Mucilage produced by sorghum (*Sorghum bicolor*) aerial roots supports a nitrogen-fixing community

Rafael E. Venado¹, Jennifer Wilker¹*, Vania Pankievicz¹#, Valentina Infante¹, April MaIntyre¹&, Emily Wolf², Saddie Vela², Fletcher Robbins¹, Paulo Ivan Fernandes-Júnior¹,³, Wilfred Vermerris⁴, and Jean-Michel Ané¹,⁵

¹Department of Bacteriology. University of Wisconsin-Madison, Madison, Wisconsin, USA
²Plant Molecular and Cellular Biology Graduate Program, University of Florida-Gainesville, Florida
³Embrapa Semiárido, Petrolina, Brazil
⁴Department of Microbiology & Cell Science and UF Genetics Institute, University of Florida- Gainesville, Florida, USA
⁵Department of Plant and Agroecosystem Sciences, University of Wisconsin-Madison, Madison, Wisconsin, USA
*Now at the Alliance of Bioversity International and CIAT, Palmira, Colombia
#Now at GoGenetics, Curitiba, Paraná, Brazil
&Now at Valent BioSciences, Libertyville, Illinois, USA

Corresponding Author:
Jean-Michel Ané
Tel: +1 608-262-6457
Email: jeanmichel.ane@wisc.edu

Total Word Count for the main body of the text 6,051 words (Introduction: 668; Materials and Methods: 1,365; Results: 2,452; Discussion: 1,566)
Number of color figures: 5
Number of tables: 0
Number of supplementary figures: 3
Number of supplementary tables: 6

Key Words: Biological Nitrogen Fixation, Sorghum, Mucilage, Diazotrophs, Microbiome, Aerial Roots.
Summary

- Sorghum (*Sorghum bicolor*) is gaining popularity as a sustainable energy crop due to its high biomass and potential for biofuel production. Some rare sorghum accessions develop many aerial roots that produce viscous carbohydrate-rich mucilage after rain.
- This aerial root mucilage is strikingly similar to that observed in specific landraces of maize (*Zea mays*) from southern Mexico, which have been previously shown to host nitrogen-fixing bacteria (diazotroph). The landraces displaying these traits can reduce nitrogen-based fertilizer input, mitigating their negative environmental impacts.
- In this study, we characterized the aerial root development of several sorghum accessions and successfully isolated more than 103 distinct diazotrophs from the sorghum mucilage.
- Using acetylene reduction and $^{15}$N gas enrichment assays, we confirmed that sorghum plants acquire nitrogen from the atmosphere through the diazotrophic associations in the mucilage.
- This sorghum symbiotic relationships with diazotrophs offer a promising avenue for nitrogen fixation, potentially diminishing reliance on synthetic fertilizers and promoting sustainable agricultural practices.
Introduction

Nitrogen (N) is essential for plant growth and development. However, atmospheric N\textsubscript{2} is relatively inert and cannot be directly utilized by most plants (Burt, 2013). Biological nitrogen fixation is the conversion of atmospheric N\textsubscript{2} into a usable form, primarily facilitated by specialized bacteria known as diazotrophs (Wagner, 2011). These bacteria synthesize nitrogenase, an enzyme that converts dinitrogen (N\textsubscript{2}) into ammonium (NH\textsubscript{4}\textsuperscript{+}), a form that plants can assimilate (Zehr \textit{et al.}, 2003). Biological nitrogen fixation is a vital process in agriculture, enabling certain plants to obtain nitrogen directly from the atmosphere. This process is crucial in enhancing soil fertility and reducing the need for synthetic fertilizers, which are significant problems for sustainable agriculture (Saikia & Jain, 2007; Werner & Newton, 2010; Kumar & Verma, 2019).

Legumes are among the plants that can form associations with diazotrophs via a specialized organ on their roots called nodules, which host bacteria that fix nitrogen in exchange for photosynthetic products (Oldroyd, 2013). Inside the nodule, the oxygen concentration is low, ensuring nitrogenase can operate efficiently. Nitrogenase also requires a significant amount of energy to catalyze the conversion of N\textsubscript{2} to NH\textsubscript{4}\textsuperscript{+} (Peters \textit{et al.}, 1995). Different approaches are underway to enable nitrogen fixation in cereals, including engineering to integrate genes required to perceive rhizobia or introducing the bacterial nitrogenase into plant cells (Oldroyd & Dixon, 2014; Pankievicz \textit{et al.}, 2019). While the complexity of these strategies makes scientific advancement challenging, the objective is worth pursuing as a long-term solution to global food security and sustainability.

Sierra Mixe maize (\textit{Z. mays} L.) landraces cultivated by Sierra Mixe farmers in Oaxaca, Mexico, secretes carbohydrate-rich mucilage from their aerial roots. This mucilage supports the communities of diazotrophs that provide 29-82\% of the plant’s nitrogen (Van Deynze \textit{et al.}, 2018; Amicucci \textit{et al.}, 2019; Bennett \textit{et al.}, 2020; Pankievicz \textit{et al.}, 2022). These landraces could serve as the foundation for nitrogen fixation in cereals without genetic engineering. The production of aerial roots is not unique to maize. Aerial roots or brace roots are present in the Andropogoneae and Paniceae tribes that include maize, sorghum (\textit{Sorghum bicolor} (L.) Moench), sugarcane (\textit{Saccharum} spp.), foxtail millet (\textit{Setaria italica} L.), and pearl millet (\textit{Pennisetum glaucum}). They serve multiple functions, including anchorage, water, and nutrient uptake (Hostetler \textit{et al.}, 2021; Sparks, 2023). Sorghum (\textit{S. bicolor}), a versatile and resilient crop, has gained significant attention as a promising bioenergy crop. Its ability to thrive in diverse climatic conditions, including
drought-prone regions, makes it an attractive option for sustainable energy production (Regassa & Wortmann, 2014; Rao et al., 2019). Sorghum's high biomass yield and rapid growth rate contribute to its potential as a bioenergetic feedstock (Rooney et al., 2007; Mullet et al., 2014). Efficient water usage and lower input requirements than other energy crops add to its potential as a sustainable crop (Moore et al., 2021). In addition, sorghum increases its biomass when inoculated with specific plant growth-promoting bacteria (PGPB), predominantly those belonging to the genera *Azospirillum*, *Paraburkholderia*, and *Herbaspirillum* (Smith et al., 1984; Pereira et al., 1989; Pacovsky, 1990; dos Santos et al., 2017; dos Reis Antunes et al., 2019; Kuramae et al., 2020).

