodes with $z \geq 2.5$, ultra-peripheral nodes with $z < 2.5$, $P \leq 0.05$ (all associations within their module), peripheral nodes with $z < 2.5$, $0.05 < P \leq 0.62$ (most of their associations within their module), connector nodes with $z < 2.5$, $0.62 < P \leq 0.80$ (many associations outside their module), and kinless nodes with $z < 2.5$, $P > 0.80$ (associations homogeneously distributed across all modules). Finally, network stability was measured by attack robustness: the relationship between node loss and network size, using the *brainGraph* package (Watson 2019). In brief, we simulated the sequential loss of microbial nodes based on various attributes and calculated the size of the remaining network at each stage, with steeper slopes representing less stable networks.

RESULTS

Sorghum soil, rhizosphere, and root networks are distinct. Our core community analysis identified 507 core soil ASVs, 449 core rhizosphere ASVs, and 420 core root ASVs in the nitrogen experiment and 683 core soil ASVs, 588 core rhizosphere ASVs, and 194 core root ASVs in the water experiment (Fig. 1). Several core ASVs (soil, rhizosphere, or root) belonging to the phyla Proteobacteria (with most ASVs in the families Burkholderiaceae, Pseudomonadaceae, and Sphingomonadaceae) and Bacteroidetes (with most ASVs in the family Chitinophagaceae) were differentially abundant in the rhizosphere and root as compared with the soil, regardless of experiment and time point (Supplementary Figure S2a). Among plant-associated samples, several core Gammaproteobacteria ASVs (e.g., family: Pseudomonadaceae and Xanthomonadaceae) and Firmicute ASVs (family: Paenibacillaceae) were more abundant in the rhizosphere, whereas Alphaproteobacteria ASVs (e.g., family: Dongiaceae, Sphingomonadaceae, and Xanthobacteraceae) were more abundant in the root (Supplementary Figure S2b).

Composition of the Grassl rhizosphere and root microbial core communities significantly changed over time, most notably with a large increase in the order Pseudomonadales in the rhizosphere midway through the growing season ($P < 0.001$; Fig. 2, Supplementary Fig. S3). Despite a significant effect of growth condition on Grassl harvest biomass ($P < 0.001$; Supplementary Fig. S4), we did not identify any differentially abundant core ASVs between growth conditions in the Grassl soil or rhizosphere but did in the Grassl root. *Pseudomonas* was more abundant in the low N root ($\log_2$ fold change [$\log_2$FC] = 22.9, $p_{adj} < 0.001$), whereas *Acinetobacter*, *Herbaspirillum*, *Pantoea* (all class: Gammaproteobacteria), *Rhizobium* (class: Alphaproteobacteria), and *Filimonas* (phylum: Bacteroidetes) were more abundant in the full N root ($\log_2$FC from 23.1 to 24.6, $p_{adj} < 0.001$). There was one ASV (*Acidibacter*, class: Gammaproteobacteria; $\log_2$FC = 7.00, $p_{adj} = 0.03$) differentially abundant in water-stressed roots.

To explore community structure, we began by assessing global network attributes for each of the networks and comparing these attributes to those of random benchmark networks (Supplementary Table S1). We found that our empirical networks had average clustering coefficients and global efficiencies higher than those of the random benchmark networks ($P < 0.01$) but that they did not significantly differ in average path length or modularity. These re-

sults confirmed that our empirical networks were likely composed of non-random clusters of bacterial ASVs and warranted further investigation.

Multiple network attributes showed that bacterial co-association patterns differed by sample type. Root networks were more modular and less efficient and had higher clustering coefficients and longer average path lengths than soil and rhizosphere networks ($P = 0.02$; Supplementary Table S1). This might be in part due to a decrease in nodes (i.e., core size) and edges connecting those nodes from the soil to the root (Supplementary Table S2). Initially, we calculated all of the cores using an 80% threshold, but this resulted in endosphere cores that were much smaller (e.g., 68 and 52 taxa) than the soil or rhizosphere cores, exacerbating the effects on modularity, efficiency, and clustering. Likely related, nodes in root

