Phyllosphere exudates select for distinct microbiome members in sorghum epicuticular wax and aerial root mucilage

Marco E. Mechan-Llontop¹,², John Mullet²,³, and Ashley Shade¹,²,⁴,⁵,⁶*

¹Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI, 48824.
²Great Lakes Bioenergy Research Center, Michigan State University, East Lansing, MI, 48824.
³Department of Biochemistry & Biophysics, Texas A&M University, College Station, TX, 77843
⁴Department of Plant, Soil and Microbial Sciences, Michigan State University, East Lansing MI 48824
⁵The Plant Resilience Institute, Michigan State University, East Lansing MI 48824
⁶Univ Lyon, CNRS, INSA Lyon, Université Claude Bernard Lyon 1, École Centrale de Lyon, Ampère, UMR5005, 69134, Ecully cedex, France. (Present address)

*Corresponding author: A. Shade; E-mail: ashley.shade@cnrs.fr

ABSTRACT

Phyllosphere exudates create specialized microhabitats that shape microbial community diversity. We explored the microbiome associated with two sorghum phyllosphere exudates, the epicuticular wax and aerial root mucilage. We assessed the microbiome associated with the wax from sorghum
plants over two growth stages, and the root mucilage additionally from nitrogen-fertilized and non-fertilized plants. In parallel, we isolated and characterized hundreds of bacteria from wax and mucilage, and integrated data from cultivation-independent and cultivation-dependent approaches to gain insights into exudate diversity and bacterial phenotypes. We found that *Sphingomonadaceae* and *Rhizobiaceae* families were the major taxa in the wax regardless of water availability and plant developmental stage to plants. The cultivation-independent mucilage-associated bacterial microbiome contained *Erwinia* *Se*, *Flavobacteriaceae*, *Rhizobiaceae*, *Pseudomonadaceae*, *Sphingomonadaceae*, and its structure was strongly influenced by sorghum development but only modestly influenced by fertilization. In contrast, the fungal community structure of mucilage was strongly affected by the year of sampling but not by fertilization or plant developmental stage, suggesting a decoupling of fungal-bacterial dynamics in the mucilage. Our bacterial isolate collection from wax and mucilage had several isolates that matched 100% to detected amplicon sequence variants, and were enriched on media that selected for phenotypes including phosphate solubilization, putative diazotrophy, resistance to desiccation, capability to grow on methanol as a carbon source, and ability to grow in the presence of linalool and β-caryophyllene (terpenes in sorghum wax). This work expands our understanding of the microbiome of phyllosphere exudates and supports our long-term goal to translate microbiome research to support sorghum cultivation.

**Keywords:** bioenergy, agriculture microbiome, bacterial isolates, plant-association, diazotroph, irrigation, fertilizer, amplicon sequencing, cultivation

**INTRODUCTION**
The phyllosphere, which includes the above-ground plant structures, has diverse surface features (Ruinen 1965; Vacher et al. 2016; Doan et al. 2020). It is a microbial habitat that is exposed to rapid environmental fluctuations and stressors, including in ultraviolet radiation, temperature, and nutrient and water availability. Thus, the diversity and functions of the phyllosphere microbiome reflects this complex habitat (Lindow and Brandl 2003; Vorholt 2012; Vacher et al. 2016). To adapt to abiotic stresses, plants produce a diversity of exudates on their external surfaces (Chai and Schachtman 2022). The secreted exudates vary in composition and structure, creating specialized phyllosphere microhabitats (Galloway et al. 2020). Exudates that accumulate in the phyllosphere include epicuticular wax on stems and leaves (Kunst and Samuels 2003), sugar-rich mucilage on aerial root structures (Bennett et al. 2020), floral nectaries (Rering et al. 2018), and extrafloral nectaries in stems and leaves (Pierce 2019). Because of their potential as locations of microbial engagement with the host, research has been initiated to explore these microbial communities that reside on phyllosphere exudates.

Plants secrete epicuticular wax on leaves, leaf sheaths, and stems for prevention of water loss under drought stress (Xue et al. 2017), reflection of solar radiation (Steinmüller and Tevini 1985), and pathogen protection (Serrano et al. 2014; Wang et al. 2020). Epicuticular waxes are enriched in long-chain hydrocarbons. The major wax components include alkanes, alcohols, esters, and fatty acids, as well as varying levels of triterpenoids, sterols, and flavonoids (von Wettstein-Knowles 1974; Kunst and Samuels 2003; Busta et al. 2021). The wax composition and quantities are affected by plant species, plant developmental stage, and environmental conditions (Yeats and Rose 2013). It has been shown that epicuticular waxes affect bacterial and fungal plant colonization in a species-dependent manner (Beattie and Marcell 2002; Tsuba et al. 2002). Also, wax accumulation and composition directly impact the phyllosphere microbial community.
diversity (Reisberg et al. 2013). A study in *Arabidopsis thaliana* reported that Proteobacteria, Bacteroidetes, and Actinobacteria were the dominant phyla associated with wax on leaves (Reisberg et al. 2013).

Plants also secrete an abundance of polysaccharide-rich mucilage on aerial roots and the above ground portion of brace roots. Brace roots support plant anchorage as well as water and nutrient uptake (Stamp and Kiel 1992; Ku et al. 2012; Reneau et al. 2020). In 2018, van Deynze et al. 2018 reported that the mucilage of aerial roots of a maize landrace harbored diazotrophic microbiota that provided almost 80% of the nitrogen needed by the host. The bacterial genera *Acinetobacter, Agrobacterium, Enterobacter, Klebsiella, Lactococcus, Pantoea, Pseudomonas, Rahnella, Raoultella, Stenotrophomonas*, and others have been found in association with the mucilage of maize. These bacteria were capable of biological nitrogen fixation (BNF), synthesizing indole-3-Acetic Acid (IAA), utilizing 1-amino-1-cyclopropanecarboxylic acid (ACC), and solubilizing phosphates. The unique polysaccharide composition of the mucilage may modulate its associated microbiota (van Deynze et al. 2018; Higdon et al. 2020b). The maize mucilage is enriched in a mixture of monosaccharides including fucose (28%), galactose (22%), arabinose (15%), glucuronic acid (11%), xylose (11%), mannose (8%), glucose (1%) and galacturonic acid (1%) (van Deynze et al. 2018; Amicucci et al. 2019). The polysaccharide composition of root mucilage may vary among maize genotypes and with changing environmental conditions (Nazari et al. 2020).

Bioenergy sorghum (*Sorghum bicolor* L. Moench) is a heat and drought-tolerant annual crop being developed for production of biomass, biofuels and bioproducts (Mullet et al. 2014; Varoquaux et al. 2019). Bioenergy sorghum confers 75%-90% greenhouse gas mitigation when used for ethanol production or biopower generation respectively (Olson et al. 2012), but excess
nitrogen fertilizer is required to grow it, resulting in the release of nitrous oxide and relatively lower carbon benefit than other biofuel feedstocks that do not have high fertilizer demands (Kent et al. 2020; Scully et al. 2021). In the 1980s, it was hypothesized that the mucilage secreted by sorghum aerial roots harbors diazotroph bacteria, as has been more recently shown in the maize landrace (Bennett et al. 2020), but this has not yet been experimentally confirmed. Although the polysaccharide composition of the sorghum aerial root mucilage is uncharacterized, it is expected that the sorghum mucilage is similar in composition to maize (van Deynze et al. 2018; Amicucci et al. 2019). Taken together, it is expected that understanding microbiome interactions on the sorghum mucilage may provide insights into microbiome-enabled solutions to optimize diazotrophic nitrogen for the host and, in parallel, reduce nitrogen fertilizer needs for bioenergy sorghum.

