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Sorghum Root Flavonoid Chemistry, Cultivar, and Frost Stress Effects on Rhizosphere Bacteria and Fungi

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ABSTRACT

Biotic stresses, including fungal infections, result in increased production of flavonoid compounds, including 3-deoxyanthocyanidins (3-DAs), in the leaf tissues of *Sorghum bicolor*. Our objectives were to determine whether sorghum genotypic variation influenced root flavonoid and 3-DA concentrations and rhizosphere microbial communities and to identify how these relationships were affected by abiotic stress. We evaluated root chemicals and rhizosphere microbiomes of five near-isogenic lines of sorghum before and after a late-season frost. Roots were analyzed for total flavonoids, total phenolics, 3-DA concentrations, and antioxidant activity. Amplicon sequencing of 16S ribosomal RNA genes and internal transcribed spacer regions was performed on rhizosphere soils. Concentrations of luteolinidin (a 3-DA) and total flavonoids differed between several lines before frost; however, these relationships changed after frost. Luteolinidin increased in three lines after frost, whereas total flavonoids decreased in all the lines after frost. Lines that differed in luteolinidin

and total flavonoid concentrations before frost were different from those after frost. Rhizosphere community compositions also differed before and after frost but only fungal community compositions differed among sorghum lines. Bacterial community compositions were highly correlated with total flavonoid and luteolinidin concentrations. Furthermore, a greater number of bacterial taxa were correlated with total flavonoids and luteolinidin compared with fungal taxa. Collectively, this study provides evidence that plant genotypic variation influences root flavonoids and rhizosphere community composition and that these relationships are affected by frost. Plant–microbe interactions and secondary metabolite production may be important components to include for selective breeding of sorghum for frost stress tolerance.

Keywords: 3-deoxyanthocyanidins, 16S rRNA, agriculture, crop, flavonoids, frost, ITS, luteolinidin, microbiome, phyllosphere, rhizosphere, sorghum

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Sorghum bicolor (L.) Moench is the fifth most valuable cereal crop globally. Not only can *S. bicolor* be used for food and feed (Reddy et al. 2012), it can also be grown as a bioenergy crop and produces more ethanol than maize when grown on marginal lands (Maw et al. 2017). Sorghum is better adapted to stresses such as drought, salinity, and heat, which may be partially due to sorghum’s higher water and nitrogen use efficiency (Reddy and Reddy 2003; Rooney et al. 2007; Schittenhelm and Schroetter 2014). Considering the multifunctionality of sorghum and its resilience against environmental stresses, it is not surprising that production of sorghum has been on the rise for the last 50+ years (FAOSTAT, data from 1960 to 2018). Increased sorghum production in the face of climate change calls for improving its tolerance to abiotic and biotic stresses that can limit productivity (Hagan et al. 2014; Hoffmann and Rooney 2014; Tetreault et al. 2019).

Although sorghum is relatively tolerant to drought and high temperatures, it is very sensitive to chilling, frost, and freezing stresses. Cold-temperature stresses on sorghum result in reduced grain size and seed germination (Roozeboom and McKinney 2009;

Staggenborg and Vanderlip 1996; Staggenborg et al. 1999). Sorghum production in southern Canada and the northern United States is particularly affected by cold-related stress. Sorghum flowering peaks in fall and early winter, when days are cooler (Singh 1985), which is a problem because cold stresses can lead to male sterility (Wood et al. 2006). Identifying genotypes of sorghum or specific genes that confer resistance to cold stresses is important for increased production at higher latitudes, and these genotypes are actively being explored (Moghimi et al. 2019; Parra-Londono et al. 2017).

Apart from apparent alterations to seed germination and grain size, low temperatures affect metabolite production in sorghum, including increased production of unsaturated fatty acids and reactive oxygen species (ROS) (Guo et al. 2016). Antioxidant synthesis is activated by ROS and includes compounds such as flavonoids and phenolics. Plant production of flavonoids and phenolics increases under stress (Guidi et al. 2016; Kawahigashi et al. 2016; Tattini et al. 2005). Flavonoid metabolites have positive correlations with protective traits in diverse crops under frost stress (Jensen et al. 2012; Schulz et al. 2016; Treutter 2006). A variety of flavonoid and phenolic metabolites are produced by sorghum through the phenylpropanoid pathway and include flavan-4-ols (Winkel-Shirley 2001). Flavan-4-ols are precursor molecules for 3-deoxyanthocyanidins (3-DAs) and include specific metabolites such as luteolinidin and apigeninidin (Boddu et al. 2005; Grotewold et al. 1998; Ibraheem et al. 2010; Lo and Nicholson 1998; Rhodes et al. 2017). In sorghum, 3-DAs are synthesized de novo in response to both pathogenic (*Colletotrichum sublineola*, *Ascochyta sorghi*, and *Bipolaris sorghicola*) and nonpathogenic (*Cochliobolus heterostrophus*) fungal incursions, thereby conferring resistance to these fungi (Ibraheem et al. 2010, 2015; Mizuno et al. 2012; Nicholson et al. 1987).