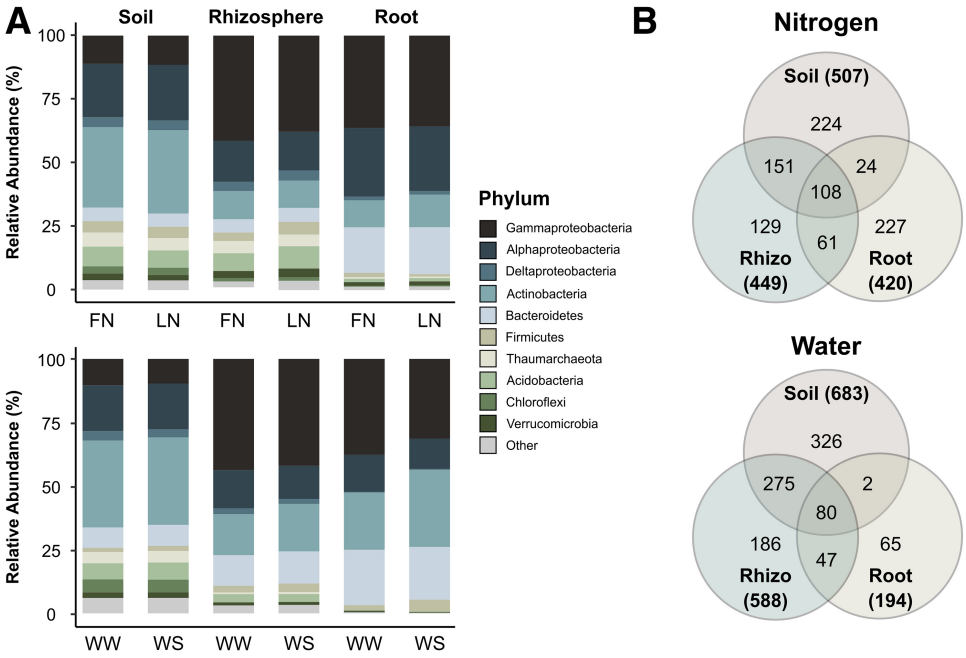
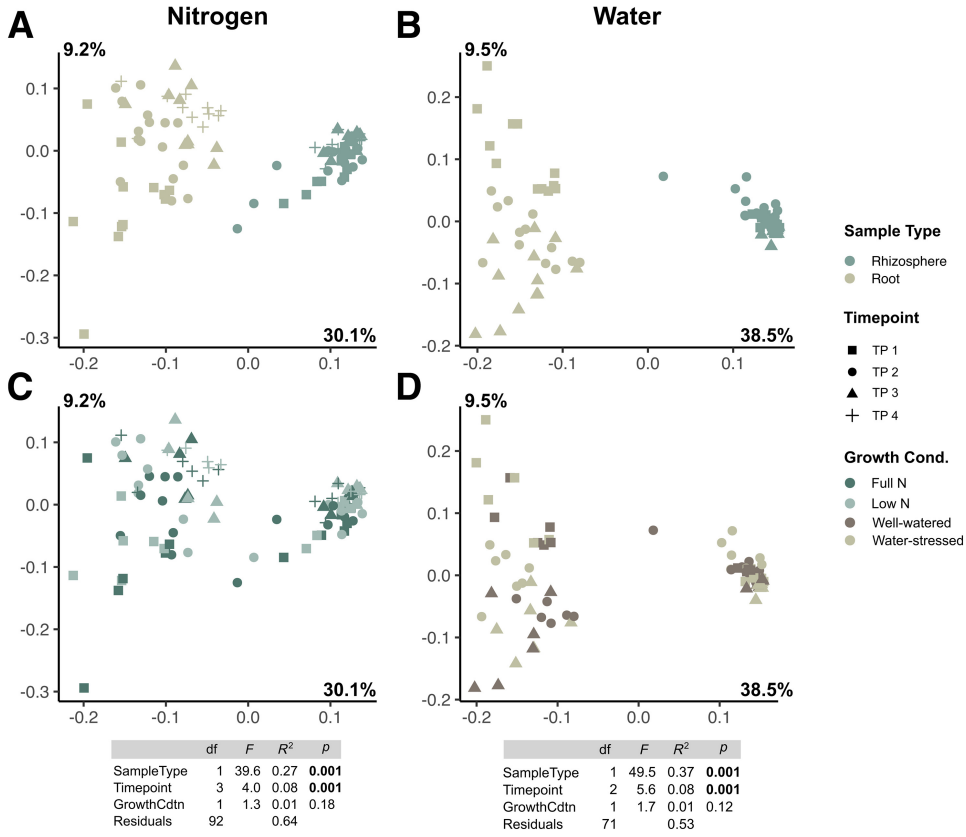


Fig. 1. Core sorghum bacterial and archaeal communities as identified via persistence method across timepoints and growth conditions. **A,** Relative read abundance of the 10 most abundant phyla for each growth condition in the six cores. **B,** Venn diagram depicting the number of shared amplicon sequence variants (ASVs) in the soil, rhizosphere, and root cores for the nitrogen experiment at Central City, NE (top), and the water experiment at Scottsbluff, NE (bottom). Numbers in parentheses represent the total number of ASVs in that core.



	df	F	R^2	p
SampleType	1	39.6	0.27	**0.001**
Timepoint	3	4.0	0.08	**0.001**
GrowthCdtn	1	1.3	0.01	0.18
Residuals	92		0.64	

	df	F	R^2	p
SampleType	1	49.5	0.37	**0.001**
Timepoint	2	5.6	0.08	**0.001**
GrowthCdtn	1	1.7	0.01	0.12
Residuals	71		0.53	

Fig. 2. Principal coordinate analyses of Grassl rhizosphere and root community composition measured by weighted UniFrac over multiple timepoints in the **A and C,** nitrogen and **B and D,** water experiment, colored by A and B, sample type or C and D, growth condition.

networks formed the fewest associations (5.8 ± 0.07), followed by rhizosphere nodes (10.5 ± 0.07) and then soil nodes (12.6 ± 0.07; $F_{(2, 5664)} = 2741.6$, $P < 0.001$; Fig. 3A). Rhizosphere and root networks also had a higher percentage of positive associations (except in the well-watered rhizosphere) and within-phylum associations than soil networks (Supplementary Table S2).