Like other plants, bioenergy sorghum accumulates high levels of epicuticular wax on stems and leaves over its development, and some functions of the wax are to exclude pathogens and prevent water loss. Sorghum epicuticular wax chemistry and structure have been extensively studied. The accumulation, and composition of sorghum epicuticular wax are affected by several factors, including plant age, genotype, water availability, and environmental stresses (Bianchi et al. 1978; Avato et al. 1984; Jordan et al. 1984; Steinmüller and Tevini 1985; Shepherd et al. 1995; Jenks et al. 1996; Bondada et al. 1996; Shepherd and Wynne Griffiths 2006; Xue et al. 2017). However, the influence of sorghum wax chemistry on bacteria colonization and community structure is unknown.

In the present study, we investigated the microbiome associated with bioenergy sorghum epicuticular wax and aerial root mucilage. Given the functions of these exudates for the host, these communities may be of interest to examine microbiome traits that support host drought.
tolerance and nutrient uptake. To begin to explore the microbial communities inhabiting these specialized phyllosphere exudates, the microbiome composition and structure of wax and mucilage was analyzed from field conditions that included management treatments expected to influence plant water and nitrogen status. Specifically, we assessed the bacterial microbiome associated with the epicuticular wax from sorghum plants at two different developmental stages that also received different amounts of water, and the bacterial and fungal microbiomes additionally associated with the aerial root mucilage from nitrogen (N)-fertilized and non-fertilized sorghum plants. In addition, we curated a bacterial isolate collection from each phyllosphere exudate. We integrate data from both cultivation-independent and -dependent approaches to gain deeper insights into the microbiome diversity and dynamics of sorghum epicuticular wax and aerial root mucilage.

We hypothesized that: 1) wax and mucilage harbor different bacterial microbiomes due to their different exudate chemistries, host functions, and compartments; 2) plant developmental stage and watering status has highest explanatory value for the wax bacterial microbiota due to the known role of wax in supporting plant drought tolerance; 3) fertilization status has highest explanatory value for the mucilage bacterial microbiota due to changes in exogenous nutrient availability that are expected to result in changes in mucilage polysaccharide composition; and 4) that the bacterial and fungal members of the mucilage microbiome exhibit similar dynamics due to expected similar host and environmental drivers.

**METHODS**
Collection of sorghum stems and recovery of epicuticular wax. We collected samples from the bioenergy sorghum (*Sorghum bicolor*) hybrid TX08001 grown at the Texas A&M University Research Farm in College Station, Texas (30°55'5.55" N, 96°43'64.6" W). Sorghum plants were grown in 5 replicate 32 rows by 30 m plots at standard planting density and fertilization (Olson et al., 2012). We sampled replicate plots 1-5 at 60 (08/03/2020) and 90 (09/02/2020) days after plant emergence (DAE). While sorghum plants at 60 DAE were irrigated to maintain non-limiting water status, plants at 90 DAE were grown without irrigation to induce water-limiting conditions until harvesting. Thus, the developmental age of the plants and their watering status are colinear and their effects cannot be separated in our study. We collected stem sections that were covered in epicuticular wax, using razor blades to destructively sample the fifth and sixth fully elongated stem node-internodes below the growing zone into sterile whirl-pak bags. In total, we collected 50 stem samples during the growing season of 2020. All samples were kept on ice for transport, shipped on dry ice to Michigan State University, and then stored at -80°C. We used sterile razor blades to carefully remove and collect the epicuticular wax from stems in sterile 1.5 ml Eppendorf tubes. Epicuticular wax samples were stored at -80°C until processing.

Collection of sorghum aerial roots and removal of the mucilage. We collected samples from the bioenergy sorghum cultivar TAM 17651 grown at the Great Lakes Bioenergy Research Center (GLBRC), as part of the Biofuel Cropping System Experiment (BCSE) in Hickory Corners, Michigan (42°23’41.6” N, 85°22’23.1” W). Sorghum plants were grown in 5 replicate 30x40 m plots arrayed in a randomized complete block design. Within each plot, nitrogen fertilizer-free subplots were maintained either in the western or eastern -most 3m of each plot. We sampled replicate plots 1-4 in both the main and nitrogen-fertilizer free subplots at 60 and 90
DAE. We used sterile razor blades to carefully collect between 3 to 5 aerial nodal roots per plant that were covered with visible mucilage into sterile 50 ml Eppendorf tubes. In total, we collected 180 aerial root samples during the growing seasons of 2020 and 2021. All samples were kept on ice for transport, and then stored at -80 °C. In the laboratory, we added 15 ml of sterile distilled water and kept the roots for 5 min at room temperature to fully hydrate the aerial root mucilage. We collected 1 ml of mucilage into sterile 1.5 ml Eppendorf tubes per sample. Mucilage samples were stored at -80 °C until processing.

**Culturing the epicuticular wax and mucilage microbiomes.** For bacterial isolation, we pooled the epicuticular wax collected from different plants, as described above, and resuspended 100 mg of wax in 1 ml of sterile distilled water. We also pooled the mucilage collected from different plants, as described above. To capture a diversity of bacteria from the wax and mucilage, we used a variety of cultivation media (**Table 1**). First, we used standard culture media with a relatively high concentration of nutrients, including Tryptic Soy Agar (TSA: casein peptone 15 gl⁻¹, soy peptone 5 gl⁻¹, sodium chloride 5 gl⁻¹, agar 15 gl⁻¹, pH 7.3) and 50TSA (1/2 dilution of TSA). We also used media with relatively lower concentrations of nutrients, including Reasoner’s 2A (R2A: yeast extract 0.5 gl⁻¹, proteose peptone N⁰3 0.5 gl⁻¹, casamino acids 0.5 gl⁻¹, glucose 0.5 gl⁻¹, soluble starch 0.5 gl⁻¹, sodium pyruvate 0.3 gl⁻¹, K₂HPO₄ 0.3 gl⁻¹, MgSO₄ x 7H₂O 0.05 gl⁻¹, agar 15 gl⁻¹), 50R2A (1/2 dilution of R2A), and M9 minimal media (Na₂HPO₄ 12.8 gl⁻¹, KH₂PO₄ 3.0 gl⁻¹, NaCl 0.5 gl⁻¹, NH₄Cl 1.0 gl⁻¹, glucose 20 gl⁻¹, 1M MgSO₄ solution 20 ml, 1M CaCl₂ solution 0.1 ml, thiamine 0.5% w/v solution 0.1 ml, agar 15 gl⁻¹). To enrich for bacteria with putative plant beneficial traits, we used selective media types, including Jensen’s medium (sucrose 20 gl⁻¹, K₂HPO₄ 1 gl⁻¹, MgSO₄ 0.5 gl⁻¹, NaCl 0.5 gl⁻¹, FeSO₄ 0.1 gl⁻¹, Na₂MoO₄ 0.1 gl⁻¹, KNO₃ 2 gl⁻¹, K₂HPO₄ 1 gl⁻¹, MgSO₄ 0.5 gl⁻¹, NaCl 0.5 gl⁻¹, FeSO₄ 0.1 gl⁻¹, Na₂MoO₄ 0.1 gl⁻¹).
0.005 gl\(^{-1}\), CaCO\(_3\) 2 gl\(^{-1}\), agar 1 gl\(^{-1}\)) and modified nitrogen-free M9 minimal media with and without 1% (w/v) D-arabinose, galactose or xylose at pH 5, 5.8 or 7 (Na\(_2\)HPO\(_4\) 12.8 gl\(^{-1}\), KH\(_2\)PO\(_4\) 3.0 gl\(^{-1}\), NaCl 0.5 gl\(^{-1}\), 1M MgSO\(_4\) solution 20 ml, 1M CaCl\(_2\) solution 0.1 ml, agar 15 gl\(^{-1}\)) for detection of putative nitrogen fixing bacteria, Pirovskaya’s agar (yeast extract 0.5 gl\(^{-1}\), dextrose 10 gl\(^{-1}\), Ca\(_3\)(PO\(_4\))\(_2\) 5 gl\(^{-1}\), (NH\(_4\))\(_2\)SO\(_4\) 0.5 gl\(^{-1}\), KCl 0.2 gl\(^{-1}\), MgSO\(_4\) 0.1 gl\(^{-1}\), MnSO\(_4\) 0.0001 gl\(^{-1}\), FeSO\(_4\) 0.0001 gl\(^{-1}\), agar 15 gl\(^{-1}\)) for detection of phosphate solubilizing bacteria, Gauze’s synthetic medium No1 (soluble starch 20 gl\(^{-1}\), KNO\(_3\) 1 gl\(^{-1}\), NaCl 0.5 gl\(^{-1}\), MgSO\(_4\) \(\times\) 7H\(_2\)O 0.5 gl\(^{-1}\), K\(_2\)HPO\(_4\) 0.5 gl\(^{-1}\), FeSO\(_4\) \(\times\) 7 H\(_2\)O 10 mg\(^{-1}\), agar 15 gl\(^{-1}\)) for isolation of Actinobacteria, King’s medium B (proteose peptone 20 gl\(^{-1}\), K\(_2\)HPO\(_4\) 1.5 gl\(^{-1}\), MgSO\(_4\) \(\times\) 7H\(_2\)O 1.5 gl\(^{-1}\), glycerol 10 ml) for isolation of fluorescent pseudomonas, and methanol mineral salts medium ((NH\(_4\))\(_2\)SO\(_4\) 2.0 gl\(^{-1}\), NH\(_4\)Cl 2.0 gl\(^{-1}\), (NH\(_4\))\(_2\)HPO\(_4\) 2.0 gl\(^{-1}\), KH\(_2\)PO\(_4\) 1.0 gl\(^{-1}\), K\(_2\)HPO\(_4\) 1.0 gl\(^{-1}\), MgSO\(_4\) \(\times\) 7H\(_2\)O 0.5 gl\(^{-1}\), Fe\(_2\)SO\(_4\) \(\times\) 7H\(_2\)O 0.01 gl\(^{-1}\), CaCl\(_2\) \(\times\) 2H\(_2\)O 0.01 gl\(^{-1}\), yeast extract 2.0 gl\(^{-1}\), agar 20 gl\(^{-1}\)) for isolation of methanol-utilizing bacteria.