Despite the similarity of sorghum’s mucilage-producing aerial roots to those in maize known to host nitrogen-fixing diazotrophs, evidence of N-fixation in sorghum has not previously been reported. Images of aerial roots of sorghum covered with mucilage, as well as a micrograph depicting *Azospirillum brasilense*, were featured in the abstract book of a conference on N-fixation in cereals hosted by the International Crops Research Institute for the Semi-Arid Tropics, India (ICRISAT) in 1984 but no data were published. The similarity between these sorghum aerial roots and those from the Sierra Mixe maize prompted us to explore N-fixation in sorghum aerial roots. To this end, we explore the diversity of aerial roots in accessions sorghum and the production of mucilage and their microbiome. We demonstrated that, like the maize landraces from the Sierra Mixe, specific sorghum accessions also acquired nitrogen from their aerial roots by the microbiome established in their mucilage.

**Materials and methods**

*Plant material and phenotyping*

Sorghum accessions were selected from the sorghum minicore (Upadhyaya et al., 2009) and augmented with accessions kindly provided by Dr. Vetriventhan Mani and Dr. Vania C. R. Azevedo from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT, Hyderabad, Telangana, India) (Supplementary Table 1). The plants were cultivated in pots containing Sungrow media in the Walnut Street Greenhouses at the University of Wisconsin-Madison with temperatures at 25 °C during the day and 17 °C at night. The plants were started on a long daylight regimen (14/10), and once the plants were 1-1.5 m tall, then were switched to short days (12/12) due to their photoperiod sensitivity. Sorghum plants were watered with municipal...
water or NPK fertilizer. At the flowering stage, when aerial roots were visible on many nodes, the root diameter of 3 roots at the top node was measured with a digital caliper, as well as the number of nodes forming aerial roots and the number of aerial roots formed on the top node. Overhead water was applied, and 30 minutes after the water application, the mucilage volume of three young and old aerial roots was quantified. The same accessions were grown in West Madison Agricultural Research Station (43.0610329663, -89.5325872441) and University of Florida North Florida Research and Education Center - Suwannee Valley near Live Oak, FL (30.313277, 82.902158) in 1.5 m rows, with 10 cm between plants, and 76 cm between rows. Only the number of nodes and root diameter were phenotyped in the field experiments.

**Bacterial isolation from sorghum**

Mucilage samples were collected from a West Madison Agricultural Research Station field trial in September 2022. The mucilage samples were inoculated in BMGM semisolid medium (Estrada de Los Santos, 2001) and incubated at room temperature for one week. After that, the microaerophilic pellicles formed were transferred to a new medium and incubated for the same period. This procedure was repeated seven times. After the last incubation period, the bacteria were inoculated in a solid BMGM medium supplemented with 0.5 g of yeast extract per liter. The bacteria were purified in the same medium and stored in glycerol at -80°C. The total collection had 164 isolates screened by clonal strains using the Box-PCR approach. Box-PCR was performed using the Box-A1 primer (5’-CTACGGCAAGGCGACGCTGACG-3’) (Versalovic et al., 1994), and the PCR products were subjected to horizontal electrophoresis with agarose gel (1.2% w/v). The identical profiles were excluded, and 103 non-clonal strains. The bacterial strains were identified by 16S rRNA sequencing. The 16S rRNA was amplified by PCR using the primers 27F (5’-AGAGTTTGATCTGGCTCAG-3’) and 1492R (5’-GGTTACCTTGTTACGACTT-3’) and sequenced in an Applied Biosystems 3500 genetic analyzer at the Functional Biosciences facilities. The bad-quality sequences were trimmed using Sequence Scanner v. 2.0, and the contigs of almost complete 16S rRNA sequences were assembled in BioEdit v. 7.7 (Hall, 1999). The sequences were compared against those deposited within the 16S rRNA reference database of GeneBank using the BLASTn tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The maximum likelihood phylogenetic tree was constructed using MEGA 11 software (Kumar et al., 2018).
**Bacterial isolates and growth conditions**

Ten strains were used for different experiments (Supplementary Table 5). *Azospirillum brasilense* FP2 and FP10 (*nifA*) were kindly provided by Dr. Fábio Pedrosa and Emanuel M. de Souza from UFPR-Brazil (Pedrosa & Yates, 1984). The *Klebsiella variicola ΔnifH* mutant was developed by Dr. Maya Venkataraman. *Azospirillum baldaniorum* Sp 245T, *Klebsiella variicola* A3, *Klebsiella michiganensis* A2, *Azorhizobium caulinodans* ORS571T, *Stutzerimonas stutzeri* A1501, *Paraburkholderia silvatlantica* SRMrh-20T, *Azotobacter vinelandii* DJ, *Herbaspirillum seropedicae* SmR1. The strains were grown in LB medium (Vervliet et al., 1975), except for *A. vinelandii*, which was grown in Burk’s sucrose medium (Toukdarian and Kennedy 1986). The cells were grown overnight at 30 °C while shaking at 130 rpm.

**Mucilage glycosyl composition**

Glycosyl composition analysis was performed by combined gas chromatography/mass spectrometry (GC/MS) of the per-O-trimethylsilyl (TMS) derivatives from the monosaccharide methyl glycosides produced by acidic methanolysis as described by (Van Deynze et al., 2018). The analysis was performed at the Complex Carbohydrate Center Research Center (CCRC) at the University of Georgia, Athens, USA.

**Free sugar quantification**

Mucilage was collected at two time points from the sorghum accession IS23992 grown under greenhouse conditions. Benedict’s reaction was used to quantify free sugars, as described in Hernandez-Lopez et al. (2020). Briefly, Benedict reagent (700 μl) was incubated with sorghum mucilage (30 μl) for 10 mins at 95 °C. Subsequently, the reaction was centrifuged at 10,000 g for 5 mins. The supernatant was read in a spectrophotometer λ = 740 nm (Bio-Rad SmartSpect™ Plus), and quantification was determined using a glucose standard curve.