Sorghum is recognized for its ability to produce flavonoids (Nielsen et al. 2004) as well as large amounts of exudates (Baerson et al. 2007). The composition of sorghum rhizosphere exudates, including type and amount, are genotype dependent (Miller et al. 2019). Flavonoids have antibacterial and antifungal properties (Górniak et al. 2019) but are also reported to serve as chemical signals to support rhizobium-legume and arbuscular mycorrhizal symbioses (Abdel-Lateif et al. 2012). Several studies have reported the effects of root exudates in shaping the composition of rhizosphere microbiomes (Broeckling et al. 2008; Cotton et al. 2019; Hu et al. 2018; White et al. 2017), and the roles of root microbiomes in mitigating abiotic stresses is increasingly being highlighted (de Vries et al. 2020). More information is needed on how flavonoids produced in sorghum roots might influence rhizosphere microbial assemblages and whether frost stress affects root flavonoid production.

In this study, we aimed to determine whether late-season frost affected the concentrations of key root chemicals and antioxidant activity of field-grown sorghum. Considering the potential for flavonoids to act as signaling compounds for rhizosphere microorganisms, we set out to determine whether rhizosphere bacterial and fungal communities differed among near-isogenic lines (NILs) of sorghum varying in 3-DA production in leaves and to assess correlations between concentrations of flavonoid compounds and abundances of microbial taxa. These NILs, which share approximately 97% of their nuclear genes, with the exception of those controlling seed or pericarp color and necrotic plant color (Pedersen and Toy 2001a, b), were also shown previously to vary in above-ground 3-DA concentrations. Given the difficulties in analyzing root exudates in field soils, we analyzed root tissue samples before and after frost for total flavonoids, 3-DAs (apigeninidin, and luteolinidin), total phenolics, and antioxidant activity. We also analyzed rhizosphere soil microbial communities using sequencing of the 16S

ribosomal RNA (rRNA) genes and internal transcribed spacer (ITS) regions. Overall this study demonstrates that near-isogenic lines can be used to exploit the power of plant genetics to better understand variability and associations of rhizosphere microbiomes.

MATERIALS AND METHODS

Root chemical analyses, rhizosphere community sample processing and analysis, and statistical analyses are provided in Supplementary File S1. Brief materials and methods are described below.

Sorghum NILs. Complete documentation of the provenance of sorghum lines has been described previously (Pedersen and Toy 2001a,b). The five NILs of *S. bicolor* (L.) Moench used in this study differ in their pericarp and plant pigmentation and also in their 3-DA content in leaf and grain. Lines N321 and N326 are classified as 3-DA nonproducers, N334 and N336 are classified as 3-DA producers, and N337 is classified as an induced producer that synthesizes 3-DA compounds after biotic stresses (I. Gaffoor and S. Chopra, unpublished). These lines are expected to share approximately 97% of their nuclear genes, except for those controlling seed or pericarp color and plant color (Pedersen and Toy 2001b), including luteolinidin and apigeninidin compounds (I. Gaffoor and S. Chopra, unpublished).

Site description, experimental design, and rhizosphere sampling. A field experiment was conducted during the 2018 growing season at the Russell Larson Research Farm of the Pennsylvania State University (40°43'N, 77°56'W, 372 m in elevation). Four-row plots per replicate of each of the five sorghum NILs were planted using a randomized complete block design with four replications. Three plants per line/replicate were excavated for root and soil sample collections. Plant rhizospheres were sampled before (52 days after planting) and 10 days (111 days after planting) after frost by digging up each plant to a depth of 15 to 20 cm and diameter of 10 to 12 cm around the plant base. In the field, roots were vigorously shaken to remove excess bulk soil. Samples were placed in a Ziploc bag, stored in a cooler with ice, and transported to the laboratory. After removing roots from the Ziploc bags, the remaining excess bulk soil was removed. We classified rhizosphere soil as the soil tightly adhering to the roots (approximately 2-mm distance from root). Sterile water (50 ml) was added to each bag and roots were manually massaged to separate the soil associated with the rhizosphere. Samples were pooled, transferred to falcon tubes, and centrifuged at 10,000 rpm for 20 min to pellet the soil. Washed root samples were frozen in liquid nitrogen and immediately ground. All samples were stored at -80°C until further analysis.

Root chemical quantification. Subsamples of sorghum roots were ground in liquid nitrogen. Chemical extraction was performed with methanol acidified with 0.1% (vol/vol) hydrochloric acid using 50 mg of ground root mL^{-1} of extractant. These extracts were then used to quantify different root chemical fractions. Total phenolic content was assessed by mixing a subsample of the extract with the Folin-Ciocalteu reagent and sodium carbonate. Absorbance values (765 nm) were compared against a gallic acid standard. Total flavonoids were quantified by adding a subsample of the extract to aluminum chloride, potassium acetate, and deionized water. Absorbance values (510 nm) were compared against a catechin standard. The two compounds measured as 3-DAs were luteolinidin and apigeninidin using absorbances of 480 and 500 nm, respectively, in original acidified methanol extracts. Finally, 2,20-diphenyl-1-picrylhydrazyl (DPPH) assays were performed by adding a subsample of the extracts to a DPPH solution diluted in methanol. Absorbance values (515 nm) were compared against a Trolox equivalent (TE) standard. All values are expressed as milligrams per gram of root.