All plates were incubated for up to 14 days. To select for anaerobic bacteria, agar plates were placed in anaerobic jars (Mitsubishi AnaeroPack 7.0L rectangular jar) containing three bags of anaerobic gas generator (Thermo Scientific AnaeroPack Anaerobic Gas generator). To enrich for bacteria resistant to desiccation, one hundred microliters of dilution \(10^{-1}\) from the wax and mucilage were inoculated on 20 ml of 50% TSB liquid culture supplemented with different concentrations of 6000 polyethylene-glycol, including –0.49 MPa (210 gl\(^{-1}\) PEG w/v), -0.73 MPa (260 gl\(^{-1}\) PEG w/v) and –1.2 MPa (326 gl\(^{-1}\) PEG w/v). To enrich for bacteria that can grow in the presence of terpenoids, 100 ml of dilution \(10^{-1}\) from the wax and mucilage were inoculated on 20 ml of 50% TSB liquid culture supplemented with 1% (v/v) of either linalool or β-caryophyllene.

Liquid cultures were incubated at 28°C for 24 h, and dilutions \(10^{-1}\) to \(10^{-4}\) were plated in
duplicate on R2A agar plates for 24 h. Well isolated individual colonies were picked with a sterile toothpick and transferred to a new R2A plate. To confirm bacterial purity, individual bacterial colonies were transferred three times on new R2A agar plates. Glycerol stock (25% v/v) of pure bacteria isolates were stored at -80°C.

**Metagenomic DNA extraction and amplicon sequencing.** Microbial DNA was extracted from 0.5 ml of mucilage and 100 mg of epicuticular wax using a DNeasy PowerSoil kit (Qiagen, Maryland, USA) according to the manufacturer’s instructions. To confirm successful DNA extraction, the metagenomic DNA was quantified using a qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA), and visualized in a 1% agarose gel. Then, the PCR amplifications and sequencing of the V4 region of the 16S rRNA bacterial or archaeal gene from the epicuticular wax and mucilage samples and the ITS1 region of the fungal rRNA gene from the mucilage samples only were performed. DNA concentrations were normalized to approximately 1 µg/µl between all samples before PCR amplification and sequencing. The V4 hypervariable region of the 16S rRNA gene was amplified using the universal primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Caporaso et al. 2011) under the following conditions: 95°C for 3 min, followed by 30 cycles of 95°C for 45 s, 50°C for 60 s, and 72°C for 90 s, with a final extension at 72°C for 10 min. The metagenomic DNA of each sample was submitted to the Genomics Core of the Research Technology Support Facility at Michigan State University for library preparation and sequencing using the Illumina MiSeq platform v2 Standard flow cell in a 2x250bp paired-end format, using their standard operating protocol.
The ITS1 region was amplified using primers ITS1f (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′) (Smith and Peay 2014) with the addition of index adapters CS1-TS-F: 5′ – ACACTGACGACATGGTTCTACA – [TS-For] – 3′ and CS2-TS-R: 5′ – TACGGTAGCAGAGACTTGGTCT – [TS-Rev] – 3’ as requested by the Genomics Sequencing Core under the following PCR conditions: 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, and 68°C for 30 s, with a final extension at 68°C for 10 min. The amplification was performed with GoTaq Green Master Mix (Promega). The PCR products were purified with ExoSAP-IT reagent, and sample sequencing was completed by the Genomics Core of the Research Technology Support Facility at Michigan State University using the Illumina MiSeq platform v2 Standard flow cell in a 2x250bp paired-end format. For quality control purposes, positive and negative controls were included throughout the DNA extraction, PCR amplification, and sequencing processes. A 75 µl aliquot of the ZymoBIOMICS Microbial Community Standard (Zymo Research, Irvine, CA, U.S.A) and 75 µl aliquot of an in-house Community Standard were included as positive controls. Sterile DEPC-treated water was included as negative control.

**Bacterial genomic DNA extraction.** Bacteria colonies that were first streaked and isolated for purity were grown on 2 ml of 50% TSB liquid culture at 28°C for 24 h. Bacteria culture was centrifuged at 5,000 rpm for 10 min. Genomic DNA of each isolate was extracted by using the Zymo – Quick DNA Fungal/Bacterial 96 kit following the manufacturer’s protocol. Total genomic DNA was quantified using a qubit 2.0 fluorometer and visualized in a 1% agarose gel. The PCR amplification of the full-length 16S rRNA gene with universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3’) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3’).
(Miller et al. 2013) was performed by using the Pfu Turbo DNA polymerase (Agilent) under the following conditions: 95°C for 2 min, followed by 24 cycles of 95°C for 30 s, 48°C for 30 s, and 72°C for 3 min, with a final extension at 72°C for 10 min. PCR products were purified with ExoSAP-IT reagent and submitted for Sanger sequencing at the Genomics Core of the Research Technology Support Facility at Michigan State University, MI, USA.

**Bacterial and fungal amplicon sequencing analysis.** Paired-end sequencing data from each sequencing experiment were processed with QIIME2 (Bolyen et al. 2019) version 2021.8.0. In brief, sequences were imported using the PairedEndFastqManifestPhred33V2 format. Sequence quality control, denoising, and generation of feature tables containing counts for the Amplicon Sequencing Variants (ASVs) were performed with the q2-dada2 plugin version 2021.8.0 (Callahan et al. 2016). Trimming parameters for the DADA2 plugin were selected with FIGARO version 1.1.2 (Weinstein et al. 2019). ASVs tables and representative sequences from each sequencing experiment were merged with the q2-feature-table plugin. ASV taxonomy (of merged ASVs) was assigned with the q2-feature-classifier plugin using the SILVA version 1.38 database (Quast et al. 2013) for bacteria and UNITE version 8.3 database (Nilsson et al. 2019) for fungi.