**Metabolomics**

Mucilage samples for metabolome analysis were analyzed at West Coast Metabolomics Center - UC Davis for analysis. Data were normalized and processed with MetaboAnalyst (https://www.metaboanalyst.ca/). This algorithm offers several different methods. Sparse Partial
Least Squares - Discriminant Analysis and Significance Analysis of Microarray were used to analyze the data.

16S rRNA amplicon sequencing

DNA was isolated from the mucilage samples from sorghum and maize using the FastDNA™ SPIN Kit for Soil (MP Biomedicals) following the manufacturer’s instructions. DNA was quantified using a Nanodrop One (ThermoScientific). The 16S rRNA region was amplified with the standard bacterial primers 335F and 769R with GoTaq Mastermix (Promega) according to the manufacturer's instructions. Amplification reaction volumes were 50 μl containing 1 μl of 10 pmol μl\(^{-1}\) primer and 4 ng of template DNA. Thermocycler conditions were as follows: 95°C for 1 min, then 35 cycles of 95°C for 10 s, 55°C for 30 s, and 72°C for 30 s, with a final step at 72°C for 5 min. PCR products were separated on an agarose gel and extracted using the GeneJET Gel Extraction Kit (Thermofisher Scientific). Novogene (Santa Clara, CA, USA) conducted library preparation and sequencing. Amplicons were sequenced with an Illumina paired-end platform generating 250 bp paired-end raw reads. OTUs abundance information was normalized using a standard sequence number corresponding to the sample with the fewest sequences. Subsequent PCoA and beta diversity analyses were performed based on these normalized data. These data were processed with QIIME2 (Version 1.7.0) and displayed with R software (Version 2.15.3).

Acetylene reduction assay (ARA)

Nitrogenase activity was evaluated in ten bacterial strains (Supplementary Table 6) grown in sorghum mucilage collected from the greenhouse. The bacteria were grown in three ml of LB media overnight at 30 °C and shaken at 180 rpm. Then 30 μl (OD\(_{600nm}\) = 0.1) were inoculated in sterile vials containing three ml of mucilage. Vials were capped and incubated for 24 hours at 30 °C. The cap was replaced with a crimp cap, and 1 ml of air was replaced with acetylene, followed by a 3-days incubation period at 30 °C. Gas chromatography was performed in a GC-2010 (Shimazu) with an HS20 autosampler by injecting one ml of air sample into the equipment. Controls included a vial with acetylene to check for traces of ethylene and A. brasilense FP2 without fixing activity. The indirect measure of the nitrogenase activity was expressed in nmol of ethylene produced per hour per mL of mucilage.
15N gas enrichment

Aerial roots from sorghum accession IS23992 were collected in sterile flasks. These roots were obtained from a greenhouse with copious amounts of mucilage. *K. variicola A3* and *K. variicola ΔnifH* mutant were grown in LB medium. Roots with mucilage were inoculated with bacteria (100 µl, OD$_{600nm}$ = 0.1) in roots with mucilage. Flasks were injected with $^{15}$N$_2$ gas to reach a concentration of 15% (v/v), and flasks were incubated for three days at room temperature (20 ºC). To determine the $^{15}$N enrichment, pheophytin was extracted from aerial roots as described by (Kahn et al., 2002; Van Deynze et al., 2018). The samples were analyzed by isotope ratio mass spectrometry (IRMS) at the soil science department at the University of Wisconsin-Madison.

Humidity experiments Greenhouse experiments

Sorghum accessions IS23992 and IS24453 were grown in the Walnut Street Greenhouses at the University of Wisconsin-Madison in Pro-Mix LP15 medium with temperatures at 28 ºC in the day and 25 ºC at night. Plants were grown under a light regime of 12-hour photoperiod from 6 am to 6 pm. Greenhouses were under high humidity (relative humidity 75%) and low humidity (relative humidity 30%) controlled with a humidifier system (Smart Fog, Reno, Nevada). The phenotypes evaluated included number of nodes with roots, the number of roots at the top node, and the root diameter. The measurements followed the previously described procedure.

Statistical analyses

All the statistical analyses were conducted in R version 4.2.1 and 2.15.3 (Foundation for Statistical Computing). ANOVA and Tukey’s HSD were conducted with the package ggpubr (Version 0.6.0) and agricolae (Version 1.3-5). Microbiome studies were performed with QIIME2 (Version 1.7.0) (Caporaso et al., 2010).

Results

Specific sorghum accessions developed aerial roots.

A collection of specific accessions selected from the ICRISAT minicore (Upadhyaya et al., 2009) collection and the more extensive ICRISAT collection exhibited the development of aerial roots at the stock node, similar to Sierra Mixe maize landrace (Van Deynze et al., 2018). Fourteen
accessions (Supplementary Table 1) were grown in a greenhouse environment and assessed for the number of nodes with aerial roots to explore the range of diversity in this trait. The number of nodes varied among the different accessions, ranging from 0 to 8 (Figure 1A and B). Furthermore, these accessions demonstrated significant mucilage secretion from the aerial roots upon exposure to water and high humidity conditions (Figure 1C). The mucilage secretion was primarily observed during the early stages of aerial root development, similar to what was documented for maize landraces (Pankievicz et al., 2022). The volume of mucilage produced also varied across the sorghum accessions, ranging from 0 to approximately 700 µl per root. The Sierra Mixe maize landraces have thicker aerial or brace roots than conventional corn varieties (Van Deynze et al., 2018). Their diameters were measured to determine if the sorghum accessions also exhibited thicker aerial roots similar to the maize Sierra Mixe landraces (Figure 1D and E). The diameter of the aerial roots ranged from 0 (when absent) to 8 mm. Except for the sorghum accession IS24453, which did not develop aerial roots, the remaining accessions displayed the presence of aerial roots, a substantial amount of mucilage secretion, and thicker roots. Accessions IS10757, IS11026, IS15170, and IS23992 produce the most mucilage and have thicker aerial roots. Our results suggest that the number of aerial roots and mucilage content variation is also observed in specific sorghum accessions.

A previous study identified a positive relationship between root diameter (thickness) and mucilage volume in different maize accessions (Pankievicz et al., 2022). A correlation analysis was conducted to investigate whether a similar correlation exists in the sorghum accessions (Supplementary Figure 1A). The results revealed a positive correlation between the number of nodes forming aerial roots, root diameter, and mucilage production volume. The extent of the correlation was higher between the diameter and mucilage volume, $r^2 = 0.4$, but low for the number of nodes and volume, $r^2 = 0.2$ (Supplementary Figure 1B). These findings collectively indicate that thicker roots are associated with higher mucilage production in the selected sorghum accessions.