We also found significant effects of stress and growth condition on node degree (stress: $F_{(3, 5664)} = 38.2$, $P < 0.001$, growth condition: $F_{(6, 5664)} = 501.7$, $P < 0.001$; Fig. 3A). Soils under stress had a lower average node degree than non-stressed soils (stress: 12.1 ± 0.1; non-stress: 13.0 ± 0.11), but rhizospheres under stress had a higher node degree than non-stressed rhizospheres (stress: 10.8 ± 0.09; non-stress: 10.2 ± 0.11). There was no difference between the average degree of stressed and non-stressed root communities.

Rearrangement of rhizosphere and root network structure under abiotic stress. Next, we used each ASV's participation coefficient (P) and within-module degree (z) to identify its role in the network as either a hub (highly connected within/across modules), connector (many associations outside module), kinless (associations homogeneously distributed across all modules), peripheral (most of their associations within module), or ultraperipheral node (all associations within module), following Guimerà and Nunes Amaral (2005) (Fig. 3B to E; Supplementary Fig. S5). In general, soil networks had a greater proportion of connector nodes, with high participation coefficients (P) and moderate within-module degree (z), suggesting they tend to bridge multiple modules ($\text{mean}_{\text{soil}} = 50.5\%$, $\text{mean}_{\text{rhizosphere}} = 41.5\%$, $\text{mean}_{\text{root}} = 15.5\%$). Rhizosphere and root networks had greater proportions of hub (high z, moderate P; $\text{mean}_{\text{soil}} = 1.1\%$, $\text{mean}_{\text{rhizosphere}} = 1.5\%$, $\text{mean}_{\text{root}} = 1.4\%$) and peripheral nodes (moderate P and z; $\text{mean}_{\text{soil}} = 46.5\%$, $\text{mean}_{\text{rhizosphere}} = 53.8\%$, $\text{mean}_{\text{root}} = 57.3\%$; Supplementary Table S3). When we looked at the identity of connector nodes, we found a higher proportion of connector nodes from the phyla Chloroflexi and Gemmatimonadota in the soil as compared with the rhizosphere and root and a higher proportion of connector nodes in the phylum Proteobacteria in the rhizosphere and root (Supplementary Fig. S7).

Within the rhizosphere, we found that the water experiment hubs had higher average node degrees than the nitrogen experiment hubs (with full N hubs having the lowest average node degrees; Supplementary Table S4). Whereas root networks in general had very few hubs, the water experiment root networks (well-watered and water-stressed) had slightly fewer hubs than the nitrogen experiment root networks, and their hubs had lower average node degrees. We also found that soil hubs had higher participation coefficients (P) than rhizosphere and root hubs ($F_{(2,54)} = 4.7$, $P = 0.01$), but there was no significant difference in within-module degree (z) between sample types (Supplementary Fig. S6). When we looked at the identity of the hubs, we found that there were no hubs shared between any of the growth conditions (Fig. 3B to E; Supplementary Fig. S5) but that they all were low abundance taxa (mean = 0.34% of ASV reads; Supplementary Table S4). Even in cases where hubs shared taxonomic identities, they represented different ASVs (e.g., both full and low N rhizosphere networks had a *Massilia* sp. hub). In plots of across-module (P) and within-module connections (z), we see that network roles of many ASVs are different under stress and non-stress for all sample types. Many of the hubs under non-stress were characterized as peripheral, and connector nodes under stress with alternative ASVs arising as hub nodes (Fig. 3B to E; Supplementary Fig. S5). For example, a *Bacillus* sp. arose as a hub under low N, whereas it was a peripheral node under full N. Instead, the hub for this same module under full N was a Xanthobacteraceae. In comparing those two modules (both modules = 6; Supplementary Fig. S8), the low N *Bacillus* hub was much more connected to all other *Bacillus* ASVs (which were more abundant under low N) and N-fixing taxa in other modules than the full N Xanthobacteraceae hub.

The ratios of positive to negative associations in our networks (sometimes referred to as negative-positive cohesion) revealed positive associations to dominate in all sample types and growth conditions (Supplementary Table S1). Similarly, most associations were between taxa from different phyla (i.e., across-phylum associations). Overall, root networks had more positive and within-phylum associations than either soil or rhizosphere networks. However, the ratio of positive to negative associations in each growth condition varied with node taxonomic identity (phylum) and the type of association (within- vs. across-phylum connections). For example, there was a higher proportion of positive within-phylum associations between Firmicutes and Actinobacteria ASVs under stress ($\text{mean}_{\text{Firmicutes}} = 80.5\%$ of 27 associations, $\text{mean}_{\text{Actinobacteria}} = 73.5\%$ of 162 associations) as compared with non-stress ($\text{mean}_{\text{Firmicutes}} = 37.5\%$ of 14 associations, $\text{mean}_{\text{Actinobacteria}} = 55\%$ of 128 associations) in the rhizosphere. Most of the associations involving Nitrospirae were negative under full N (53% of 17 associations negative), whereas 38% of 34 associations were negative under low N. Finally, most Verrucomicrobia nodes were positively associated with taxa from other phyla (mean = 63.3% of 825 associations), but of their few within-phylum associations, most were negative (mean = 70.3% of 20 associations).