The ASV table, taxonomy table, and sample metadata files were imported into R version 4.1.3 for data visualization and statistical analysis. Diversity and statistical analyses were performed using the phyloseq (McMurdie and Holmes 2013) and vegan (Dixon 2003) packages. Treatments compared were: exudate (wax, mucilage) for bacterial microbiomes; fertilization status (fertilized, unfertilized), year of sample collection (2020, 2021), and developmental stage (60 DAE, 90 DAE) for mucilage bacterial and fungal microbiomes; and developmental
stage/water availability (60 DAE, 90 DAE) for wax bacterial microbiomes. A Wilcoxon rank
sum test with continuity correction was used to test for differences in alpha diversity across
treatments. Permutated analysis of variance (PERMANOVA) and permuted analysis of beta-
dispersion (PERMDISP) were used to assess differences in beta diversity structure across
treatments by centroid and dispersion. Differential abundance analysis was performed with the
DESeq2 package (Love et al. 2014). Each dataset (bacterial/fungal, wax/mucilage) was
subsampled independently to ensure maximum coverage for comparisons over time and across
field treatments. The exception was when testing hypothesis 1 (differences in wax and mucilage
bacterial microbiome), and in this case both datasets were subsampled to an even 2,500
sequences per sample for comparison.

Full-length 16S rRNA gene Sanger sequencing analysis: Culturing phyllosphere exudate
microbiota. To generate a consensus sequence of the full-length 16S rRNA gene from each
bacterial isolate, sequences were imported into Geneious version 2021.2.2
(https://www.geneious.com/). High-quality forward and reverse sequences were aligned and
trimmed to generate a consensus sequence. Then, the consensus sequence was searched with
BLAST for taxonomic classification. CD-HIT version 4.8.1 (Li and Godzik 2006) was used to
remove redundant 16S rRNA sequences. To identify bacterial isolates that match 100% to the
identified ASVs from the culture-independent approach, a local BLAST search was performed.
In summary, a local BLAST database was created with all non-redundant 16S rRNA sequences
from our bacterial collection using the makeblastdb command and the -dbtype nucl option. A
BLAST search was carried out to identify related sequences in the representative sequences
(ASVs dna-sequences.fasta) file generated from the DADA2 denoising step with the blastn
command, and the following options: "6 qseqid sseqid pident length mismatch gapopen qstart qend
sstart send evalue bitscore".

Comparison with publicly available plant-associated bacterial genomes. We retrieved 637 plant-associated (PA) bacterial genomes that were classified as non-root associated from the (Levy et al. 2017) study. High-quality bacterial genomes were annotated with Prokka (Seemann 2014) using an in-house python script and annotated 16S rRNA gene copies were identified (available on GitHub, see Data availability statement). For bacteria with multiple 16S rRNA copies, CD-HIT version 4.8.1 (Li and Godzik 2006) was used to remove redundant sequences (99% similarity) and one 16S rRNA sequence was conserved, totaling 433 unique PA sequences. All 16S rRNA sequences from the PA bacterial genome dataset were concatenated in a single fasta file with the cat command. CD-HIT was used to remove redundant sequences (100% similarity) from the 16S rRNA concatenated file. All non-redundant 16S rRNA sequences from both the sorghum bacterial collections and the publicly available PA bacteria were merged in a single fasta file. Sequence alignment was performed with MAFFT v7.407 (Katoh et al. 2002). Alignment trimming was performed with trimAl (Capella-Gutiérrez et al. 2009). A maximum-likelihood (ML)-based phylogenetic tree was built with IQ-TREE 2.2.0-beta version (Minh et al. 2020). ModelFinder version (-m TEST option) (Kalyaanamoorthy et al. 2017) was used to select the best model for the phylogenetic tree construction. Branch support was assessed using 1,000 ultrafast bootstrap approximations (-bb 1000 option) (Hoang et al. 2018). Phylogenetic diversities were calculated as the total tree length, that represents the expected number of substitutions per site. Phylogenetic tree was edited with iTOLs version 6.5.8 (Letunic and Bork 2021).
**Data and code availability.** The data analysis workflows for sequence processing and ecological statistics are available on GitHub (https://github.com/ShadeLab/Sorghum_phyllosphere_microbiome_MechanLlontop_2022.git). Raw sequencing data has been deposited in the Sequence Read Archive NCBI database under BioProject accession number PRJNA844896 (including 16S rRNA and ITS amplicons). Full-length 16S rRNA sequence data has been deposited in the GenBank with accession numbers ON973084-ON973283.

**RESULTS**

**Sequencing summary.** In total, we sequenced the bacterial 16S rRNA V4 region from 48 epicuticular wax samples from the 2020 growing season, as well as the bacterial 16S rRNA V4 region from 179 mucilage samples and the fungal ITS region from 173 mucilage samples that were collected across two growing seasons in 2020 and 2021. We obtained 8,648,839 bacterial sequences from the wax, and 20,606,039 bacterial and 20,181,404 fungal sequences from the mucilage. After quality control, removal of chimeras, and denoising, 7,930,768 quality bacterial reads were obtained from the wax samples, and 19,880,634 bacterial and 12,157,819 fungal sequences were obtained from mucilage (Table 2). For wax, the total number of sequences per sample after the denoising process with DADA2 into Amplicon Sequence Variants (ASVs) ranged from 1,722 to 272,108. After the removal of nonbacterial and unassigned sequences, a total of 2,386,033 sequences remained, with sequencing reads per wax sample ranging from 138 to 206,128. We removed wax samples with fewer than 1000 sequences, and the remaining 42 epicuticular wax samples were rarefied to 1,303 sequences for further analysis (Figure 1A).

Given the observed richness (12 to 93 ASVs per sample) by these cultivation-independent
methods, Figure 1A shows that the wax bacterial microbiome was covered with the given sequencing effort.

For root mucilage, the number of bacterial sequences per sample after the denoising ranged from 222 to 330,853. After the removal of nonbacterial and unassigned sequences, a total of 12,956,774 sequences remained, with sequencing reads per sample ranging from 110 to 235,069. We removed samples with fewer than 20,000 sequences, and the remaining 158 samples were rarefied to 20,519 sequences for comparative analysis (Figure 1B). Given the observed richness (49 to 555 ASVs per sample) by these cultivation-independent methods, Figure 1B shows that the mucilage bacterial microbiome was covered with the given sequencing effort. The number of fungal sequences per mucilage sample after the denoising ranged from 78 to 119,207. After the removal of non-fungal and unassigned sequences, a total of 12,297,453 sequences remained, with sequencing reads per sample ranging from 32 to 119,207. We filtered mucilage samples with fewer than 30,000 ITS sequences, and the remaining 171 samples were rarefied to 33,975 sequences for comparative analysis (Figure 1C). Similarly, given the observed richness by these cultivation-independent methods (47 to 237 ASVs per sample), Figure 1C shows that the mucilage fungal microbiome was covered with the given sequencing effort.

Hypothesis 1: Wax and mucilage harbor different bacterial microbiomes

Compositional differences in the bacterial microbiomes of the epicuticular wax and mucilage were apparent at the family level of taxonomic resolution (Figure 2A and B) as well as at the genus level (Supplementary Figure S1A and B). Wax and mucilage bacterial microbiomes had different richness (observed taxa Wilcoxon rank \( p<0.001 \), Supplementary Table 1) and
different structures (PERMANOVA R-squared= 0.14, \( p=0.001 \)). Thus, Hypothesis 1 was supported. However, there were no differences detected in the dispersions of wax and mucilage bacterial microbiome structures (PERMDISP F=0.69, \( p=0.43 \)).

**Hypothesis 2: Plant developmental stage/watering status has highest explanatory value for the wax bacterial microbiota**

Altogether, we identified 534 bacterial ASVs in epicuticular wax. Wax bacterial microbiome samples collected from sorghum plants at 60 DAE and 90 DAE had different richness (observed taxa Wilcoxon rank \( p=0.03 \)) ([Supplementary Table 1](#)). There was higher variation in the community structure in the epicuticular wax on plants at 90 DAE compared with plants at 60 DAE (PERMDISP F=17.92, \( p=0.001 \)). There was a small but significant influence of sorghum developmental stage on the epicuticular wax community structure (PERMANOVA R-squared=0.06, \( p= 0.003 \), **Figure 3A, Table 3**).