ITS and 16S rRNA sequencing and processing. Frozen rhizosphere soil samples were thawed prior to DNA extraction by Wright Labs, LLC for Illumina amplicon sequencing of ITS regions and 16S rRNA genes. Reads were processed following the DADA2 pipeline in R to assign amplicon sequence variants (ASVs) (Callahan et al. 2016). Taxonomy was assigned with UNITE for ITS sequences (accessed on 12 December 2018) (UNITE Community 2019) and Silva for 16S rRNA sequences (accessed on 3 December 2018) (Quast et al. 2013). General statistics regarding how many sequences were processed and passed certain steps in DADA2 can be found in Supplementary Table S1.

Statistical analyses. *Root chemicals.* Linear mixed-effect modeling was performed to determine whether root chemical concentrations were different between lines, time, or the combination of lines–time, with block as the random variable. Models were built using lme4 in R and, if a factor was significant ($P < 0.05$), then pairwise comparisons using emmeans were performed with Tukey P value adjustments (Lenth 2020). Because apigeninidin and luteolinidin were found to be highly correlated (Pearson correlation > 0.99), only luteolinidin concentrations were used for the analyses.

Diversity metrics. α Diversity was calculated for both ITS and 16S rRNA sequences from rarefied sequence counts. Richness (observed ASVs) and Shannon Diversity were calculated, and linear mixed-effect models were created as described above. β -Diversity metrics used to assess bacterial community composition included Bray-Curtis and weighted UniFrac, while Bray-Curtis alone was used for fungal ITS compositional analyses. Global permutational multivariate analyses of variance (PERMANOVAs) were performed with Adonis and pairwise PERMANOVAs were conducted with the RVAideMemoire package (Hervé 2019).

Differential abundance and correlational analyses. Analyses to identify taxa that were differentially abundant across time and lines were performed using DESeq2 after converting the phyloseq object into a DESeq object (Love et al. 2014). Correlations between ITS or 16S β diversity and concentrations of root chemicals were assessed using Mantel tests with vegan (Oksanen et al. 2019). The abundances of taxa were correlated with chemical variables using Kendall correlations and correlations of ≥ 0.50 or ≤ -0.5 were considered as positively or negatively correlated, respectively, with the chemical variable. In several cases, producer lines (N334 and N336) and nonproducer lines (N321 and N326) were analyzed separately.

Raw sequencing data were submitted to NCBI under the Bio-project PRJNA601153. Code for analyses and ASV tables with taxonomy, sequence count, and raw metadata used for this project are available at <https://github.com/maracashay/Sorghum-Rhizosphere-ChopraBruns>. Full descriptions of the methods and analyses can be found in Supplementary File S1.

RESULTS

Root chemical concentrations change after frost and between lines. Results from the linear mixed-effects models indicated that all variables were affected by time (frost), while DPPH was affected by line, and luteolinidin and total flavonoid concentrations were influenced by the interaction between time and line (Supplementary Table S2). Mean concentrations for all lines before and after frost can be found in Supplementary Table S3). Mean activity of DPPH before frost were 0.256 (standard error [SE] 0.02) and 0.300 (SE 0.02) TE mg g⁻¹ after frost, while total phenolic concentrations before frost were 4.62 (SE 0.37) and 2.77 (SE 0.26) mg g⁻¹ after frost. Assessment of DPPH activity by line indicated higher activity

in line N326 compared with N334, with mean activities of 0.35 (SE 0.01) and 0.20 (SE 0.01) mg g⁻¹, respectively.

Analysis of luteolinidin (log₁₀ mg g⁻¹ root) by line–time indicated that the lines N321 (mean of -0.94 SE 0.02) and N326 (mean of -0.940 SE 0.16) had significantly lower concentrations than the producer lines and induced line (means between -0.14 and 0.40 mg g⁻¹) (Fig. 1A). After frost, N326 (mean of -0.22 SE 0.07) had lower concentrations of luteolinidin compared with N336 and N337 (means of 0.43 SE 0.12 and 0.29 SE 0.06, respectively) (Fig. 1A). Lines N321, N326, and N336 had increased concentrations of luteolinidin after frost compared with before frost (Fig. 1A).

Concentrations of total flavonoids were also different between lines and time. Line N321 had lower total flavonoids (mean of -0.29 SE 0.12) compared with the producer lines and induced line before frost (means between 0.07 and 0.30) (Fig. 1B). Line N326 (mean of -0.12 SE 0.01) was also lower than N334 (mean of 0.30 SE 0.10) before frost (Fig. 1B). After frost, concentrations of total flavonoids were lower in the nonproducer lines N321 and N326 (means of -0.56 SE 0.03 and -0.68 SE 0.09, respectively) and one producer line N336 (mean of -0.55 SE 0.07) compared with the producer line N334 (mean of -0.17 SE 0.01) and the induced line N337 (mean of -0.21 SE 0.01) (Fig. 1B). All five lines had lower total flavonoid concentrations after frost compared with before frost (Fig. 1B).

Sequencing overview. In total, 6,088 ASVs were identified from 16S rRNA sequencing; of these, 17 were not assigned to the kingdom's Bacteria or Archaea and were subsequently removed. After quality filtering and processing of ITS sequences, approximately 913 ASVs were identified and all were assigned to the kingdom Fungi. Bacterial (16S rRNA gene) reads ranged from 20,244 to 124,359 per sample, with a mean sequence count of 58,008 reads. Two fungal samples had to be removed due to low sequence count (count of 0). Fungal ITS reads ranged from 38,744 to 334,410 per sample, with a mean sample count of 144,707 reads. Each sample was normalized via rarefaction for α -diversity estimates. Bacterial samples were rarefied to a depth of 20,000 and fungal samples were rarefied to a depth of 38,000 sequences per sample.