To further compare the influence of hub nodes on surrounding network structure, we created subsets of each network containing the hub nodes and their first-degree neighbors (Fig. 4). These networks revealed that both stress rhizosphere networks (low N and water-stressed) had more nodes and edges between them than non-stress rhizosphere networks despite little difference in the number of hub nodes and a difference in experimental location. Stress rhizosphere network hubs also had more connections to putatively plant growth-promoting taxa on average compared with non-stress rhizosphere hubs (Supplementary Table S3), and overall, there were more associations between plant growth-promoting nodes (Fig. 4B and D; Supplementary Fig. S8). When we examined shared membership between these first-degree neighbor networks, we found 33 ASVs shared between the full N and low N rhizosphere networks (i.e., 30% of low N first-degree neighbor network taxa) and 20 ASVs shared between well-watered and water-stressed rhizosphere networks (i.e., 18% of drought first-degree neighbor network taxa). Most shared taxa belonged to the phylum Proteobacteria in the orders Rhizobiales and Betaproteobacteriales, which were proportionally more abundant among shared taxa (e.g., Betaproteobacteriales represented 44% of shared vs. 18% of non-shared Proteobacteria) than among non-shared taxa. We also identified a single ASV (*Massilia* sp.) shared across all first-degree neighbor networks.

Rhizosphere networks show significant stability despite abiotic stress. To examine the stability of each network, we sequentially removed nodes from each network based on one of three connectivity attributes (decreasing betweenness centrality, degree, or at random) and measured the size of the remaining largest grouping of connected nodes (Supplementary Fig. S9). Overall, these attacks revealed that soil networks were the most stable to node loss, followed by rhizosphere networks and then root networks. Additionally, networks from the nitrogen experiment (conducted in Central City) were the least stable and networks from the water experiment (conducted in Scottsbluff) the most stable for both the soil and rhizosphere, which likely reflects the increased size of the water experiment core. Even though soil and rhizosphere drought networks were less stable than watered networks, this difference only manifested when more than 60% of the nodes were removed.