The sorghum epicuticular wax microbiome was dominated by the Proteobacteria (84% mean relative abundance) and Bacteroidetes (11%) bacteria phyla. The bacterial classes Alphaproteobacteria (54%), Gammaproteobacteria (30%), and Bacteroidia (11%) were in highest abundance. Sphingomonadaceae (25%), Rhizobiaceae (21%), and Xanthomonadaceae (7%) were the major bacterial families in sorghum epicuticular wax (**Figure 2A**). At the genus level, *Sphingomonas* (28%), *Rhizobium* (12%), *Aureimonas* (10%), and *Acinetobacter* (5%) were the dominant taxa in wax ([Supplementary Figure 1](#)). Differential abundance analysis showed that only one ASV (ASV ID #5438e751393c2dda98fe3d99c26da1) from the Microbacteriaceae family was more abundant on the wax of plants at 60 DAE (by 3.08-fold, DeSeq \( p = 0.01 \)), and that one ASV (ASV ID #8f820a46efec19477f4485d1c436764) assigned to *Pseudoxanthomonas*
genera was more abundant on the wax of plants at 90 DAE (by 4.49-fold, DESeq \( p = 0.01 \)).

Taking these results together, Hypothesis 2 was weakly supported with a small, significant difference in wax bacterial microbiome by plant stage and two taxa that were distinguishing between the stages.

Hypothesis 3: Fertilization status has highest explanatory value for the bacterial mucilage microbiota

Altogether, 12,047 bacterial ASVs were identified in aerial root mucilage. There was no difference in richness between mucilage samples collected from sorghum plants at 60 DAE and 90 DAE (observed species Wilcoxon rank \( p = 0.82 \), Supplementary Table 1), and also no difference between mucilage samples from nitrogen-fertilized plants as compared with unfertilized plants. (observed species Wilcoxon rank \( p = 0.15 \), Supplementary Table 1). There was different beta dispersion in community structure by plant developmental stage (PERMDISP \( F=19.56, p=0.001 \)) but not by fertilization status (PERMDISP \( F=1.83, p=0.187 \)). The mucilage bacterial microbiome structure was better explained by developmental stage than fertilization status (PERMANOVA R-squared= 0.14 and 0.03, respectively, both \( p = 0.001 \)) (Figure 3B).

The aerial root mucilage bacterial microbiome was dominated by the Proteobacteria (61% mean relative abundance) and Bacteroidota (36%) bacteria phyla. The bacterial class Gammaproteobacteria (40%), Bacteroidia (34%), and Alphaproteobacterial (21%) were the most abundant. Erwiniaeaceae (23%), Rhizobiaceae (14%), Flavobacteriaceae (12%), Pseudomonadaceae (9%), and Sphingomonadaceae (6%) were the major bacterial families in mucilage (Figure 2B). A differential abundance analysis identified 25 ASVs enriched in the mucilage at 60 DAE and 72 ASVs significantly enriched in plants at 90 DAE (Figure 4, DESeq
Taking these results together, Hypothesis 3 was not supported, and the bacterial microbiome of the mucilage was not highly sensitive in structure or dispersion to fertilization given this study’s field conditions, nor were there notable distinguishing taxa by plant fertilization status.

**Hypothesis 4: The bacterial and fungal members of the mucilage microbiome exhibit similar dynamics.**

Altogether, 5,641 fungal ASVs were identified in aerial root mucilage. There were differences in richness between mucilage samples collected from sorghum plants during the 2020 and 2021 growing seasons (observed species Wilcoxon rank \( p = 0.008 \)), and also between mucilage samples from nitrogen-fertilized plants compared with unfertilized plants (observed species Wilcoxon rank \( p < 0.01 \)). However, no difference was observed between mucilage samples from plants at 60 DAE vs. 90 DAE (Supplementary Table 1). The mucilage fungal microbiome structure was strongly influenced by year of collection (PERMANOVA R-squared= 0.51, \( p < 0.001 \)). Fungal community structure was weakly influenced by developmental stage (PERMANOVA R-squared= 0.02, \( p < 0.05 \)), but not by fertilization status (PERMANOVA, \( p > 0.05 \)) (Figure 2C).

The mucilage fungal microbiome was dominated by the Ascomycota (76%) and Basidiomycota (23.7%) phyla. The Dothideomycetes (50%), Sordariomycetes (24%), and Tremellomycetes (14%) fungal classes were the most abundant. Cladosporium (22%), Nectriaceae (17%), Didymellaceae (14%), Bulleribasidiaceae (9 %), Pleosporaceae (8%) were the dominant fungal families in the mucilage. The genera *Cladosporium* exhibited higher abundance in the 2020 growing season (34%) compared with 2021 (14%). In contrast, we found
an enrichment of the genera *Epicoccum* in 2021 (18%) compared with the 2020 growing season (0.02%) *(Supplementary Figure 1)*. Taking these results together, Hypothesis 4 was not supported because the bacterial microbiome of mucilage was more sensitive to plant development and consistent across sampling years than the fungal, while the fungal microbiome also exhibited greater variability between years.

**Cultivation-dependent bacterial taxonomic and phenotypic diversity of sorghum phyllosphere wax and mucilage.**

Bacterial culture collections from the epicuticular wax and aerial root mucilage were constructed by enriching bacteria with putative plant-beneficial traits *(Table 1)*. In total, 500 bacteria from the wax and 800 bacteria from the mucilage were isolated, and then a subset of 200 isolates from both the wax and mucilage were taxonomically identified by sequencing the full-length 16S rRNA gene *(Supplementary Table 2)*. These isolates were chosen to represent the range of different cultivation conditions employed and, additionally, to maximize distinguishing phenotypes (morphology, color, etc) to avoid redundancy in the collection *(Figure 5)*. The wax bacterial collection was dominated by the Proteobacteria, followed by Actinobacteria, and Bacteroidetes phyla, and the mucilage bacterial collection was dominated by the Proteobacteria, followed by Actinobacteria, Firmicutes, and Bacteroidetes phyla *(Supplementary Table 2)*.

Forty-eight ASVs matched with 100% sequence identity to strains in the isolate collections *(Supplementary Table 2)*. Most of the bacterial families found in the sorghum wax and mucilage had representatives among the isolate collection *(Figure 6)*. Families such as Beijerincklaceae, Chitinophagaceae, Oxalobacteraceae were not captured by our wax bacterial
cultivation efforts. Families observed using cultivation-independent techniques but that were not captured by our mucilage cultivation efforts included Cytophagaceae and Oxalobacteraceae.

To understand potential novelty and redundancy represented by the diversity of our wax and mucilage bacterial collections, we compared the full-length 16S rRNA genes with those extracted from the bacterial genomes of previously described non-root-associated, plant-associated (PA) bacteria (Levy et al. 2017), assigned as non-root-associated. 637 bacterial genomes were retrieved from a publicly available database (see Methods) to provide a reference of context for our 200 sorghum phyllosphere isolates. The final data set contained 527 non-redundant full-length 16S rRNA sequences: 94 new 16S rRNA genes from our sorghum wax and mucilage collections, and 433 rRNA genes from the published plant-associated bacterial genomes (Figure 7).

DISCUSSION

We investigated the microbiota associated with bioenergy sorghum phyllosphere exudates, specifically from epicuticular wax on stems and leaves and from mucilage on aerial roots.