Communities change with time and between the lines. *α and β Diversity.* Both richness and Shannon diversity differed between time points for bacterial rRNA datasets but not across line or line–time. No differences in richness or Shannon diversity were observed for fungal ITS datasets (Supplementary Table S4; $P < 0.05$). Before frost, bacterial communities had a mean richness of 879 (SE 47) and a mean Shannon Diversity of 5.25 (SE 0.10), which were higher than richness and Shannon Diversity after frost, with means of 587 (SE 32) and 4.60 (SE 0.18), respectively. Frost also had a significant impact on the β diversity of both the bacterial and fungal communities (Table 1; $P < 0.05$). Only fungal diversity was different across sorghum lines (Table 1; $P < 0.05$), and results from the pairwise PERMANOVAs indicated differences in fungal ITS sequence composition between N326 and all other lines. The two lines classified as non-3-DA-producers (N321 and N326) had a different ITS composition compared with N334, a line classified as a 3-DA-producer (adjusted $P < 0.05$).

Differential abundance. Comparisons of rhizosphere samples before and after frost revealed a large shift in community composition for both bacteria and fungi (Table 1; Supplementary Fig. S1). Over half of the bacterial phyla showed significant changes in abundance based on differential abundance analyses (Supplementary Table S5). At the class level, 27.2 and 45.4% of bacterial and fungal taxa, respectively, were differentially abundant (Supplementary Table S5). These changes decreased at lower taxonomic levels but were still quite high, with 26 and 21.1% of bacterial and

fungal genera, respectively, being differentially abundant before and after frost.

Few bacterial taxa were differentially abundant across lines (Supplementary Table S6). Of 6,061 total bacterial and archaeal ASVs, only 7 to 13 ASVs differed across lines. Of 488 prokaryotic genera detected, no more than 4 differed across lines. No bacterial

or archaeal taxa above genus level differed in abundances across lines. In contrast, many fungal taxa were differentially abundant across lines, ranging from 30 to 90 ASVs and accounting for 3.3 to 9.8% of all ASVs (Supplementary Table S6). Fungal taxa at higher taxonomic classifications were also found to be differentially abundant across lines, except at the phylum level.

Fig. 1. Root chemical concentrations across line–time for **A**, luteolinidin and **B**, total flavonoids. Values are expressed in milligrams per gram of root and transformed using log10 to meet linear mixed-effects modeling assumptions. Letters and asterisks denote differences between lines and time identified through emmeans with Tukey *P* value adjustments. Lowercase letters indicate significant differences between lines for Before and After sampling times (adjusted *P* < 0.05). Asterisks next to lines indicate significant differences between luteolinidin or total flavonoid root concentrations between the Before and After samples (adjusted *P* < 0.05).

TABLE 1 Community composition for the internal transcribed spacer (ITS) region and 16S ribosomal RNA gene sequences using Bray-Curtis or weighted UniFrac ^a						
wUniFrac	Sum of squares	Mean squares	DF	F value	R ²	Pr (>F)
16S						
Line	0.046	0.011	4	1.451	0.101	0.134
Time	0.138	0.138	1	17.527	0.305	<0.001*
Line–time	0.033	0.008	4	1.032	0.072	0.414
Residuals	0.236	0.008	30	...	...	...
Bray-Curtis						
Line	0.713	0.178	4	1.093	0.900	0.301
Time	1.704	1.704	1	10.473	0.215	<0.001*
Line–time	0.646	0.162	4	0.993	0.081	0.481
Residuals	7.944	0.163	30	...	...	...
ITS						
Bray-Curtis						
Line	1.201	0.300	4	2.494	0.205	<0.001*
Time	0.807	0.807	1	6.700	0.138	<0.001*
Line–time	0.484	0.121	4	1.005	0.083	0.457
Residuals	3.371	0.120	28	...	...	...

^a Significant factors are denoted with an asterisk (*) (*P* < 0.05). Analyses were performed with permutational multivariate analyses of variance.

In several cases, patterns of differential abundance across sorghum lines did emerge for some bacterial and fungal genus-level assignments (Table 2). For example, the bacterial genera *Delftia*, *Rhodoferrax*, and *Methylosorus* had different abundances in the nonproducer lines (N321 and N326) compared with the 3-DA producer line, N336. Genus *Comamonas* was higher in N321 and N326 compared with producer lines N334 and N336, as well as the induced line N337 (Table 2). Abundance of the genus *Fusarium* was higher in the nonproducer line N326 compared with the producer lines and the induced line. The 3-DA-producing line N334 supported lower numbers of both the fungal genus *Corynespora* and the bacterial genus *Rosenbergiella* in the rhizosphere compared with all other lines (Table 2). Genera *Sporobolomyces* and *Acephala* had exactly opposite abundance patterns, where *Sporobolomyces* was lower in the nonproducer N321 and in the producer line N336 compared with the three other lines. The genus *Acephala* was higher in N321 and N336 compared with the other three lines (Table 2).