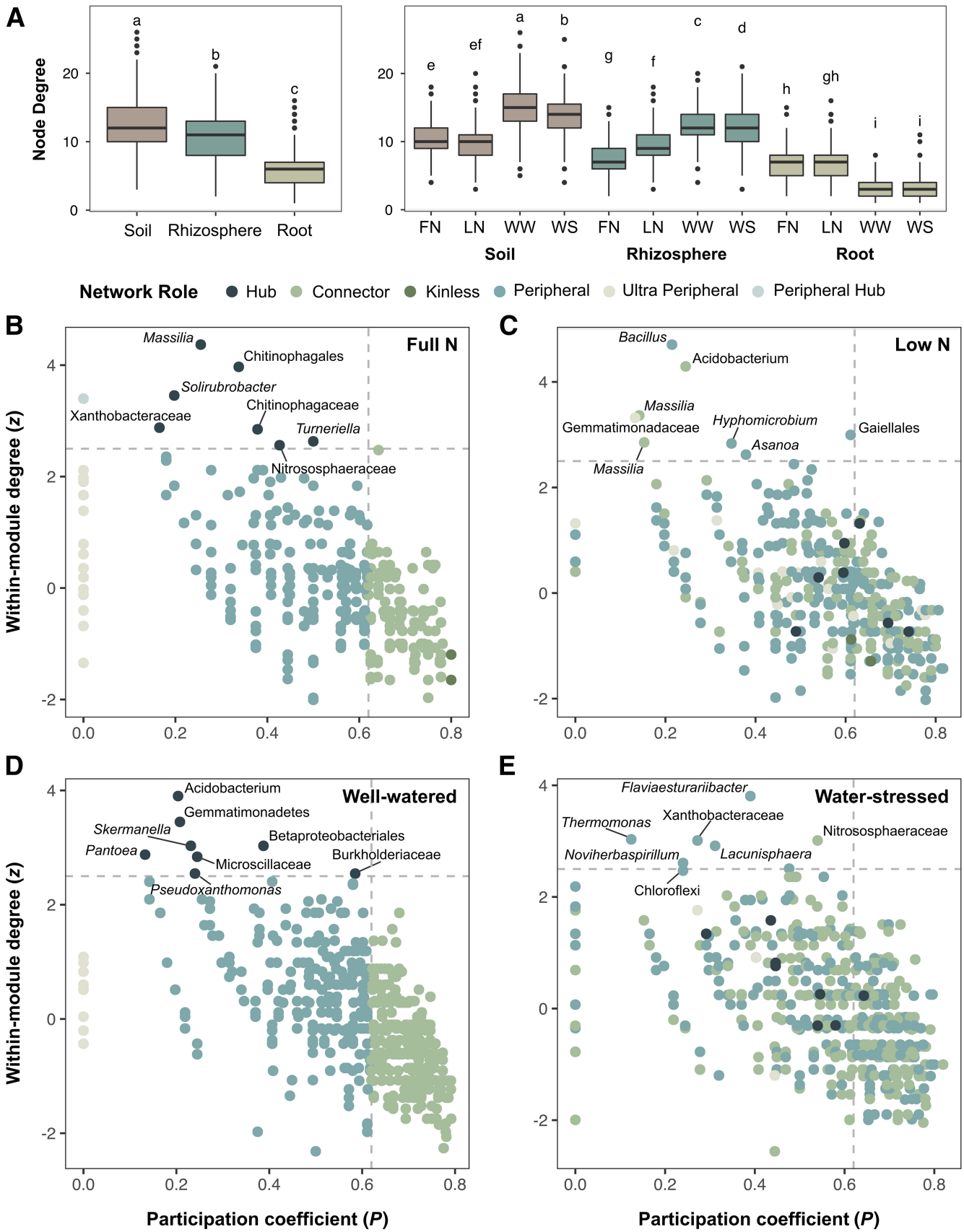


Fig. 3. Connectivity in soil, rhizosphere, and root samples. **A,** Node degree by sample type and growth condition (FN = Full N, LN = Low N, WW = Well-watered, WS = Water-stressed). **B to E,** Network roles of each node in the sorghum rhizosphere under B, full N; C, low N; D, well-watered; and E, water-stressed conditions. The node location in each plot is based on its participation coefficient (across-module connectivity) and *z*-score within-module degree. Node color represents each node's network role in the experiment-associated non-stressed treatment. Hubs in each network are labeled with the lowest taxonomic designation available. Network roles in soil and root are in Supplementary Figure S5.

To explore how our choice of core threshold (80%) influenced stability, we recalculated network stability for the rhizosphere using a relaxed threshold (50%). While increasing the size of the core increased network stability overall, it also confirmed our finding that stressed networks were as or more stable than non-stressed networks (Supplementary Fig. S10). In the root, the well-watered network was the least stable and the full N network the most stable, although it is worth noting that the water experiment root core community had fewer ASVs (nodes) than all other core bacterial communities, which can affect stability.

DISCUSSION

In this study, we explored the diversity, composition, and structure of bacterial communities around *Sorghum bicolor* roots, with a focus on the bioenergy genotype Grassl. In line with our