To assess the consistency of previous findings (Figure 1), the same set of fourteen sorghum accessions was cultivated at two different locations: the University of Florida North Florida Research and Education Center-Suwannee Valley in 2021 (Live Oak, Florida, USA) and the West
Madison Agricultural Research Station in 2022 (Madison, Wisconsin, USA). During the experiments, only the number of nodes with aerial roots and the root diameter were recorded (Supplementary Figure 2). It was evident that the environmental conditions played a significant role, as not all accessions developed aerial roots in both environments. The sorghum accessions generally produced more aerial roots in Florida than in Wisconsin. In Florida, approximately half of the accessions grew nodes with aerial roots; in Wisconsin, less than a quarter of the accessions did (Supplementary Figures 2A and C). Furthermore, greater aerial root diameters were recorded in Florida compared to Madison (Supplementary Figures 2B and D). These results emphasize the environment's substantial impact on the number of nodes and root diameter. Under controlled greenhouse conditions, most accessions produced thicker aerial roots, whereas plant development was more heterogeneous in the more variable field environment.

Humidity influences the number of nodes in sorghum.

The growth and development of field crops are influenced by many environmental factors, including humidity, light intensity, and temperature (Ford & Thorne, 1974). Our research focused on investigating the impact of moisture on the development of aerial roots, particularly how high humidity and water availability trigger mucilage production in the aerial roots of sorghum. Based on our previous findings in greenhouse and field conditions, we selected two sorghum accessions for evaluation: IS23992, known for producing many aerial roots, and IS24453, which lacks aerial roots under field and low humidity greenhouse conditions (Figure 1 and Supplementary Figure 2). We cultivated these accessions under high humidity (75%) and low humidity (30%) conditions. Significantly, we observed an increase in the number of nodes with aerial roots when grown under high humidity for both sorghum accessions, with a more pronounced effect observed in IS23992 (Figure 2A). However, the number of aerial roots at the top node was only significantly affected by humidity in IS24453 (Figure 2B). To determine whether the increase in the number of nodes with aerial roots in humid conditions was due to differences in root diameter, we measured the diameter of aerial roots at the top node under both humidity conditions. Interestingly, the aerial root diameter did not significantly differ between the two accessions (Figure 2C). These findings highlight the crucial role of humidity in the development of aerial roots and the control of node formation in sorghum accessions.
Carbohydrates are the primary molecules of sorghum mucilage.

The mucilage produced by the Sierra Mixe landraces consists of an intricate polysaccharide composed of several monomeric units, including arabinose, fucose, galactose, glucuronic acid, mannose, and xylose (Van Deynze et al., 2018; Amicucci et al., 2019). In the maize landraces, water triggered the expression of plant genes related to synthesizing carbohydrates (Pankievicz et al., 2022). To determine the sorghum mucilage (Figure 3A) composition, gas chromatography/mass spectrometry (GC/MS) analysis was conducted. The chemical composition analysis revealed the same monosaccharides were present in sorghum mucilage as in maize mucilage (Van Deynze et al., 2018). However, differences in the proportion of specific monosaccharides were observed (Figure 3B). Specifically, the monosaccharides arabinose, galactose, glucuronic acid, and mannose had higher ratios in sorghum mucilage than maize mucilage. Galactose accounts for more than 50% of the total sugars in sorghum mucilage. The amount of fucose in sorghum mucilage was approximately half that found in maize mucilage. These results indicate that the mucilage in the two species consists of the same monosaccharides but that the structure likely differs based on the observation that the proportions in which the monosaccharides are present differ.

As shown in Pankievicz et al. (2022), the exposure of aerial root to water induced the expression of genes associated with carbohydrate synthesis/degradation. To investigate this degradation process, we assessed the levels of free sugars in sorghum mucilage at two time points: one and five hours after its production from accession IS23992, cultivated in the Walnut Street greenhouse. Using Benedict's reaction (Hernández-López et al. 2020), we observed that the free sugar concentration increased over time, rising from an average of 86 mM at one hour to 109 mM after five hours (Figure 3C). This finding suggests that sorghum aerial roots on these roots produce enzymes breaking the mucilage polysaccharide into simple sugars. We cannot exclude the contribution of microbes on these roots to this mucilage degradation process as the aerial roots were not germ-free, but very few microbes are isolated from sorghum mucilage when grown in this greenhouse.

To explore the differences in the soluble metabolites in the mucilage between maize and sorghum (Supplementary Table 2), we performed a metabolomics analysis using gas chromatography
coupled with time-of-flight mass spectrometry (GC/TOF-MS). We analyzed our data with the sparse Partial Least Squares - Discriminant Analysis (sPLS-DA) algorithm to reduce the number of variables (metabolites) and produce robust and easy-to-interpret results. The principal component analysis revealed two clusters where samples were grouped based on the genotype (Figure 3C). We use a Significance Analysis of Microarray (SAM) to identify differentially expressed proteins. The top ten most abundant metabolites include six unknown metabolites as well as octadecanoic, montanic acid (octacosanoic acid), pinitol (1D-chiro-inositol), and N-acetyl aspartate diethyl ester (Supplementary Table 3). These metabolomic differences may contribute to the functional differences observed in sorghum and maize mucilage.

Aerial root mucilage from sorghum contains a distinctive type of border cell. Plant mucilage is also produced at the root cap of underground roots (Hawes & Lin, 1990; Brigham et al., 1995; Knee et al., 2001). Within the mucilage, a specific type of cell called border cells (BCs) are responsible for mucilage production (Hawes & Pueppke, 1986; Pankievicz et al., 2022). To investigate the presence of border cells in sorghum mucilage, we examined mucilage derived from both underground and aerial sorghum roots (Supplementary Figures 3A and C). BCs derived from underground roots exhibited a distinct and elongated morphology similar to those observed in BCs from pea (Pisum sativum L.), maize, and alfalfa (Medicago sativa L.) (Hawes & Pueppke, 1987; Woo et al., 2004; Zhang et al., 2014). In contrast, border cells from aerial roots appeared much larger and elongated, with irregular shapes and sizes (Supplementary Figure 3C). The BCs in sorghum aerial roots resembled those found in mucilage from maize aerial roots (Pankievicz et al., 2022). These larger BC may contribute to the abundant mucilage production by maize and sorghum aerial roots.