The chemistry of epicuticular wax that covers sorghum stems has been extensively characterized (Bianchi et al. 1978; Jordan et al. 1984; Jenks et al. 2000; Farber et al. 2019a, 2019b), but there is still much to learn about its microbial residents and their colonization dynamics. Thus, we decided to characterize the wax microbiota from stems of field-grown bioenergy sorghum plants at 60 DAE and 90 DAE. We chose these two-time points because they represent different developmental stages, and, in our field conditions, they also had different water availability. During the vegetative stage, sorghum plants at 60 DAE have all leaves...
developed and fully expanded. At 90 DAE in the upper mid-west, plants have transitioned to the reproductive stage, seed development is in progress and nutrients are being relocated to the kernel. In the southwestern U.S., sorghum plants are in extended vegetative growth stage, with floral initiation expected at 120 DAE. The major lineages we detected in the epicuticular stem wax, including *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria*, agree generally with reports from *Arabidopsis thaliana* and *Sorghum bicolor* epicuticular leaf wax (Reisberg et al. 2013; Sun et al. 2021). Furthermore, we also observed changes in the relative abundances of several taxa at 60 DAE compared with plants at 90 DAE, which could be associated with changes in the composition of the epicuticular wax as the plant grows (Avato et al. 1984; Jenks et al. 1996), though more work is needed to characterize changes in the chemical composition of the wax alongside the structural changes in the microbiome to understand their relationship more fully. It has been suggested that microbes in wax may be able to metabolize wax components and use them as a carbon source (Ueda et al. 2015). Our study enriched several bacterial isolates that were able to grow with linalool and beta-caryophyllene, two of the terpenes found in sorghum wax. To gain further insight into epicuticular wax microbiome assembly and dynamics, next steps could expand this research not only by including samples from different growing seasons, but also by including sorghum genotypes that are mutants in wax production (Jenks et al. 1994, 2000; Peters et al. 2009; Punnuri et al. 2017).

For decades it has been suggested that the sorghum aerial root mucilage harbors diazotroph bacteria (Wani 1986; Bennett et al. 2020). We hypothesized that fertilization would strongly influence the phyllosphere mucilage microbiota due to changes in exogenous nutrient availability and changes in mucilage polysaccharide composition. However, our cultivation-independent data (16S rRNA amplicons) suggest that that differences in nitrogen fertilization had
no notable influence on the microbiome structure for both bacterial and fungal communities. In contrast, plant developmental stage strongly affected the mucilage bacterial microbiome structure. Similar evidence of microbiome seasonality has been found in other studies of different surfaces of the phyllosphere microbiome (Copeland et al. 2015; Grady et al. 2019; Xiong et al. 2021; Smets et al. 2022). We also observed several putative diazotroph bacteria in the sorghum mucilage that were isolated anaerobically and on nitrogen-free media, including *Curtobacterium*, *Pantoea*, *Pseudomonas*, *Strenotrophomonas*, which were reported as lineages that could colonize the maize mucilage (van Deynze et al. 2018; Higdon et al. 2020b, 2020a).

Regarding the fungal microbiome in the mucilage, we found that the year of collection had the highest explanatory value. With two years of field data, there is not enough information to understand if the fungal community is responsive to other covariates (e.g., weather) or more stochastically assembled every year. Fungal community members likely have different responses than bacterial members to changing environmental conditions, including temperature, moisture, solar radiation, and precipitation (Jackson and Denney 2011; Copeland et al. 2015; Wagner et al. 2016; Gomes et al. 2018). We can deduce that the bacterial and fungal communities did not have strong relationships or co-dependencies based on their structures, and likely have different dominating drivers. However, the possibility of redundant functional relationships between different bacterial and fungal mucilage members cannot be eliminated.

We combined both culture-independent and dependent approaches to improve our understanding of the microbiome diversity in phyllosphere exudates. Due to the chemical composition, plant DNA contamination, and low bacterial biomass associated with the wax and mucilage, a metagenomic sequencing approach would have been challenging to pursue with the sorghum phyllosphere (Sharpton 2014; van Deynze et al. 2018). Sequencing the V4 16S rRNA
and the ITS1 regions allowed us to deeply characterize bacterial and fungal communities in sorghum phyllosphere exudates, albeit with limited taxonomic resolution that can be provided by the amplicons (to approximately the genus level Poretsky et al. 2014) as well as limited functional insight (Langille et al. 2013; Turner et al. 2013). Thus, we decided to culture wax and mucilage bacteria by using a variety of isolation media and growing conditions that we expected to enrich for plant-beneficial bacterial phenotypes. In the end, we were able to capture representatives of most of the bacterial families and genera that we observed in our culture-independent approach. These isolates can now be used to test directly for plant beneficial properties and microbe-plant interactions in the laboratory.

In summary, we report a characterization of microbiome structure of energy sorghum phyllosphere exudates, epicuticular wax and aerial root mucilage under multiple field conditions and across two seasons for mucilage. We found that the wax and mucilage harbor distinct bacterial communities, suggesting niche specialization in the sorghum phyllosphere, and captured several key bacterial lineages in a parallel cultivation effort. Additionally, we found that fungal communities and bacterial communities in the mucilage are responsive to different drivers, with bacterial communities most distinctive by developmental stage and fungal communities most distinctive by year of sample collection. Next steps are to use the ecological dynamics from the cultivation-independent sequencing and apparent phenotypes of the bacterial isolates to understand the roles of these exudate microbiome members for plant performance.

**ACKNOWLEDGMENTS**

This work was supported by the Great Lakes Bioenergy Research Center, U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research under Award
Number DE-SC0018409. Support for field research was provided by the Great Lakes Bioenergy Research Center, U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research (Awards DE-SC0018409 and DE-FC02-07ER64494), by the National Science Foundation Long-term Ecological Research Program (DEB 1637653) at the Kellogg Biological Station, and by Michigan State University AgBioResearch. JM acknowledges field support from graduate students at TAMU. AS acknowledges support from Michigan State University AgBioResearch.

The authors declare no conflict of interest.

LITERATURE CITED


https://doi.org/10.1371/journal.pone.0239677.

https://doi.org/10.1371/journal.pone.0239081.


Jenks, M. A., Rich, P. J., Rhodes, D., Ashworth, E. N., Axtell, J. D., and Ding, C.-K. 2000. Leaf sheath cuticular waxes on bloomless and sparse-bloom mutants of Sorghum bicolor. Phytochemistry. 54:577–584 Available at:


https://doi.org/10.2135/cropsci1984.0011183X002400060038x.


https://doi.org/10.1021/acs.est.0c01676.


Letunic, I., and Bork, P. 2021. Interactive Tree Of Life (iTOl) v5: an online tool for phylogenetic tree display and annotation. Nucleic Acids Res. 49:W293–W296 Available at: https://doi.org/10.1093/nar/gkab301.

Letunic, I., and Bork, P. 2021. Interactive Tree Of Life (iTOl) v5: an online tool for phylogenetic tree display and annotation. Nucleic Acids Res. 49:W293–W296 Available at: https://doi.org/10.1093/nar/gkab301.


Biofuels, Bioproducts and Biorefining. 6:640–655 Available at: https://doi.org/10.1002/bbb.1357.


Pierce, M. P. 2019. The ecological and evolutionary importance of nectar-secreting galls. Ecosphere. 10:e02670 Available at: https://doi.org/10.1002/ecs2.2670.


Table 1. Solid media and their enrichment objectives (target phenotypes) used in this study to culture bacteria from the sorghum wax and mucilage. Dilutions from $10^{-1}$ to $10^{-4}$ were plated for each condition, for each exudate.