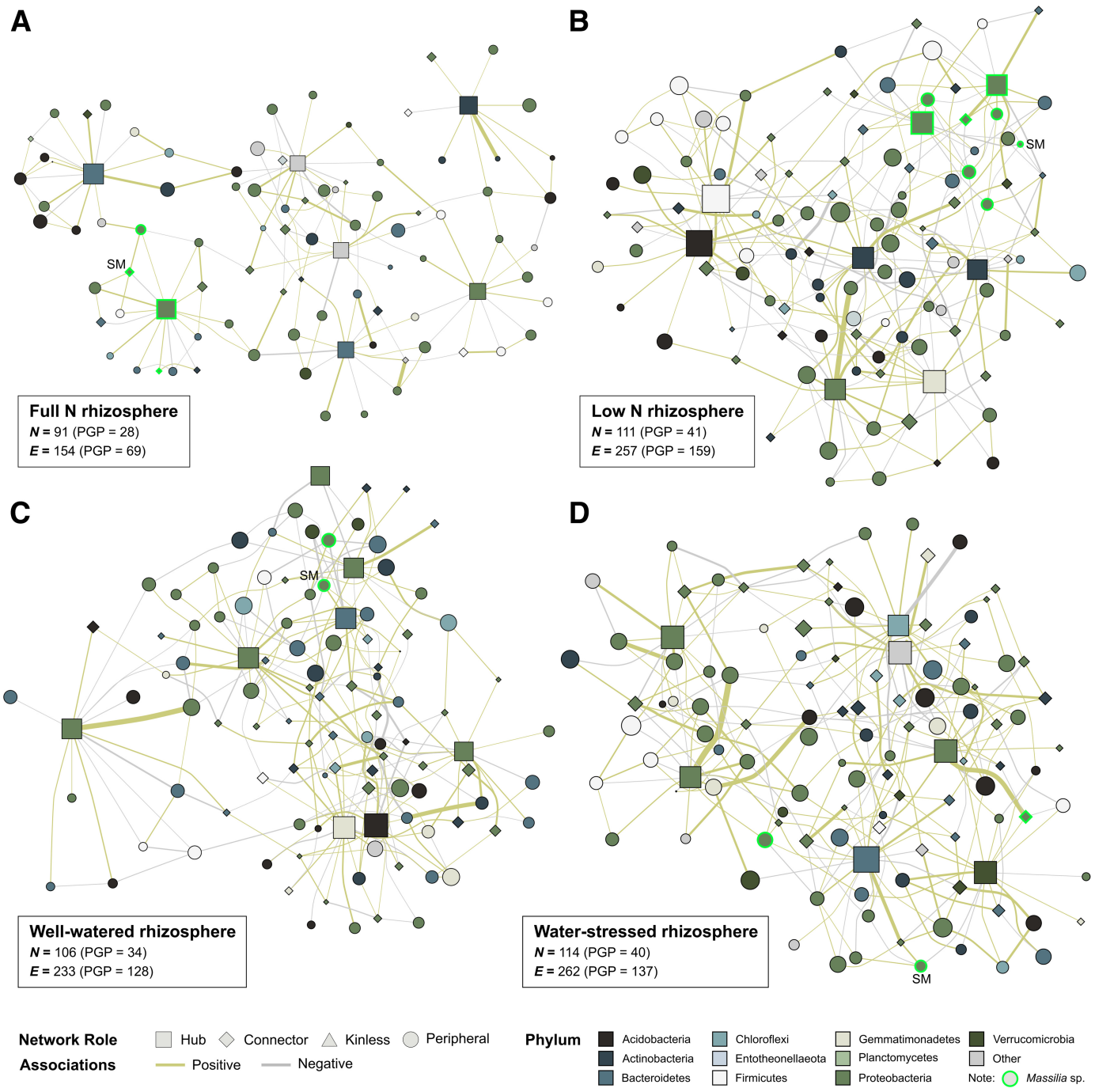


Fig. 4. Hubs and first-degree neighbors of rhizosphere networks. **A,** Full N; **B,** low N; **C,** well-watered; and **D,** water-stressed conditions. Node (*N*) shape denotes network role, node size corresponds to connectivity, and node color identifies phylum. Edge (*E*) thickness corresponds to the effect size of covariance for each association, and edge color identifies the direction of the association: positive or negative. Boxes below each network give the total number of *N* and *E* for each network, as well as *N* and *E* exclusively between putatively plant-growth promoting (PGP) taxa in parentheses. A bright green node outline indicates *Massilia* amplicon sequence variants (ASVs), with the single *Massilia* ASV shared across all networks indicated by "SM."

hypotheses, our analysis revealed that both plant compartment (soil, rhizosphere, or root) and abiotic stress are important predictors of bacterial network structure, especially regarding complexity and modularity. We found that abiotic stress can lead to substantial reorganization of microbial associations with consequences for network stability. Below, we discuss the potential ecological mechanisms responsible for these relationships and their implications for plant-microbe symbioses under increased environmental stress.

Differences between soil, rhizosphere, and root bacterial communities. Despite their proximity, we found several differences between the core communities of soil, rhizosphere, and roots. The rhizosphere and root core bacterial communities had fewer ASVs than the soil core community, which likely reflects the chemically modified niche space available to colonizing soil microbes in the presence of plant cells or root metabolites (Bakker et al. 2014; Nuccio et al. 2020; Whalley et al. 2005) (Fig. 1; Supplementary Table S1). Although it is difficult to compare cores across experiments because they were conducted at different locations, we also found that the root core bacterial community in the water experiment was smaller than that for the nitrogen experiment. A smaller root core is representative of (i) fewer overall reads detected in the root, (ii) lower diversity (although not significantly different between experiments; Supplementary Fig. S1d), and (iii) higher dissimilarity between samples (i.e., there are fewer taxa that are present in at least 50% of samples; Fig. 2B and D; Supplementary Fig. S3). One possible reason that the water root core is proportionally smaller than the nitrogen root core is that water stress was not only more selective (e.g., lower ASV richness and increased relative abundance of specific taxonomic groups such as Actinobacteria) but also resulted in highly dissimilar bacterial communities at the ASV level.

In agreement with studies conducted across plant species, we found that most differentially abundant taxa in the rhizosphere and root cores were from the phyla Proteobacteria and Bacteroidetes, especially several genera known to have plant growth-promoting traits (Fitzpatrick et al. 2018; Ling et al. 2022) (Fig. 1A; Supplementary Fig. S2; Supplementary Data File S1). For example, we found that ASVs from the family Pseudomonadaceae were highly abundant in the rhizosphere, especially midway through the growing season (Supplementary Fig. S3). The association of pseudomonads with the rhizosphere has been well documented (García-Salamanca et al. 2013; Haney et al. 2015; Lugtenberg and Kamilova 2009; Lugtenberg et al. 2001; Melnyk et al. 2019). Our previous experiments in sorghum suggest that the abundant *Pseudomonas* population in the rhizosphere consists of a taxonomically broad group of lineages that are enriched by specific plant-derived carbon sources (Chiniquy et al. 2021). Given the strong influence of time on the composition of the rhizosphere and root, we built our networks to reflect a consensus of microbe–microbe relationships across the sorghum growth cycle rather than at a single point in time.

In line with our hypothesis that plant compartment influences not only microbial diversity but also network structure, we found that soil nodes were on average more connected than rhizosphere and root nodes (Fig. 3A), which is likely due to the higher diversity of bacterial taxa in the soil core community leading to an increased probability for potential associations (Fan et al. 2018; Mendes et al. 2014). The higher diversity and efficiency of soil networks may explain why they were more robust to both targeted (i.e., nodes are removed in order from most to least connected) and at-random attacks than rhizosphere and root networks, even when a comparable fraction of the total nodes was removed. Our observation that soil networks had more associations than rhizosphere and root networks is in agreement with some studies (Fan et al. 2018; Mendes et al. 2014) and in disagreement with others (Shi et al. 2016; Yan et al. 2017). This may be due to our use of the core community rather than all ASVs to generate consensus networks that represent the net-associations between taxa across several time points. Many studies sample only at a single time point later in plant development and so may be able to capture temporally-specific patterns in network structure with an increased sample size. Unfortunately, our sample size was not large enough to further divide our dataset to generate networks for each timepoint via SPIEC-EASI, but we recommend exploring temporal dynamics in network structure in future studies. Differences between studies could also be associated with the plant species used.

Additionally, the lower connectivity in the rhizosphere and root observed here (despite lowering the threshold to 50% to include a comparable number of root taxa in the core) could at least partially be attributed to their greater modularity (i.e., denser connections between taxa within the same module rather than spread evenly across the network) compared with the soil (Supplementary Table S1). Greater modularity of rhizosphere and root networks is likely related to the greater compositional heterogeneity we observed in plant-associated habitats. As seen in microbial metabolic networks (Parter et al. 2007), modularity might arise as a beneficial network trait in variable environments, such as plant-associated habitats, where bacteria are responding to abiotic and biotic changes as the host develops. Increased modularity may reflect the plant's influence over their bacterial community favoring more specialized, compartmentalized microbe–microbe associations (Chomicki et al. 2020) that consequently prevent the proliferation of disturbances throughout the network.