Border cells detached from the root cap can remain active for several weeks (Hawes & Pueppke, 1986; Vicré et al., 2005). To determine the viability of sorghum border cells, we used a live-dead staining protocol on both types of sorghum mucilage collected after 72 hours. We used a combination of SYTO™ 13 and propidium iodine, which are permeant and non-permeant cell dyes, respectively (Boulos et al., 1999; Ullal et al., 2010). Most cells were viable, as indicated by only SYTO™ 13 in the nuclei. Additionally, BCs from underground roots displayed regular and well-defined nuclei, while BCs from aerial roots exhibited irregular nuclei (Supplementary...
Figures 3B and D). To quantitatively assess the morphological differences between the two types of BCs, we measured their length from one cellular pole to another and their cell area. Significant differences were observed in both measurements. BCs from aerial roots generally are twice the size compared to BCs from underground roots. In maize, BC length in aerial roots varied depending on the growth stage, with younger aerial roots having longer BCs and older aerial roots having smaller BCs (Pankievicz et al., 2022). The BCs from sorghum aerial roots in our study were collected at a young growth stage, which explains the twofold size difference compared to underground BCs. Although both types of BCs maintain the function of mucilage production, the observed morphological differences could account for the variation in the amount of mucilage secreted by these cells.

Sorghum mucilage hosts a wide range of diazotrophs.

A diverse community of diazotrophs can become established in the mucilage of the Sierra Mixe maize landraces (Van Deynze et al., 2018). To explore the microbial diversity within sorghum mucilage, we conducted a study where we isolated and sequenced bacteria using metabarcoding bacterial 16S rRNA. We focused on mucilage samples from two experimental environments: the West Madison Agricultural Research Station University of Wisconsin and the University of Florida North Research & Education Center - Suwannee Valley. Additionally, we included mucilage samples from Sierra Mixe landraces as references (Supplementary Table 4). To analyze the bacterial composition, we processed the variable region of the 16S rRNA and organized it into Operational Taxonomic Units (OTUs). The OTUs provided information on the samples' relative abundance of different taxa. This approach allowed us to identify any outliers that significantly differed from the rest of the samples and visually compare the differences among them (He et al., 2015). We performed principal coordinate analysis to examine the beta diversity, representing the samples' diversity. Our findings indicated a distinct separation among samples based on the location and plant origin of the mucilage. The most significant difference was observed between the sorghum and maize samples (Figure 4A). Furthermore, we investigated the bacterial phylum in sorghum and maize mucilage. Our analysis revealed that the phylum Pseudomonadota was dominant in the mucilage, followed by Cyanobacteriota and Bacillota (Figure 4B). The profiles of samples from Wisconsin were relatively similar regardless of the mucilage's origin, while samples from Florida exhibited enrichment in Cyanobacteria, with a similar abundance as
Pseudomonadota (Figure 4B). The isolation of bacterial diazotrophs using the N-free semisolid BMGM medium revealed a high diversity of potential diazotrophs. We obtained 103 non-clonal strains that were identified within 34 different species (Supplementary Table 5) with the prevalence of Pseudomonadota (Gammaproteobacteria - 32, Alphaproteobacteria - 30, Betaproteobacteria - 5), Actinomycetota (28), Bacteroidota (3), and Bacillota (1) (Figure 4C). The nitrogenase activity of one representant strain of each one of the 34 species was evaluated by the acetylene reduction assay and the results indicated the Agrobacterium pusense BM_20.4, Bacillus amyloliquenfaciens BM_7.6, Pseudomonas turukhanskensis BM_14.7, Microbacterium aerolatum BM_14.4, Agrobacterium fabacearum BM_6.8, Microbacterium neimengense BM_10.2, Enterobacter cancerogenus BM_14.3, Epilithonimonas hungarica BM_19.2, Pseudomonas lutea BM_5.2, and Pseudacidovorax intermedius BM_2.1 showed higher nitrogenase activity than the reference diazotrophs Klebsiella variicola A3 and Azospirillum brasiliense FP2 (Figure 4D). Only eight bacterial strains showed no nitrogenase activity at all.

Mucilage enables biological nitrogen fixation in sorghum.

In the Sierra Mixe maize landraces, the mucilage creates a suitable environment for diazotrophs, contributing to nitrogen acquisition for the plant. This environment provides low oxygen levels and carbohydrates (Van Deynze et al., 2018). We tested whether the mucilage could serve as a proper medium for nitrogen fixation by an acetylene reduction assay (ARA). Nine diazotrophs were isolated from sorghum mucilage collected from Wisconsin and Florida fields (Supplementary Table 6). As a negative control, we included A. brasiliense FP10, a strain that lacks nitrogenase activity but does not impede growth compared to the wild type (Pedrosa & Yates, 1984). To prevent endogenous diazotrophic bacteria in the sorghum mucilage, we collected mucilage from sorghum plants grown in the greenhouse. We specifically selected the sorghum accession IS23992 because it produced numerous nodes with aerial roots in the greenhouse and the field (Supplementary Figure 2). Additionally, this accession consistently had an average of 250 µl of mucilage (Figure 1). The acetylene reduction assay (ARA) results indicated significant nitrogenase activity in mucilage inoculated with the strains Azotobacter vinelandii and Klebsiella variicola (Figure 5A). However, the remaining seven strains exhibited almost no nitrogenase activity, with only Klebsiella michiganensis showing some ARA activity. The negative control also displayed low activity, possibly due to a small fraction of diazotrophs in the mucilage. This
result supports the notion that mucilage is a medium to facilitate the nitrogen fixation activity of specific diazotrophs in the aerial roots of sorghum.

To validate the plant's ability to take up and assimilate nitrogen, we conducted a $^{15}$N$_2$ gas enrichment experiment. Aerial roots with mucilage were collected from the greenhouse-grown plants of the IS23992 accession. Based on the acetylene reduction assay results, we inoculated the roots with *Klebsiella variicola A3* (wild type) and used *Klebsiella variicola A3 ΔnifH* as a negative control. Isotope-ratio mass spectrometry (IRMS) was used to measure the $^{15}$N content in pheophytin derived from chlorophyll isolated from the aerial roots of sorghum. Roots inoculated with *Klebsiella variicola* exhibited significant incorporation of $^{15}$N$_2$ gas compared to the negative control (Figure 5B). These results confirmed that mucilage from the aerial roots of sorghum supports diazotrophs capable of providing nitrogen to the plant.