Differential abundances of ASVs from bacterial and fungal communities were observed when comparing producer lines (N334 and N336) with nonproducer lines (N321 and N326) and are shown in Figures 2 and 3. Of the four bacterial ASVs that were differentially abundant between the producer and nonproducer lines, three were higher in the nonproducers and belonged to the family *Pseudomonadaceae*. Producer rhizospheres had a higher abundance of one ASV that was assigned to the genus *Chujaibacter* (Fig. 2).

In contrast to the four bacterial ASVs that were differentially abundant between producer and nonproducer lines, 27 fungal ASVs

were differentially abundant (Fig. 3). Rhizosphere communities of 3-DA producers had 18 ASVs that were more abundant and 9 ASVs that were less abundant compared with nonproducer communities. Approximately 18 of the 27 fungal ASVs belonged to genera *Mortierella*, *Septophoma*, or *Fusarium* (Fig. 3). Interestingly, some *Mortierella* and *Fusarium* ASVs were higher in producers, whereas other ASVs from these genera were higher in the nonproducers. In contrast, all ASVs assigned to *Setophoma* were higher in the producer lines (Fig. 3).

Communities and root chemistry relationships. *Community diversity.* Mantel tests were performed to assess correlations between dissimilarities in rhizosphere community compositions and root contents of total flavonoids, luteolinidin, and total phenolics (Table 3; $P < 0.05$). Correlations between bacterial composition and root chemicals were highest when Bray-Curtis dissimilarity was assessed by total flavonoid with an Mr value of 28.2 and a $P = 0.001$ (Table 3). When all variables were included (total flavonoid + total phenolics + DPPH + luteolinidin), the Mr value was slightly lower at 26.5 but still significant for Bray-Curtis. Correlations between phylogenetic distances (weighted Uni-Frac) and root chemical variables were slightly lower compared with Bray-Curtis correlations for 16S rRNA gene communities (Table 3). Fungal community dissimilarity based on Bray-Curtis was only correlated to total flavonoids (Mr of 16.9 and $P = 0.013$) and all variables (Mr of 12.1 and $P = 0.049$) (Table 3). Fungal community dissimilarities were not correlated to luteolinidin or total phenolics because the bacterial dissimilarities were correlated.

TABLE 2
Genera identified from the 16S and internal transcribed spacer (ITS) datasets that are differentially abundant among sorghum lines (DESeq analyses; adjusted $P < 0.05$)^a

Row A	N321				N326			N334		N336
Row B	N326	N334	N336	N337	N334	N336	N337	N336	N337	N337
Genera										
16S										
<i>Delftia</i>	...	...	H	H	...	H	...	H	H	...
<i>Rosenbergiella</i>	...	H	...	...	H	...	H	L	L	...
<i>Rhodoferrax</i>	...	...	H	...	...	H	...	...	...	...
<i>Methylosorus</i>	...	...	H	...	...	H	...	...	...	...
<i>Candidatus Nitrocosmicus</i>	...	...	...	...	...	...	...	...	L	...
<i>Duganella</i>	...	...	...	...	...	...	...	...	H	...
<i>Ramlibacter</i>	...	...	...	...	...	...	...	H	...	...
<i>Comamonas</i>	...	H	H	H	H	H	H	...	...	...
ITS										
<i>Sporobolomyces</i>	L	L	...	L	...	H	...	H	...	L
<i>Corynespora</i>	...	H	...	...	H	...	...	L	L	...
<i>Cystofilobasidium</i>	L	L	L	L	...	...	...	...	...	...
<i>Fusidium</i>	H	...	...	...	...	...	...	...	...	...
<i>Fusarium</i>	...	...	...	...	H	H	H	...	...	...
<i>Setophoma</i>	...	...	...	...	L	...	...	...	...	...
<i>Acephala</i>	H	H	...	H	...	L	...	L	...	H
<i>Ramichloridium</i>	...	...	...	H	...	...	H	...	H	H

^a Symbols: H in columns indicates that the genus is higher in the line shown in row A compared with the line shown in row B, and L in columns indicates that the genus is lower in the row A line than in the row B line. Nonproducer lines are designated in roman, producer lines in italics, and the induced line in bold.

Individual taxon correlations. Abundances of very few fungal taxa were correlated with root chemical variables, whereas abundances of many bacterial taxa were correlated (Table 4). Only three and two fungal ASVs were correlated with total flavonoids in the producer and luteolinidin and total phenolics in the nonproducer lines (Table 4). When assessing correlations between bacterial taxa associated with the producer and nonproducer lines separately, 18 ASVs were found to be correlated with total flavonoids in the 3-DA producers and 52 ASVs were correlated with luteolinidin in the nonproducers. Associations between luteolinidin, total flavonoids, total phenolics, and bacterial taxon abundances were observed from ASV to phylum level (Table 4).