Bacterial hub identity is influenced by environmental stress. Highly connected taxa, known as hubs, have been proposed to represent putative keystone taxa in the community and have been identified in a variety of microbial systems (Berry and Widder 2014; Costello et al. 2012; Herren and McMahon 2018; Trosvik and de Muinck 2015; van der Heijden and Hartmann 2016). Unlike dominant taxa whose influence on community function is exclusively tied to their abundance, keystone taxa can be rare but are defined by their outsized influence on community function based on their unique roles (Banerjee et al. 2018; Power et al. 1996). In agreement with this concept, we found a negative relationship between node degree and an ASV's average relative abundance (Supplementary Fig. S11). Additionally, all bacterial taxa nodes identified as hubs had low relative abundances (mean = 0.34%; Supplementary Table S3). It was only when we considered within-module degree that we saw a weak positive correlation between abundance and connectivity, suggesting that high abundance taxa may have limited influence beyond their module. Overall, this provides further evidence suggesting that less abundant taxa can be as or more important for network structure as more abundant taxa (Herren and McMahon 2018; Lyons and Schwartz 2001; Shi et al. 2016; Wang et al. 2020).

Interestingly, the stressed and non-stressed networks for each experiment, built from the same core ASVs, exhibited no shared hubs (Fig. 3B to E; Supplementary Fig. S5). This finding agrees with our hypothesis that stress would lead to shifts in the taxonomic identity of network hubs. It suggests that putative keystone taxa in this system are context-dependent, playing a central role only under certain environmental conditions or locations (Lu et al. 2013; Lupatini et al. 2019; Shi et al. 2016). In fact, this shift in hub identity also influenced the composition of hub first-degree neighbors in the rhizosphere, with little shared membership between stress and non-stress first-degree neighbor networks (Fig. 4). These sub-networks represent the most central and interconnected parts of the rhizosphere core community. Keystone taxa and their

accompanying biotic interactions with other organisms have been identified as important predictors for community stability (Herren and McMahon 2018); thus, a change in keystone ASVs and their first-degree neighbors could indicate a shift in whole-community function between conditions even if changes in composition are subtle (Banerjee et al. 2016). It is worth mentioning that the only ASV shared across all our networks was identified as a *Massilia* sp., which dominated the rhizosphere during early plant development but was at much lower abundance at all other time points. Previous research in a variety of plant species suggests that this fast-growing, copiotrophic genus, as well as various other genera within the Oxalobacteraceae families, are strongly tied to early plant developmental stages, likely due to the exudates released during that time (Green et al. 2007; Li et al. 2014; Ofek et al. 2012; Xiong et al. 2021). Although the high connectivity of *Massilia* ASVs in the rhizosphere core does not appear to be directly influenced by environmental conditions, their role is still context-dependent with respect to time; they likely play a critical role in initial rhizosphere assembly.

None of the hubs identified in our soil, rhizosphere, or root networks was a kinless or network-wide hub (represented by $z \geq 2.5$, $P \leq 0.62$; Fig. 3B to E; Supplementary Fig. S5), defined by homogenous distribution of links among all modules (Guimerà and Nunes Amaral 2005). This suggests that hubs in the sorghum bacterial community influence narrow biological processes within their module (Banerjee et al. 2018). For example, both the full N and low N networks have many putatively nitrogen-fixing taxa (Supplementary Fig. S8) associated within module #6 with a hub identified as a Xanthobacteraceae under full N and a *Bacillus* sp. under low N. In this instance, differences in the identity of hubs between environmental conditions may simply result from functional redundancy in the community, as the two ASVs seemed to co-occur in a compositionally similar module regardless of growth condition. However, under low N, this module was much more connected to all other *Bacillus* ASVs (which were also more abundant under low N) and N-fixing taxa in other modules. *Bacillus* sp. have been identified to have many plant growth-promoting functions, of which at least some species are capable of enhancing plant nitrogen acquisition and growth under low N (Huang et al. 2015; Mania et al. 2016; J. Wang et al. 2021; Zhang et al. 2017), and their role as a hub/connector, increased abundance, and increased association under low N suggest that, as in other plant species, they play an important role in sorghum's ability to deal with abiotic stress.

Generalist-specialist shifts in the presence of a host and under stress. Previous studies suggest that connector nodes are more conserved than peripheral nodes given their role in inter-module communication and thus overall network stability (Guimerà and Nunes Amaral 2005). Additionally, connectors have been seen as analogous to generalists and peripherals as specialists (Montoya et al. 2006; Zhou et al. 2011). A greater proportion of rhizosphere and root ASVs were identified as peripheral nodes, whereas a greater proportion of soil ASVs were identified as connector nodes (Supplementary Table S2). Overall, the increase in modularity and peripheral nodes in the rhizosphere and root could signify that taxa are more functionally, spatially, or temporally compartmentalized, which could lead to fewer, but more specialized, associations between ASVs (Hernandez et al. 2021; Li et al. 2021; Zhang et al. 2018).