**Discussion**

Nitrogen availability limits the performance of economically important cereal crops, including sorghum and maize. Both species belong to the Andropogoneae tribe and share a common ancestor dating 12-16 million years ago (Swigonová et al., 2004). Among them, sorghum is a more versatile and drought-resistant crop, yielding higher biomass, making it a promising candidate for biofuel production (Mathur et al., 2017). Biological nitrogen fixation is a suitable method of overcoming nitrogen deficiencies to minimize the requirement for synthetic fertilizer applications for production. While these symbiotic relationships are well studied in legumes, cereal crops lacked a suitable model until the recent discovery of biological nitrogen fixation in aerial root mucilage in maize landraces, specifically Sierra Mixe (Van Deynze et al., 2018). Here, we determined that specific sorghum accessions harbor the ability to host diazotrophs enabling the fixation of atmospheric nitrogen via their mucilage-producing aerial roots. We also elucidated the similarities and differences in aerial root development, mucilage production, composition, and microbial diversity between sorghum landraces.

Some sorghum accessions produce abundant and thick aerial roots under humid conditions

Variations in the number of nodes with aerial roots among different genotypes of sorghum was studied earlier. For example, the Chinese landrace Sansui produces 6-8 aerial roots, while the elite
cultivar Jiliand only has a single node (Li et al., 2014). Our study further demonstrated that specific sorghum accessions displayed varying numbers of nodes with aerial roots (Figure 1A). Additionally, we observed differences in diameter and mucilage production in these accessions, and a positive correlation among these three traits confirmed a similar development as the Sierra Mixe (Van Deynze et al., 2018). Recently, this finding was reinforced by a study involving 59 sorghum accessions that also produce mucilage from aerial roots (Xu et al., 2023). The presence of aerial roots in other members of the Poaceae family, such as sugarcane and foxtail millet, suggests a common evolutionary origin (Hostetler et al., 2021).

High relative humidity levels significantly impact plant growth and the production of various phytohormones, including abscisic acid and ethylene (Arve & Torre, 2015). In the case of rice, elevated humidity has been observed to stimulate the formation of lateral roots (Chhun et al. 2007). This effect is attributed to the increased presence of ethylene, a plant hormone that promotes lateral root development (McDonald & Visser, 2003; Visser & Voesenek, 2005). By providing high humidity, we observed more nodes with aerial roots independently of the sorghum genotype (Figure 2). We speculate that this response is due to an ethylene-driven mechanism triggered by the elevated humidity, leading to an increased number of nodes with aerial roots. Although the exact mechanism of how water influences hormone regulation remains unclear, it is evident that humidity plays a crucial role in determining the number of nodes with aerial roots, possibly through a water-sensing mechanism that regulates phytohormone production.

Some sorghum accessions produce abundant mucilage from aerial roots.

Plants secrete mucilage from various tissues to adapt to abiotic and biotic stresses. Mucilage is commonly found in diverse plant parts, including roots, seeds, leaves, and stems. Notably, in underground roots, the task of producing and releasing mucilage and other components is carried out by specialized cells known as border cells, which utilize large vesicles. In the case of the maize landraces, the mucilage from their aerial roots resembles the mucilage commonly found at the tips of underground roots in maize. Packievicz et al. (2022) demonstrated the presence of border cells in the mucilage from the aerial roots of Sierra Mixe, and they noted differences in size compared to border cells derived from underground roots. Our data corroborated these findings, showing a
significant distinction between border cells in underground and aerial roots (Supplementary Figure 3).

Carbohydrates constitute the main components of both underground and aerial root mucilage (Osborn et al., 1999; Van Deynze et al., 2018; Amicucci et al., 2019; Nazari et al., 2020). In our research, we investigated the carbohydrate composition of sorghum mucilage, and the results show the presence of similar monosaccharides, albeit in slightly different proportions (Figure 3). These findings are consistent with a prior study on maize genotypes, which revealed changes in the carbohydrate profile based on their geographic locations and agroecological systems (Nazari et al., 2020). Interestingly, our analysis did not identify the presence of glucose or fructose as reported in the sorghum mucilage from the aerial root in accessions 59 and SL36 (Xu et al., 2023). Instead, the monosaccharide profile closely resembled the Sierra Mixe landraces (Van Deynze et al., 2018). This discrepancy suggests that environmental factors significantly influence maize and sorghum mucilage's carbohydrate and metabolite composition. However, the genetic aspect cannot be disregarded entirely, as evidenced by our principal component analysis, which displayed a clear separation based on species (Figure 3).

Sorghum aerial roots mucilage hosts specific microbial communities containing diazotrophs

Numerous studies have documented the predictable variation in plant microbiome composition based on host and environmental factors (Lundberg et al., 2012; Edwards et al., 2015; Hartman et al., 2017; Crosbie et al., 2022). Our microbiome investigation revealed that location and host influence microbial composition in the aerial root mucilage (Figure 4). Certain plant enzymes capable of breaking down mucilage into simple sugars are expressed in the Sierra Mixe landraces and may play a role in nurturing their microbiomes. Simultaneously, the microbiome contributes to mucilage degradation through specialized enzymes (Pankievicz et al., 2022). *Pseudomonadota, Acidobacteriota, Cyanobacteriota,* and *Bacillota* were the most prevalent phyla in sorghum, aligning with findings from other sorghum microbiome studies (Xu et al., 2018). Moreover, specific phyla have been proposed as potential biomarkers for certain biological processes (Bhagat et al., 2021). Our study sheds light on the microbial profiles of sorghum and maize for nitrogen fixation and offers valuable resources for engineering these microbes to enhance nitrogen fixation. These resources will serve as a foundation for investigating microbial community interactions and
their roles in facilitating nitrogen fixation. Such insights could pave the way for possible inoculation strategies in the future.