Several bacterial taxa were associated with both total flavonoids from producer lines and luteolinidin from nonproducer lines, and these overlaps were identified from ASVs through phyla (Fig. 4; Supplementary Fig. S2). At the genus level, 15 genera were shared between 3-DA-producer and nonproducer lines and included *Asticcaacaulis*, ‘*Candidatus* Ovatusbacter’, *Cytophaga*, *Dyadobacter*, *Ellin6067*, *Luteimonas*, *Luteolibacter*, *Methylotenera*, *Niastella*, *Porphyrobacter*, *Ramlibacter*, *Reyranelia*, *Sphingomonas*, *Thermomonas*, and the *Burkholderia/Caballeronia/Paraburkholderia* group. Interestingly, the type of correlation was the opposite between luteolinidin and total flavonoids; all correlations with luteolinidin were negative and nearly all of the correlations with total flavonoids were positive. At the genus level, all 33 genera were negatively correlated with luteolinidin in the nonproducers, whereas 19 of the 20 genera correlated with total flavonoids were positively correlated in the 3-DA-producer lines. Phyla that were positively correlated with total flavonoids in the producer lines and negatively correlated with luteolinidin from the nonproducer lines included *Acidobacteria*, *Armatimonadetes*, and *Bacteroides* (Fig. 4).

DISCUSSION

Frost stress affects sorghum cultivar root chemistry. This study was designed to determine whether frost stress affected sorghum cultivar root chemistry and whether changes to root chemical dynamics had an impact on rhizosphere microbial

communities. In earlier studies, 3-DA production by sorghum was assessed for phytoalexin activity, because 3-DAs are produced and translocated at the sites of fungal infection in sorghum foliar tissues (Nielsen et al. 2004; Snyder et al. 1991). Biosynthesis of 3-DAs has also been hypothesized to be due to abiotic stresses and root exudation patterns can change in response to stress, including both abiotic and biotic stresses (Canarini et al. 2019; Edayilam et al. 2018; Gargallo-Garriga et al. 2018). Herein, we did find increased concentrations of luteolinidin, a compound within the 3-DA class of flavonoids, after frost but this change was dependent on the specific *S. bicolor* line analyzed (Fig. 1). In a study comparing irrigation treatments, 3-DA production was found to be highest in sorghum grain grown under drought stress (Wu et al. 2017). However, we also found decreases in total flavonoids and total phenolics after frost, which supports the findings of Shefflin et al. (2019). In their study, concentrations of phenylalanine, a precursor molecule of flavonoids, 3-DAs, and other defense compounds such as salicylic acid, were lower in sorghum roots grown under nitrogen stress. Lower concentrations of 3-DA precursor molecules after a stress event may indicate that these molecules have, in fact, been synthesized to 3-DA compounds. Changes in flavonoid exudation patterns can affect a plant’s ability to attract rhizobia or fungi, and these can shape rhizosphere communities by acting as either a carbon source or a toxic compound for certain groups of microorganisms (Broeckling et al. 2008; Shaw et al. 2006). Further understanding of how root exudation patterns change in response to stress and how those patterns relate to plant health is important for determining how climate change will affect sorghum crop yield and grain quality.

Rhizosphere microbial communities affected by frost. Plant stress in the form of frost not only affects root exudation patterns of plants, as previously mentioned, but can also directly affect soil microorganisms’ physiology by slowing metabolisms and disrupting cell membranes (Schimel et al. 2007). Microbial biomass and respiration decrease during repeated freeze-thaw cycles by 33.3 and 30%, respectively (Larsen et al. 2002; Pesaro et al. 2003; Schimel and Clein 1996). Studies have proposed a communitywide shift toward bacterial dominance following freeze-thaw (Nieminen and Setälä 2001; Larsen et al. 2002). Although we did not determine the ratios of bacteria/fungi in these rhizosphere communities, we did determine that bacterial but not fungal diversity decreased following frost. Frost affected bacterial composition more strongly ($R^2 = 21.5$ for Bray-Curtis and $R^2 = 30.5$ for wUniFrac) than fungal composition ($R^2 = 13.8$) (Table 1). Considering the broad-level changes to both bacterial and fungal taxa (27%+ at class level) before and after frost, it is clear that frost has a significant impact on rhizosphere microbial communities (Supplementary Table S5; Supplementary Fig. S1). However, because frost also affected root exudation patterns, we cannot rule out the possibility that frost had an indirect effect on rhizosphere communities or some combination of direct and indirect effects.

Differences in microbiome diversity between sorghum lines. In a study by Schlemper et al. (2018), a significant interaction was observed between sorghum line and soil type that drove differences in bacterial but not fungal communities. Other studies have also observed effects of soil type on sorghum bacterial rhizosphere community compositions (Marschner et al. 2004; Oberholster et al. 2018), which may be exacerbated in the earlier stages of sorghum growth (Schlemper et al. 2017). We had originally hypothesized that the rhizosphere communities for bacterial and fungal communities would be distinct between the nonproducing, producing, and induced lines. However, no differences were observed for α -diversity metrics across lines and there were no interactive effects of line–time (Supplementary Table S4). We also found no

Fig. 2. Differentially abundant 16S ribosomal RNA gene amplicon sequence variants (ASVs) between producer and nonproducer lines (DESeq analyses; adjusted $P < 0.05$). Taxonomic classifications of ASVs are provided.

differences in bacterial community composition across lines but there was a significant difference in fungal composition (Table 1). Bacterial community composition has been suggested to be less sensitive to specific cultivars and more sensitive to environmental variables such as the amount of NH_4^+ , NO_3^- , and carbon, and soil texture (Ramond et al. 2013). In contrast to our experiment and the experiment by Ramond et al. (2013), Schlemper et al. (2017) did find cultivar-driven bacterial rhizosphere community composition when studying *Striga*-resistant sorghum cultivars. Because the sorghum cultivars used in this study were NILs, it may be that these plant genotypes are not different enough to elicit large-enough changes in bacterial communities; however, fungal communities may be more sensitive to small changes in plant genotypes.