<table>
<thead>
<tr>
<th>Media</th>
<th>Target phenotype</th>
<th>Temperature (°C)</th>
<th>Oxygen condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reasoner’s 2A agar (R2A)</td>
<td>General diversity</td>
<td>25, 37</td>
<td>Aerobic, anaerobic</td>
</tr>
<tr>
<td>50% R2A</td>
<td>General diversity</td>
<td>25, 37</td>
<td>Aerobic, anaerobic</td>
</tr>
<tr>
<td>Tryptic Soy Agar (TSA)</td>
<td>General diversity</td>
<td>25, 37</td>
<td>Aerobic, anaerobic</td>
</tr>
<tr>
<td>50% TSA</td>
<td>General diversity</td>
<td>25, 37</td>
<td>Aerobic, anaerobic</td>
</tr>
<tr>
<td>M9 minimal medium</td>
<td>General diversity</td>
<td>25, 37</td>
<td>Aerobic, anaerobic</td>
</tr>
<tr>
<td>King’s B medium</td>
<td><em>Pseudomonas</em> species</td>
<td>25, 37</td>
<td>Aerobic, anaerobic</td>
</tr>
<tr>
<td>Nitrogen-free Jensen’s medium</td>
<td>Nitrogen fixation</td>
<td>25, 37</td>
<td>Aerobic, anaerobic</td>
</tr>
<tr>
<td>M9 minimal medium nitrogen-free, 1% xylose</td>
<td>Nitrogen fixation</td>
<td>25, 37</td>
<td>Aerobic, anaerobic</td>
</tr>
<tr>
<td>M9 minimal medium nitrogen-free, 1% galactose</td>
<td>Nitrogen fixation</td>
<td>25, 37</td>
<td>Aerobic, anaerobic</td>
</tr>
<tr>
<td>M9 minimal medium nitrogen-free, 1% arabinose</td>
<td>Nitrogen fixation</td>
<td>25, 37</td>
<td>Aerobic, anaerobic</td>
</tr>
<tr>
<td>M9 minimal medium nitrogen-carbon free</td>
<td>Nitrogen fixation</td>
<td>25, 37</td>
<td>Aerobic, anaerobic</td>
</tr>
<tr>
<td>Pirovskaya’s agar</td>
<td>Phosphate solubilization</td>
<td>25, 37</td>
<td>Aerobic, anaerobic</td>
</tr>
<tr>
<td>50% Tryptic Soy Broth, 1% linalool*</td>
<td>Resistance to/utilization of terpenoids</td>
<td>28</td>
<td>Aerobic</td>
</tr>
<tr>
<td>50% Tryptic Soy Broth, 1% β-caryophyllene*</td>
<td>Resistance to/utilization of terpenoids</td>
<td>28</td>
<td>Aerobic</td>
</tr>
<tr>
<td>50% Tryptic Soy Broth, 6000 Polyethylene Glycol*</td>
<td>Osmotic tolerance</td>
<td>28</td>
<td>Aerobic</td>
</tr>
<tr>
<td>Gauze’s synthetic medium N-1</td>
<td>Actinobacteria species</td>
<td>25</td>
<td>Aerobic</td>
</tr>
<tr>
<td>Methanol Mineral Salts Medium</td>
<td>Methylotrophs</td>
<td>25</td>
<td>Aerobic</td>
</tr>
</tbody>
</table>

*After initial enrichment in liquid media, turbid cultures were diluted and plated onto R2A to isolate colonies.
Table 2. Sequencing summary of sorghum epicuticular wax and aerial root mucilage microbial communities characterized in this study.

<table>
<thead>
<tr>
<th></th>
<th>Wax (16S rRNA)</th>
<th>Mucilage (16S rRNA)</th>
<th>Mucilage (16S rRNA)</th>
<th>Mucilage (ITS1)</th>
<th>Mucilage (ITS1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2020</td>
<td>2020</td>
<td>2021</td>
<td>2020</td>
<td>2021</td>
</tr>
<tr>
<td>Number of samples</td>
<td>48</td>
<td>99</td>
<td>80</td>
<td>92</td>
<td>81</td>
</tr>
<tr>
<td>Raw Read Pairs</td>
<td>8,648,839</td>
<td>12,783,054</td>
<td>10,034,885</td>
<td>10,403,184</td>
<td>9,778,220</td>
</tr>
<tr>
<td>QC reads</td>
<td>7,930,768</td>
<td>10,809,135</td>
<td>9,071,499</td>
<td>6,200,571</td>
<td>5,957,248</td>
</tr>
<tr>
<td>% Chloroplast/</td>
<td>70%</td>
<td>24%</td>
<td>48%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Mitochondria/</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>unassigned of QC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>reads</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Permuted multivariate analysis of variance (PERMANOVA) to test for microbiome differences in beta diversity.

<table>
<thead>
<tr>
<th>Dataset</th>
<th>Exudate</th>
<th>Variable tested</th>
<th>Degrees of freedom</th>
<th>PseudoF</th>
<th>R-squared</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>Mucilage, wax</td>
<td>Exudate</td>
<td>1</td>
<td>35.51</td>
<td>0.14</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Mucilage</td>
<td>Development</td>
<td>1</td>
<td>25.22</td>
<td>0.14</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Mucilage</td>
<td>Fertilization</td>
<td>1</td>
<td>4.26</td>
<td>0.03</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Mucilage</td>
<td>Year</td>
<td>1</td>
<td>3.36</td>
<td>0.02</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Mucilage</td>
<td>Fertilization*Development</td>
<td>1</td>
<td>1.78</td>
<td>0.01</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Mucilage</td>
<td>Development*Year</td>
<td>1</td>
<td>2.78</td>
<td>0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>Mucilage</td>
<td>Fertilization*Year</td>
<td>1</td>
<td>1.64</td>
<td>0.01</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>Wax</td>
<td>Development</td>
<td>1</td>
<td>2.75</td>
<td>0.06</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

| Fungi   | Mucilage        | Development              | 1                   | 3.25    | 0.02      | <0.05   |
|         | Mucilage        | Fertilization            | 1                   | 2.20    | 0.01      | 0.07    |
|         | Mucilage        | Year                     | 1                   | 176.38  | 0.51      | <0.001  |
|         | Mucilage        | Fertilization*Development| 1                   | 0.57    | 0.00      | 0.68    |
|         | Mucilage        | Development*Year         | 1                   | 5.03    | 0.01      | <0.01   |
|         | Mucilage        | Fertilization*Year       | 1                   | 4.29    | 0.01      | <0.05   |
Figure Legends

Figure 1. Sequencing effort and alpha diversity for sorghum epicuticular wax and aerial root mucilage. Amplicon sequencing variants (ASVs) were defined at 100% identity of 16S rRNA gene or ITS1 gene for bacterial and fungal datasets, respectively. Subsampled read depth is indicated by the red, vertical, dashed line. Top panel: Rarefaction curves of quality-controlled sequences. Bottom panels: Observed taxa (No. ASVs, a.k.a. richness) and phylogenetic diversity (PD) metrics. A) Epicuticular wax bacterial samples were rarefied to 1,303 reads per sample. B) Aerial root mucilage bacterial samples were rarefied to 20,519 reads per sample. C) Aerial root mucilage fungal samples were rarefied to 33,975 reads per sample.

Figure 2. Relative abundances of bacterial families in sorghum epicuticular wax (A) and aerial root mucilage (B) at 60 and 90 days after plant emergence; and relative abundances of fungal families in mucilage (C) from samples collected in 2020 and 2021. Only families with relative abundances >0.03 are shown.

Figure 3. Principal Coordinates Analysis (PCoA) based on Bray-Curtis dissimilarities for bacterial microbiome from sorghum epicuticular wax (A), bacterial microbiome from aerial root mucilage (B) and fungal microbiome from mucilage (C). DAE is days after plant emergence.

Figure 4. Differential abundance analysis for amplicon sequencing variants (ASVs) defined at 100% sequence identity. Differentially enriched bacterial ASVs in the aerial root mucilage of plants at 60 and 90 DAE are shown. The fold change is shown on the x-axis and bacterial genera
are listed on the y-axis. Each colored dot represents a separate ASV annotated within a bacterial
Class.