Sorghum benefits triggered by the inoculation of diazotrophic bacteria were reported several years ago (Wong & Stenberg, 1979; Pal & Malik, 1981; Morgenstern & Okon, 1987), and the delivering of nitrogen from the air to the host plants by biological fixation was confirmed in the last few years in inoculated plants (dos Santos et al., 2017) and in plants associated with the native communities (Barros et al., 2020). However, those reports have no information about the plants' aerial roots or mucilage production. Our results indicated production of aerial roots and mucilage is a crucial aspect of nitrogen fixation in sorghum, indicating the ability of the plants reported by Santos et al. (2017) and Barros et al. (2020) to produce aerial roots and mucilage.

Microbial communities can be well established in plant mucilages due to the abundance of carbon, and the diazotrophs could benefit from the microaerophilic conditions. The mucilage viscosity is very close to that observed in nitrogen-free semisolid media, which is a classical approach to isolating diazotrophs from non-legumes (Baldani et al., 2014), indicating the abundance of diazotrophs in sorghum mucilage. The existence of efficient diazotrophs colonizing sorghum aerial root mucilage was confirmed in the present study with the application of a nitrogen-free semisolid medium approach, indicating a wide diversity of diazotrophs and corroborating the 16S rRNA amplicon sequence data. The highlights of unusual bacteria with the highest nitrogenase activity, such as Agrobacterium spp. and Epilithonimonas hungarica, for example, indicate that, under the conditions assessed, the diazotrophic community of sorghum mucilage is different from those communities from sorghum underground roots since other studies using the same medium and approach could not identify these diazotrophs (Silva et al., 2018; dos Reis Antunes et al., 2019).

Nitrogen fixation occurs in sorghum aerial root mucilage

Nitrogen fixation in cereals can reduce part of our dependence on synthetic nitrogen-based fertilizers. Our research has shown that mucilage is crucial in creating a favorable environment for diazotrophs to thrive (Figure 5A), enabling plants to acquire nitrogen (Figure 5B). To achieve nitrogen fixation in cereals, synthetic biology offers promising approaches through transferring nitrogenase genes to plants, engineering a root nodule symbiosis, or remodeling associative

---

The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

bioRxiv preprint doi: https://doi.org/10.1101/2023.08.05.552127; this version posted August 6, 2023.
diazotrophs. Some progress has been made in expressing \textit{nif} genes responsible for encoding nitrogenase components in plant cells (Allen \textit{et al.}, 2017; Xiang \textit{et al.}, 2020). However, challenges remain, particularly related to the stability of this enzyme in plants, which is sensitive to oxygen and requires high energy levels (Rutten & Poole, 2019; Lindström & Mousavi, 2020). Another approach involves the formation of nodule-like structures to host bacteria, necessitating the transfer of several genes involved in nodule organogenesis and infection mechanisms, but this is a very long-term perspective (Oldroyd & Dixon, 2014; Pankievicz \textit{et al.}, 2019). The genetic remodeling of associative diazotrophs to generate ammonium excreting bacteria is a short-term promising approach, but using genetically engineered bacteria in the field is currently allowed in only a handful of countries. While exploring these approaches is valuable, we should recognize the huge potential within plant natural diversity to find and breed for better hosts for diazotrophs using non-transgenic approaches. Some maize landraces of Sierra Mixe have demonstrated their ability to acquire nitrogen via aerial roots. Similar traits in sorghum accessions indicate that exploring natural diversity in other cereals for nitrogen fixation is a promising avenue. Doing so can expedite our efforts to introduce nitrogen fixation into cereals for developed and developing countries worldwide. Established sorghum breeding programs could breed for aerial development and mucilage secretion. These natural traits hold considerable value to reduce our dependence on nitrogen fertilizers for cereal crops, reducing costs for growers, the contamination of streams and groundwater, the degradation of coastal zones, and greenhouse gas emissions.

\textbf{Acknowledgments}

We thank Dr. Sanhita Chakraborty and Dr. Kimberly Gibson for their valuable input and engaging discussions. Additionally, we thank Alyssa Davis and Julio Quiñones for their technical support and Ben Broughton and Mike Boyette at the UF North Florida Research & Education Center-Suwannee Valley for managing the field plots. We thank Maya Venkataraman and Dr. Brian F. Pfleger for providing the \textit{K. variicola} \textit{ΔnifH} strain. Funding for this project was provided to J.M.A. by the United States Department of Energy (DOE) grant #DE-SC0021052 and the United States Department of Agriculture (USDA) #2020-67013-32675.

\textbf{Competing interests}

The authors declare that they have no conflict of interest.
Author contributions

JMA, REV, JW, VP, VI, AM, PIF, and WV designed and performed experiments. JW, VP, VI, SV, EW, and FR performed field experiments. JMA and WV provided reagents and material. REV, JMA, PIF, WV wrote the manuscript. All authors reviewed the manuscript and approved it.

ORCID

April MacIntyre: ORCID: 0000-0002-1867-6661
Emily Wolf: ORCID: 0000-0002-0331-3788
Fletcher Robbins: ORCID:
Jean-Michel Ané: ORCID: 0000-0002-3128-9439
Jennifer Wilker: ORCID: 0000-0003-2329-5457
Paulo Ivan Fernandes-Júnior: ORCID: 0000-0002-6390-3720
Rafael E. Venado: ORCID: 0000-0002-5020-0348
Saddie Vela: ORCID: 0000-0001-9504-7159
Vania Pankievicz: ORCID: 0000-0003-3192-9691
Valentina Infante: ORCID: 0009-0009-6705-1754
Wilfred Vermerris: ORCID: 0000-0002-4582-3436

Data Availability

Not applicable
References


Morgenstern E, Okon Y. **1987**. Promotion of plant growth and NO3- and Rb+ uptake in
Sorghum bicolor × Sorghum sudanense inoculated with Azospirillum brasilense-Cd. Arid Soil Research and Rehabilitation 1: 211–217.