More important than overall differences in community structure across cultivars may be changes in individual taxa or lineages of taxa between the cultivars. Several taxa that may be important in promoting plant growth, suppressing pathogenic microbes, or nutrient cycling in these sorghum rhizospheres were identified as being differentially abundant across lines or between the producer and nonproducer lines (Table 2; Figs. 2 and 3). These include ‘*Candidatus Nitrocosmicus*’, *Chujaibacter*, *Comamonas*, *Delftia*,

Duganella, *Pseudomonas*, *Ramlibacter*, *Rhodoferax*, *Corynespora*, *Fusarium*, *Fusidium*, *Hymenoscyphus*, *Mortierella*, and *Setophoma terrestris*.

Bacteria belonging to the genus *Pseudomonas* were found to be higher in the nonproducer lines compared with the producer lines (Fig. 2). In other studies, some members of the genus *Pseudomonas* can be pathogenic to sorghum, while others can directly increase sorghum yield or produce compounds that inhibit the growth of soilborne plant pathogens (Capper and Higgins 1993; Sajeli Begym et al. 2014; Vidhyasekaran and Muthamilan 1995; Weller 2007). Genus *Ramlibacter* has also been implicated as suppressing soil-borne diseases for sweet pepper (L.-N. Zhang et al. 2019) and was found to be significantly lower in the induced line compared with the producer line, N334. Although not specific to sorghum, other genera, including *Comamonas*, *Delftia*, and *Duganella*, have all either been identified as potentially plant-growth promoting through the production of compounds like indole-acetic acid (Tsolakidou et al. 2019; Zhang et al. 2016) or correlated with the availability of nutrients like N, P, and S (Schmalenberger et al. 2008; Tang et al. 2019; Tayyaba and Muhammad 2015; L.-N. Zhang et al. 2019). Both *Comamonas* and *Delftia* were higher in the nonproducer lines compared with the producer line, N336, while *Duganella* was

Fig. 3. Differentially abundant internal transcribed spacer amplicon sequence variants (ASVs) between producer and nonproducer lines (DESeq analyses; adjusted $P < 0.05$). Taxonomic classifications of ASVs are provided.

significantly lower in the induced line, N337, compared with the producer line, N334 (Table 2). Two other genera that might also be important for controlling the availability of nitrogen that have differential abundances between the lines include ‘*Ca. Nitrocosmicus*’ and *Chujaibacter*, which have been associated with nitrogen cycling reactions, including ammonia oxidation and nitrification, respectively (Alves et al. 2019; Kong et al. 2019; Lehtovirta-Morley et al. 2016; Rodriguez-Sanchez et al. 2018; Sauder et al. 2017).

Of the fungal taxa that were identified as being differentially abundant across the lines or between producer and nonproducer lines, several have plant-growth-promoting properties or could be used as biocontrol inoculants. *Mortierella* spp. can solubilize phosphorous and are often found associated with rhizospheres of plants (Gkarmiri et al. 2017; Qiao et al. 2019; Zhang et al. 2011; T. Zhang et al. 2019). Several ASVs assigned to *Mortierella* were differentially abundant in the producer lines, while other *Mortierella* ASVs were higher in the nonproducer lines (Fig. 3). Species within the fungal genus *Fusidium* produce an antibiotic (fusidic acid) and an antifungal compound (fusidilactone) and are known to

be endophytes of plants (Godtfredsen et al. 1962; Gunatilaka 2006). Herein, the genus *Fusidium* was more abundant in line N321 compared with line N326, both of which are nonproducers (Table 2).

There were four fungi identified as being differentially abundant that were assigned to taxa known to be pathogenic to plants: *Botrytis cinerea*, *Corynespora* sp., *Fusarium* sp., and *Setophoma terrestris*. Of these fungi, only *Fusarium* has been identified as a potential pathogen of sorghum, causing stalk rot, grain mold, and seedling blight of sorghum (Castor and Frederiksen 1980; Frederiksen 1986; Jardine and Leslie 1992; Petrovic et al. 2009; Reed et al. 1983). Although we did not see any evidence of disease on the sorghum, it is interesting to note the differences in abundance across the sorghum lines. Sorghum line N326 had a higher abundance of *Fusarium* compared with N334, N336, and N337 (Table 2); six *Fusarium* ASVs were more abundant in the nonproducers and one was more abundant in the producer lines (Fig. 3). Genera *Botrytis* and *Corynespora* and *Setophoma terrestris* cause diseases in other crops such as canola, cucumber, cotton, onion, soybean, squash, and tomato (Hansen 1929; Ikeda et al. 2012;

TABLE 3
EcoDist (Mantel test) results correlating dissimilarity of environmental variables (Euclidean) to dissimilarity in community composition for 16S and internal transcribed spacer (ITS) sequences (Bray-Curtis or weighted UniFrac)^a

Sequence	All variables		Total flav ^b		Luteolinidin		Total phenolics		DPPH ^c	
	Mr	P	Mr	P	Mr	P	Mr	P	Mr	P
Bray-16S	26.5	0.001*	28.2	0.001*	20.9	0.001*	14.0	0.004*	0.9	0.417
UniFrac-16S	22.1	0.003*	16.4	0.009*	20.1	0.003*	10.6	0.032*	8.3	0.089
Bray-ITS	12.1	0.049*	16.9	0.013*	7.4	0.120	10.8	0.350	1.8	0.59

^a Significant correlations are denoted with an asterisk (*) ($P < 0.05$).