**Figure 5.** Taxonomic diversity of the subset of bacteria cultivated from sorghum epicuticular
wax and aerial root mucilage that were selected for 16S rRNA gene sequence analysis based on
representation of different cultivation conditions and colony phenotypes. A) Bacterial isolates
cultured at 25°C under aerobic conditions, B) Bacterial isolates cultured at 37°C under aerobic
conditions, C) Bacterial isolates cultured at 25°C under anaerobic conditions, and D) Bacterial
isolates cultured at 37°C under anaerobic conditions.

**Figure 6.** Overlap in bacterial diversity found in the sorghum epicuticular wax and aerial root
mucilage based on culture-independent and culture-dependent approaches. Relative abundance at
the family level > 0.01 are shown.

**Figure 7.** Phylogenetic diversity in the sorghum epicuticular wax and aerial root mucilage.
Maximum Likelihood phylogenetic tree (IQTREE, under UNREST+FO+I+G4 model) is based
on the 16S rRNA gene alignment from nonredundant sorghum bacterial isolates and Levy et al.
2017 genomes.
**Supplementary Information**

**Supplementary Figure S1.** Relative abundances of bacterial genera in sorghum epicuticular wax (A) and aerial root mucilage (B) at 60 and 90 days after plant emergence; and relative abundances of fungal families in mucilage (C) from samples collected in 2020 and 2021. Only genera with relative abundances >0.03 are shown.

**Supplementary Table S1.** *Excel file.* Tests for differences in bacterial and fungal alpha diversity (richness, *a.k.a.* number of observed taxa) between exudates (mucilage, wax) and, within each exudate, between categories of development (60 v. 90 DAE), fertilization (nitrogen-fertilized, unfertilized), and year (2020, 2021) using the Wilcoxon rank sum test with continuity correction.

**Supplementary Table S2.** *Excel file.* Bacterial isolates from wax and mucilage and their taxonomy based on full-length 16S rRNA gene Sanger sequencing. The isolates that shared 100% sequence identity to short-read bacterial ASVs (Amplicon Sequencing Variants) are indicated and mapped to the ASV ID.
A) Bacterial alpha diversity

- Observed taxa
- Phylogenetic

B) Bacterial alpha diversity

- Observed taxa
- Phylogenetic

C) Fungal alpha diversity

- Observed taxa
- Phylogenetic

Epicuticular.Wax

Mucilage
### Epicuticular Wax - Bacterial Microbiome

<table>
<thead>
<tr>
<th>Family</th>
<th>60 Days after emergence</th>
<th>90 Days after emergence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burkholderiaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Caulobacteriaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Chitinophagaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Comamonadaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Cytophagaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Elsteraceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Erwiniaaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Flavobacteriaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Hymenobacteriaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Legionellaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Microbacteriaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Moraxellaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Morganellaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Oxalobacteriaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Paenibacillaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Pseudomonadaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
</tbody>
</table>

### Aerial Root Mucilage - Bacterial Microbiome

<table>
<thead>
<tr>
<th>Family</th>
<th>60 Days after emergence</th>
<th>90 Days after emergence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetobacteriaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Bacteriovoracaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Beijerinckiaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Beijerinckiaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Bionectriaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Bullenbasidiaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Ceratobasidiaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Cladosporiaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Coniothyriaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Conioscyphaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Corticiaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Cyphellophoraceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Dermateaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Didymellaee</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Didymosphaeriaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Entolomataceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Filobasidiaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Glomeraeaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Heliotraceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Hyaloscyphaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
</tbody>
</table>

### Aerial Root Mucilage - Fungal Microbiome

<table>
<thead>
<tr>
<th>Family</th>
<th>2020</th>
<th>2021</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bionectriaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Bullenbasidiaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Ceratobasidiaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Cladosporiaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Coniothyriaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Conioscyphaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Corticiaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Cyphellophoraceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Dermateaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Didymellaee</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Didymosphaeriaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Entolomataceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Filobasidiaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Glomeraeaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Heliotraceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
<tr>
<td>Hyaloscyphaceae</td>
<td>[Graph of relative abundance]</td>
<td>[Graph of relative abundance]</td>
</tr>
</tbody>
</table>

---

**Bacterial Families**
- Burkholderiaceae
- Caulobacteriaceae
- Chitinophagaceae
- Comamonadaceae
- Cytophagaceae
- Elsteraceae
- Enterobacteriaceae
- Erwiniaaceae
- Flavobacteriaceae
- Hymenobacteriaceae
- Legionellaceae
- Microbacteriaceae
- Moraxellaceae
- Morganellaceae
- Oxalobacteriaceae
- Paenibacillaceae
- Pseudomonadaceae
- Rhizobiaceae
- Rhodanobacteraceae
- Rubtalcaceae
- Sphingobacteriaceae
- Sphingomonadaceae
- Xanthomonadaceae
-Weeksellaceae
- Xanthobacteraceae

**Fungal Families**
- Bionectriaceae
- Bullenbasidiaceae
- Ceratobasidiaceae
- Chrysozymaceae
- Cladosporiaceae
- Conioscyphaceae
- Coniothyriaceae
- Corticiaceae
- Cyphellophoraceae
- Dermateaceae
- Didymellaee
- Didymosphaeriaceae
- Entolomataceae
- Filobasidiaceae
- Glomeraeaceae
- Heliotraceae
- Hyaloscyphaceae
- unidentified
Mucilage microbiome
Differentially abundant ASVs: 60 vs 90 DAE

Log2FoldChange

Class
- Alphaproteobacteria
- Gammaproteobacteria
- Bacteroidia
- Bdellovibrionia
- Verrucomicrobiae
- Polyangia
- Actinobacteria
- Abditibacteria

TAXA
- Legionella
- Flavisolibacter
- Adhaeribacter
- Abditibacterium
- Aureimonas
- Quadrisphaera
- Unclassified Rickettsiaceae
- Pantoea
- Hymenobacter
- Methylobacterium-Methylorubrum
- Sphingomonas
- Unclassified Chitinophagaceae
- Pseudoxanthomonas
- Bld19
- Roseomonas
- Brevundimonas
- Duganella
- Luteolibacter
- Caulobacter
- Verrucomicrobiun
- Herbaspirillum
- Rhodopseudomonas
- Edaphobaculum
- Unclassified Alphaproteobacteria
- Unclassified Comamonadaceae
- Peredibacter
- Chiayivirga
- Mucilaginibacter
- Methylophilus
- Chryseobacterium
- Taibaella
- Dyadobacter
- Bdellovibrio
- Pedobacter
- Lacibacterium
- Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium
- Pseudomonas
- Novosphingobium
- Filimonas
- Acinetobacter
- Unclassified Oxalobacteraceae
- Unclassified Rhizobiaceae
- Flavobacterium
- Variovorax
- Sphingobium

Page 41 of 45
Anaerobic conditions
25 °C
37 °C

Aerobic conditions
25 °C
37 °C

R2A
M9_minimal
Jensen_Nitrogen_Free
M9_Nitrogen-Carbon_Free

Pirovskaya
M9_Nitrogen-Carbon_Free

Gauze_N-1
R2A_terpenes

King_B
M9_Nitrogen_Free

M9_Nitrogen-Carbon_Free

TSAR2A
M9_minimal
Jensen_Nitrogen_Free
M9_Nitrogen_Free
M9_Nitrogen-Carbon_Free

R2A_terpenes
Gauze_N-1

Pirovskaya
R2A
M9_minimal
M9_Nitrogen_Carbon_Free

Gauze_N-1
R2A_terpenes

King_B
M9_Nitrogen_Free

M9_Nitrogen_Carbon_Free

Number of bacterial isolates selected for whole genome sequencing

Cultivation media

Family
Acetobacteraceae
Bacillaceae
Caulobacteraceae
Chitinophagaceae
Comamonadaceae
Cytophagaceae
Enterobacteriaceae
Erwiniaceae
Flavobacteriaceae
Gordoniaceae
Methylbacteriaceae
Microbacteriaceae
Micrococcaceae
Moraxellaceae
Nocardiaceae
Nocardioidaceae
Pseudomonadaceae
Rhizobiaceae
Sphingobacteriaceae
Sphingomonadaceae
Weeksellaceae
Weisseallaceae
Xanthomonadaceae