We also found that connector and peripheral roles were significantly reorganized under abiotic stress, with many ASVs switching from peripherals to connectors and vice versa (Supplementary Fig. S7). Generalist-specialist shifts have been rarely reported in microbial network-based studies (Lu et al. 2013), but their presence here under stress further suggests the context-dependent nature of network roles. These shifts may reflect the flexibility and diversity of metabolic functions that individual microbial taxa can exhibit to be more competitive for either environmental resources or host habitat under stressful conditions such as nutrient and water limitation (Chen et al. 2021; Grimbergen et al. 2015). Interestingly, we did not find a clear pattern in the percentage of nodes identified as connectors under stress (i.e., the percentage of nodes identified as connectors did not always shift in the same direction under stress; Supplementary Table S2). It seems that stress played a more important role in determining the identity of generalist (connectors) and specialist (peripheral) nodes rather than their overall percentages in the bacterial network.

The role of increased positive associations under stress. In addition to the reorganization of network roles under stress, we found differences in the type and number of associations between taxa in those networks. First, we found that networks under stress had more associations between taxa overall and between those taxa identified as putatively plant growth promoting (Fig. 4; Supplementary Fig. S8). However, contrary to what we hypothesized, network associations were more likely to be positive under stress than non-stress (Supplementary Table S2). Although previous research suggests that negative associations should dominate microbial networks, especially when species are more similar (Berry and Widder 2014; Nemergut et al. 2013; Verster and Borenstein 2018), we found many instances in which species from the same phylum were more likely to share positive associations with one another. These associations could be driven by environmental filtering, which is not mutually exclusive from their interactions with other microbes. Studies have shown that rhizosphere microbes often exhibit strong positive associations because they are ecologically or functionally similar and thus share niche space (Chaffron et al. 2010; Edwards et al. 2015; Mendes et al. 2014). As Loftus et al. (2021) pointed out, the increase in positive associations found in our and other recent studies (Kurtz et al. 2015; Shi et al. 2016) may be due to our use of network methods that are robust to the compositionality (i.e., many zeroes in the abundance matrix) inherent in microbiome data. Failing to account for compositionality (which naturally exhibits a negative correlation bias), may lead researchers to infer more negative associations than actually exist (Gloor et al. 2017).

Experimental studies have shown that like many macroorganisms, microbes may switch from competitive to facilitative interactions when exposed to stress (Michalet and Pugnaire 2016; Piccardi et al. 2019; Stachowicz 2001; Zhou et al. 2021). For example, some groups of microbes may engage in metabolic cross-feeding and/or biofilm formation to more efficiently use resources and energy under stress—a theory that has been substantially embraced within the last decade (Ebrahimi et al. 2019; Goldford et al. 2018; Hallam and McCutcheon 2015; Pacheco et al. 2019; Ren et al. 2014; Shank et al. 2011). In studies of plant-plant interactions and, more recently, microbe–microbe interactions, this phenomenon has been referred to as the Stress Gradient Hypothesis (Bertness and Callaway 1994; Hernandez et al. 2021). Still, it is important to remember that our networks are undirected, and their connections represent the net association between taxa; thus, positive associations may not always indicate cooperation. For instance, positive associations could indicate parasitism or commensalism between taxa or could simply reflect coexistence despite shared niche preferences by partitioning niche space or tolerating low resource levels (Kehe et al. 2021; Loftus et al. 2021).

Although increased positive interactions have been suggested to enhance diversity and productivity, they also may decrease stability (Allesina and Tang 2012; Hernandez et al. 2021; Kehe et al. 2021). This is because they can create positive feedback loops, or interdependence, between taxa—when one community member's

abundance is negatively impacted, so are the abundances of the taxa reliant on them (Coyte et al. 2015). Interestingly, although our stressed networks had more positive associations, they were not necessarily less stable than their respective non-stressed networks (Supplementary Fig. S9). This is likely to be due to the high modularity/compartmentalization inferred in the stressed networks, which, as mentioned earlier, can prevent the proliferation of positive feedback loops throughout the entire network (Guimerà et al. 2004; Loftus et al. 2021). Additionally, as seen in the soil networks, networks under stress were more connected, which could signal more redundancy in their network connections and act as "insurance" against network collapse. Finally, some research suggests that high levels of nitrogen fertilizers could negatively affect microbial community stability (Kavamura et al. 2018), but more research is needed to confirm if that is the case in our system.

CONCLUSION

Environmental stressors, such as nutrient and water limitation, do not seem to have pronounced effects on overall sorghum core soil, rhizosphere, or root community composition, but there were significant changes in select Gamma- and Alphaproteobacteria. Despite similar composition, communities associated with plants undergoing nitrogen or water stress did exhibit notable changes in network structure, particularly substantial changes in the identity of network hub (putative keystones), connector (generalists), and peripheral (specialists) taxa. Stressed networks also showed an increased proportion of positive associations, which could represent coexistence despite shared niche preferences or even cooperation (e.g., metabolic cross-feeding), but mechanistic studies are needed to parse these specific relationships. Although an increase in the proportion of positive associations has been shown to be destabilizing, we did not find that stressed networks were less stable than non-stressed networks, likely due to their increase in connectivity (i.e., redundancy) and compartmentalization. Together, these findings support the idea that both sorghum and its bacterial community can be resilient to environmental stressors.

Data availability. All sequence data used in this study are available at NCBI under BioProject PRJNA945936.

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