^b Total flavonoids.

^c DPPH = 2,20-diphenyl-1-picrylhydrazyl.

TABLE 4
Number of Kendall correlations between 16S or internal transcribed spacer (ITS) taxa abundances and root chemical concentrations and 2,20-diphenyl-1-picrylhydrazyl (DPPH) activity that were ≥ 0.50 or ≤ -0.5 ^a

Group	All five lines				Producers				Nonproducers			
	Flav	Lute	Phen	DPPH	Flav	Lute	Phen	DPPH	Flav	Lute	Phen	DPPH
16S												
ASVs	1	0	2	0	18	0	11	1	10	52	22	0
Genera	1	0	1	0	20	0	9	0	9	33	17	2
Families	0	0	0	0	17	0	7	0	10	24	14	1
Orders	0	0	0	0	12	0	3	0	6	21	13	0
Classes	0	0	0	0	4	1	3	0	2	6	4	0
Phyla	0	0	0	0	4	0	3	0	2	4	3	0
ITS												
ASVs	0	0	0	0	3	0	0	0	0	2	2	0
Genera	0	0	0	0	2	0	0	0	0	2	1	0
Families	0	0	0	0	1	0	0	0	0	1	1	0
Orders	0	0	0	0	2	0	0	0	0	1	2	0
Classes	0	0	0	0	1	0	0	0	0	0	0	0
Phyla	0	0	0	0	0	0	0	0	0	0	0	0

^a Flav = total flavonoids, Lute = luteolinidin, Phen = total phenolics, and ASVs = amplicon sequence variants.

MacKenzie et al. 2018; Prins et al. 2000; Yang et al. 2017; F. B. Zhang et al. 2019). Nonproducer lines had higher abundances of six ASVs assigned to *Fusarium* spp. and one ASV assigned to *Corynespora cassicola* (Fig. 3). In contrast, producer lines had higher abundances of five ASVs assigned to *S. terrestris*, one ASV assigned to *B. cinera*, and one ASV assigned to *Fusarium culmorum* (Fig. 3). It is unclear why the abundances of these pathogenic fungi (not to sorghum) are different between producer and nonproducer lines. Further exploration of activity and function of these plant–fungal rhizosphere interactions should be performed to determine how these lines of sorghum are influencing fungal communities in soils.

Root chemistry and sorghum rhizosphere microbial communities. Cultivar-dependent exudation patterns from sorghum roots have been suggested to influence rhizosphere microbial community composition (Funnell-Harris et al. 2008). Studies have observed differences in exudation patterns of certain compounds, including flavonoids, important for attracting symbiotic fungi and altering plant defense systems (Steinkellner et al. 2007). In this study, we identified correlations between total flavonoids and luteolinidin in sorghum roots and bacterial rhizosphere abundances (Fig. 4). More specifically, bacteria in the rhizospheres of non-producers were negatively affected by luteolinidin, which indicates that luteolinidin may have an antibacterial effect. Correlations between bacteria and total flavonoids of producers were almost entirely positive. Because luteolinidin is a flavonoid compound, and luteolinidin and total flavonoids had an opposite effect on bacterial

abundances, we cannot rule out the possibility that other metabolites produced by sorghum may be driving some of the differences in rhizosphere community compositions across these sorghum lines.

Conclusion. In this study, root concentrations in *Sorghum bicolor* of luteolinidin and total flavonoids were different not only after frost but were also different between the NILs. Rhizosphere microbial communities were different before and after frost, leading us to hypothesize that frost did affect microbial communities; however, it is unclear whether this was a direct or indirect effect. Fungal communities and individual taxon abundances were different between sorghum lines, while relatively few bacterial taxa were different between sorghum lines. In contrast, abundances of bacteria rather than fungi were sensitive to root chemical variables, specifically total flavonoids, and luteolinidin. Taxa from the nonproducer lines were negatively affected by luteolinidin, while taxa from the producer lines were positively correlated with total flavonoids. These findings have important implications for understanding plant–microbe interactions and how sorghum rhizosphere communities differ between NILs. Many taxa that were differentially abundant across lines have been implicated as plant-growth promoters, potential biocontrols for soilborne diseases, or plant pathogens. Future studies should incorporate activity or functional analyses of microbial communities and plant gene expression analyses to elucidate how the production of root chemicals by sorghum influences rhizosphere communities.

Fig. 4. Venn diagrams showing the taxa correlated with total flavonoids in the producer lines (blue) and luteolinidin in the nonproducer lines (orange). Taxa included in the overlapping area are those that are correlated with both total flavonoids in the producer lines and luteolinidin in the nonproducer lines. Correlations were calculated using Kendall correlations and only correlations ≤ 0.50 or ≤ -0.5 between taxa and total flavonoids and luteolinidin are represented.

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