Figure 1. Sorghum screening of selected accessions. Different sorghum accessions display a diversity in traits associated with aerial roots and mucilage production. A) Number of nodes in different sorghum accessions. B) Representative image of selected sorghum accessions. Scale bar = 28 cm. C) Volume of mucilage (µl) produced by different sorghum accessions. D) Diameter (mm) of aerial roots in different sorghum accessions. E) Aerial roots of the sorghum accessions in D. Scale bar = 28 cm
Figure 2. Effect of humidity in sorghum accession IS23992 and IS24453. Boxplots of different phenotypes measured in the sorghum accessions. A) Number of nodes with aerial roots. B) Number of aerial roots at top node. C) Average root diameter on the top node. A Wilcoxon test was performed in R (Ver 4.2.1) with the package ggpubr (Ver 0.6.0). Significant levels * p-value ≤ 0.05, ** p-value ≤ 0.01 and n.s. no significant (n = 8).
**Figure 3. Sorghum aerial root mucilage composition.** Sorghum release mucilage from their aerial roots A) Production of mucilage is triggered by water and under high humidity environments. Scale bar (5 cm) B) Monosaccharides composition of sorghum and maize mucilage. Both mucilages have the same monosaccharides but in different proportions. C) Reducing sugar in sorghum mucilage from the accession IS23992. Wilcoxon test was performed with significant level *** p-value ≤ 0.001 (n = 8). D) Principal component analysis using Sparse Partial Least Squares. Sorghum and maize samples cluster based on similarities.

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>Amount S. bicolor (%)</th>
<th>Amount Z. mays (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose (Ara)</td>
<td>17.47</td>
<td>12.27</td>
</tr>
<tr>
<td>Fucose (Fuc)</td>
<td>13.31</td>
<td>40.68</td>
</tr>
<tr>
<td>Xylose (Xyl)</td>
<td>2.26</td>
<td>2.92</td>
</tr>
<tr>
<td>Glucuronic acid (GlcA)</td>
<td>8.06</td>
<td>3.09</td>
</tr>
<tr>
<td>Mannose (Man)</td>
<td>7.23</td>
<td>2.92</td>
</tr>
<tr>
<td>Galactose (Gal)</td>
<td>51.68</td>
<td>38.12</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

1 Data obtained from 1.64 mg of sample
2 Data from Van Deynze et al. 2018
Figure 4. Microbiome profile of sorghum mucilage. Community profile showing the relative abundance present in sorghum and maize. Samples S1 to S4 and M1 to M6 were collected from the field experiments at the University of Wisconsin (UW-Madison) and samples S5 to S7 were collected at University of Florida (UF). A) Principal coordinate analysis plot of sorghum and maize samples based on 16S amplicons and OTU. C) Unrooted maximum-likelihood phylogenetic tree of 103 bacteria isolated from sorghum aerial roots mucilage in BMGM N-free semisolid medium. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. This analysis involved 103 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion). There were 908 positions in the final dataset. Numbers at nodes are bootstrap values (1000 replications, values < 50% are not shown). Colors: Red: Gammaproteobacteria, Purple: Betaproteobacteria, Blue: Alphaproteobacteria, Brown: Bacillota, Green: Actinomycetota, Yellow: Bacteroidota. D) Nitrogenase activity of 34 isolated from sorghum aerial roots mucilage in BMGM N-free semisolid medium (n=3). Bars are the error mean deviation. Klebsiella varicola A3 ΔnifH. Species codes (alphabetical order): Agrobacterium divergens, Agrobacterium fabaceae, Agrobacterium larrymoorei, Agrobacterium pusense, Azospirillum brasilense, Azospirillum humidicucens, Azospirillum palustre, Bacillus amyloliquefaciens, Enterobacter cancerogenus, Epilithonimonas hungarica, Herbaspirillum seropedicae, Klebsiella michiganensis, Klebsiella oxioca, Klebsiella varicola, Microbacterium aerolatum, Microbacterium binotii, Microbacterium hominis, Microbacterium reimengense, Microbacterium oleivorans, Microbacterium testaceum, Novosphaingobium kaempferiae, Phytobacter diazotrophicus, Pseudoacidovorax intermedius, Pseudomonas bharatica, Pseudomonas campii, Pseudomonas lutea, Pseudomonas sediminis, Pseudomonas turukhanskensis, Pseudoxanthomonas winnipegensis, Rhizobium cellulolyticum, Siphonobacter intestinalis, Stenotrophomonas lactitubi, Stenotrophomonas nataldociola, Stenotrophomonas rhizophila, Stenotrophomonas terrae.
Figure 5. Biological nitrogen fixation in aerial roots from sorghum. A) Acetylene reduction assay in mucilage generated in the greenhouse. Mucilage was used as media to grow ten strains Azospirillum brasilense FP2, Azospirillum baldaniorum, Azorhizobium caulinodans, Azotobacter vinelandii, Herbaspirillum seropedicae, Klebsiella michiganensis, Klepsiella variicola, Paraburkholderia silvatlantica, Pseudomonas stutzeri (fixing strains) and Azospirillum brasilense FP10 (non-fixing strains). Tukey’s honestly significant difference (HSD) test was performed in R and significant groups are displayed as lower case (n = 6). B) Analysis of $^{15}$N enrichment in aerial roots of sorghum accession IS23992. Aerial roots were inoculated with K. variicola ΔnifH (negative control) and K. variicola (positive control). Wilcoxon test was performed with significant level *** p-value ≤ 0.001 (n = 8).
Supplementary Figure 1. Trait correlations. A) Scatter plot shows the correlation between the number of nodes, mucilage volume and diameter. A low positive correlation is observed among all traits. B) Correlation matrix for number of nodes, mucilage volume and diameter.
Supplementary Figure 2. Sorghum screening of selected genotypes in two locations. A) Number of nodes in different sorghum accessions grown at University of Florida during 2021. B) Diameter (mm) of aerial roots in different sorghum accessions grown at University of Florida. C) Number of nodes in different sorghum accessions grown at University of Wisconsin during 2022. D) Diameter (mm) of aerial roots in different sorghum accessions grown at University of Wisconsin during 2022. * Data from these accessions were obtained under low nitrogen regimen.
Supplementary Figure 3. Border cells in sorghum mucilage. A) Border cells from sorghum underground roots (S=scale bar 100 μm). B) Border cells from sorghum aerial roots (scale bar 100 μm). C) Live-Dead viability staining in border cells from sorghum underground roots (scale bar 100 μm). D) Live-Dead viability staining in border cells from sorghum aerial roots (scale bar 100 μm). E) Length comparison between border cells in μm. F) Area comparison between border cells in μm². AR (aerial roots) and UR (underground roots). The p-values were ** < 0.01 and *** < 0.001 based on a Wilcoxon test (n = 20)